

Supporting information

Study participants and plan. Prior to inclusion in the study, all participants provided written informed consent. Inclusion criteria were: 1) adult patient (≥ 18 years) with at least 6 weeks of CSU who remained symptomatic despite the use of up to $\times 4$ H_1 -antihistamines and 2) an urticaria activity score (UAS) during a 7-day period (UAS7) of ≥ 16 (on a scale ranging from 0 to 42, with higher scores indicating greater activity, based on patient diary entries completed twice daily) during the 7 days before the first treatment with omalizumab. Exclusion criteria were: 1) a clearly defined underlying cause for chronic urticaria, 2) routine administration (i.e., daily or every other day for ≥ 5 consecutive days) of systemic glucocorticoids, anti-inflammatory, immunomodulatory, or immunosuppressive agents within the previous 30 days, 3) the use of any H_2 -antihistamine or leukotriene-receptor antagonist within 7 days preceding the screening visit, 4) treatment with omalizumab within the previous year, or a known hypersensitivity to omalizumab, 5) a history of cancer, or 6) women who were pregnant or nursing or unable to use an effective method of contraception during dosing with omalizumab.

The study plan is shown in Figure S1. Because the study aim was to identify two groups with the most discordant response to omalizumab, response was assessed only in subjects who completed UAS7 diary scores over the entire 12-week study period. To quantitate the response to omalizumab of each subject, the area under the curve (AUC) for the weekly UAS7 values over the 12-week study period from a baseline value of zero (UAS7 AUC) was computed using the trapezoidal method and expressed as UAS7 $\times$ week unit (Table S1). All peaks above a set score of zero were counted. For the UAS7 AUCs, the medians, 25th and the 75th percentiles were also determined. Because the study aim was to identify two groups with the most discordant response to omalizumab, response was assessed only in subjects who completed UAS7 diary scores over the entire 12-week study period. Responders were defined as those subjects with an AUC for the weekly UAS7 over the 12-week treatment period that was equal to or less than the 75th percentile and had at least a 90% improvement of baseline UAS7 at one of their weekly UAS7 diary scores. Non-response (NR) was defined as subjects who a) failed to achieve a 90% improvement of their baseline weekly UAS7, or b) had an AUC for the weekly UAS7 over the 12-week study period that was above the 75th percentile (i.e., top quartile). Responders were further characterized into two groups: those with complete response (CR) and those with partial response (PR) by determining the AUC for the weekly

UAS7 over the last half of the study to identify individuals with a durable therapeutic response (Table S1, Figure S1). Baseline characteristics including blood eosinophil and basophil levels of the study participants are shown in Table S2 and Figure S2.

FIGURE S1. Study plan and clinical response to omalizumab. The study plan is shown in Panel **A**. Omalizumab injections were on Days 0, 30, and 60 (arrows) with UAS7 scores and blood obtained for microRNA (miRNA) analysis as indicated. Twenty-seven subjects were screened (Day -14 visit) and 6 either failed screening or elected not to enter the treatment phase; 21 subjects received omalizumab on Day 0 with first follow-up on Day 14. Subject 18 withdrew on Day 30 because of worsening urticaria symptoms and did not receive omalizumab then. Twenty subjects received omalizumab on Days 30 and 60. Subject 26 received cyclosporine A for urticaria exacerbation between Days 60 and 90 with no UAS7 scores recorded after Day 60. The area under the curve (AUC) for weekly UAS7 over the last 6 weeks of omalizumab therapy for each subject in the complete responders (CR), partial responders (PR), and non-responders (NR) is shown in panel **B**. Weekly UAS7 scores over the 12-week study period for CR, PR, and NR are shown in panels **C**, **D**, and **E** respectively.

FIGURE S2. Relationship between eosinophil and basophil levels and response to omalizumab. Panel **A** shows Welch's ANOVA comparing the baseline eosinophil levels between the three response groups: complete responders (CR) (n=6), partial responders (PR) (n=9), and non-responders (NR) (n = 4). Welch's ANOVA comparing the mean and 95% confidence intervals for basophil levels between CR (n = 6), PR (n=9), and NR (n=4) (Panel **B**). Panel **C** shows the response to omalizumab measured by the area under curve (AUC) for the weekly urticaria activity scores (UAS7) over the 12-week study period as a function of baseline basophil and eosinophil levels assessed by linear regression model. Baseline basophil levels were negatively correlated with the UAS7 AUC. Shown are regression lines and 95% confidence intervals.

TABLE S1. Clinical response classification by area under the curve (AUC) of UAS7 scores[†]

Participant	90% Improvement	AUC 0-12 wk	AUC 7-12 wk	Response
Subject 1	Yes	21	0	CR
Subject 8	Yes	23.5	0	CR
Subject 17	Yes	57.5	0	CR
Subject 25	Yes	102	0	CR
Subject 10	Yes	60	3	CR
Subject 12	Yes	43.5	3.5	CR
Subject 22	Yes	115	11	PR
Subject 7	Yes	82.5	15.5	PR
Subject 4	Yes	81.5	17.5	PR
Subject 15	Yes	88.5	18	PR
Subject 21	Yes	162	28	PR
Subject 5	Yes	127.5	30.5	PR
Subject 27	Yes	171	31.5	PR
Subject 19	Yes	148	38.5	PR
Subject 16	Yes	137.5	43	PR
Subject 11	No	243.5	75	NR
Subject 9	No	274.5	118.5	NR
Subject 3	No	314	122.5	NR

Subject 24	No	340.5	142	NR
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[†]AUC UAS7 response is expressed as UAS7 x week unit. AUC 0-12 wk, area under the curve for the UAS7 over the 12-week of the study; AUC 7-12 wk, area under the curve for the UAS7 over weeks 7-12 of the study period; CR, complete responder; PR, partial responder; NR, non-responder.

TABLE S2. Baseline characteristics of study participants[†]

Variable	All Subjects	Responders	Non-Responders	P value
Subject (n)	19	15	4	
Age (yrs)	48 ± 13.8	49.4 ± 10.3	42.7 ± 24.6	0.63
Gender – Female	12 (63%)	8 (53.3%)	4 (100%)	0.25
Weight (kg)	90.8 ± 26.7	94.5 ± 27.7	77 ± 19.1	0.18
Baseline UAS7	34 ± 5.3	33.3 ± 5.1	36.7 ± 5.6	0.32
Angioedema (+)	9 (47%)	6 (40%)	3 (75%)	0.30
Eosinophils/μL	136.3 ± 81.4	140.6 ± 87.3	120 ± 60.7	0.60
Basophils/μL	26.1 ± 14	28.7 ± 14.8	16.5 ± 2.5	0.008

[†]Values are presented as n (%) for categorical variables and mean ± SD for continuous variables. Statistical differences between responders and non-responders were analyzed using Fisher exact test for categorical variables or unpaired two-tailed Welch's t-test for continuous variables.

TABLE S3. Change in the levels of EV microRNAs by Day 90[†]

MicroRNA	Complete Responders		Non-Responders	
	Log FC	FDR	Log FC	FDR
miR-3976	-3.661	0.033	1.874	0.65
miR-6499-5p	-2.369	0.31	4.496	0.008
miR-7848-3p	-0.721	0.88	4.606	0.001
miR-4494	-1.272	0.84	1.407	0.76
miR-450a-2-3p	-2.476	0.32	4.506	0.016
miR-6877-3p	0.411	0.89	3.561	0.005
miR-141-3p	-1.249	0.84	-1.728	0.63

[†]FC, fold change; FDR, false discovery rate.

MicroRNA isolation and microRNA seq profiling. Extracellular vesicles (EV) were isolated from the plasma of the study participants using Invitrogen's Total Exosome Precipitation Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). MicroRNAs were isolated from the EV using the QIAGEN® miRNeasy Serum/Plasma Kit (QIAGEN Sciences, Germantown, MD) and quality assessed by the QIAGEN® QIAseq microRNA. MicroRNA seq profiling was performed at QIAGEN Sciences (Germantown, MD). Raw reads were de-multiplexed and FASTQ files for each sample were generated using Illumina bcl2fastq software. Reads and Unique Molecular Index (UMI) counts were generated using QIAGEN GeneGlobe pipeline. The UMI counts for each microRNA were read into R (r-project.org) using Bioconductor edgeR package and filtered to remove microRNAs that were either unexpressed or expressed at a consistent, unreliably low level. Samples were normalized using a trimmed mean of M-values and counts converted to log counts/million (logCPM) using the voomWithQualityWeights function from the Bioconductor limma package. to estimate observation

and sample-level weights for use in a conventional linear model and control for heteroscedasticity. For each subject, there were repeated measurements estimated using the `limma duplicateCorrelation` function and controlled for by a linear mixed model when making comparisons.

Statistics. Assuming a standard deviation of 0.65, which was derived from previously published experimental data^{1,2} and assuming biologically meaningful changes in microRNA expression at levels of absolute fold change greater than 1.5, a sample size of 10 was chosen for $\alpha \leq 0.05$ and $\beta \leq 0.2$ for absolute fold change ≥ 1.5 . Descriptive statistics were performed to determine the mean and standard deviation (SD) of continuous variables and the number (n) and relative frequency (%) of categorical variables. The Kolmogorov-Smirnov test and histogram analyses were used to determine whether continuous variables were normally distributed. A two-tailed t-test and Analysis of Variance (ANOVA) with Dunnett T3 correction for multiple comparisons were used for the comparisons of continuous variables between the different groups as appropriate. Welch's correction was applied when appropriate to account for unequal sample sizes. Fisher's exact test was used for group comparison of categorical variables. For miR-Seq data statistical analyses, comparisons between groups were made using empirical Bayes adjusted contrasts. Multiplicity was controlled at a false discovery rate (FDR; Benjamini-Hochberg correction) of 0.05 (i.e., the expected maximum proportion of false positives is estimated to be 5%). Heatmaps for differentially expressed microRNAs were generated after conversion to z-scores. To test for a different temporal outcome of UAS7 score over time, we used a linear mixed model that included dichotomized microRNA expression (high or low at baseline), a time coefficient (treatment week), and an interaction between the two; we tested for the significance of interaction by dropping the interaction term from the model and computing a likelihood ratio test (LRT), which tested for a difference at one or more time points. Analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 8.3 (GraphPad Software, San Diego, CA).

Ingenuity pathway analysis (IPA). QIAGEN® IPA microRNA Target Filter was employed using high stringency filters to predict the corresponding mRNA targets of differentially expressed exosomal microRNAs. Only mRNA targets that were experimentally validated or had high confidence predictions were selected using the TarBase, miRecords, and Target Scan (human) databases and literature findings curated within the QIAGEN® Knowledge Base. Predicted mRNA targets were analyzed with the IPA core analysis feature to identify significantly enriched canonical

pathways. Significance of canonical pathways was determined using a right-tailed Fisher's exact test. Benjamini-Hochberg procedure was applied to control False Discovery Rate (FDR). IPA Grow tool visualized the relationship between individual microRNAs and their predicted canonical pathway targets. QIAGEN® IPA Molecule Activity Predictor tool predicted the upstream and/or downstream effects of activation or inhibition of molecules in a pathway given one or more neighboring molecules with known activity. Predictions were based on an algorithm that incorporates calculation of a maximum absolute z-score to predict activity of molecules, diseases, and biological functions.

References

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2. Ach RA, Wang H, Curry B. Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods. *BMC Biotechnol*. 2008;8:69.