Serum MicroRNA-21 as a Biomarker for Allergic Inflammatory Disease in Children

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ABSTRACT

MicroRNAs (miRs) have emerged as useful biomarkers for different disease states, including allergic inflammatory diseases such as asthma and eosinophilic esophagitis (EoE). Serum miRs are a possible non-invasive method for diagnosis of such diseases. We focused on microRNA-21 (miR-21) levels in serum, in order to assess the feasibility of using this gene as a non-invasive biomarker for these diseases in the clinic, as well as to better understand the expression pattern of miR-21 in allergic inflammation. We used quantitative PCR (QPCR) to assay miR-21 and other control miRs in esophageal biopsies from EoE patients and serum samples from EoE and asthma patients. Serum levels of miR-21 were significantly elevated in patients with asthma, whereas serum miR-21 levels were not associated with the presence of allergen-specific IgE (i.e. atopy). Esophageal biopsies showed a large elevation of miR-21 in EoE and an increase in miR-21 in EoE serum. Control U6 miR did not vary between asthma and control patients, however EoE serum had significantly decreased U6 microRNA compared to controls. The decreased U6 in EoE sera did not completely account for the relative increase in miR-21 in the sera of EoE patients. We report for the first time that miR-21 is elevated in the sera of both asthma and EoE patients. We find no relation between serum miR-21 levels and atopy. Our results thus suggest miR-21 is a novel biomarker for human allergic inflammatory diseases.

Introduction:

Micro-RNAs (miRs) are short 19-25 nucleotide long transcripts that are important repressors of gene expression at the post-transcriptional level (1). MiRs are commonly elevated in cancer, autoimmunity and inflammation (2, 3), and thus miRs have been extensively examined as sensitive biomarkers for disease. Due to the increased prevalence of allergic disease in recent decades (4), there is great interest in developing new approaches for analyzing and treating allergic diseases such as asthma. MicroRNA-21 (miR-21) expression is increased in allergic airway inflammation in mice, an asthma-like disease (5-7). Mir-21 expression is also increased in the allergic disease eosinophilic esophagitis (EoE) (8). Therefore, miR-21 appears to be a biomarker for Th2-type

allergic disease. Functionally, miR-21 can promotes Th2 responses by two different pathways. First, miR-21 can repress IL-12 gene expression, thus inhibiting Th1 responses and favoring Th2 responses (5, 6). Second, miR-21 can directly promote Th2 differentiation in a T cell intrinsic manner, by increasing Gata3 and IL-4 expression at early points after T cell activation (9).

Since miRs are useful biomarkers for disease, miRs found in serum or "circulating miRs" represent a powerful method to assess disease in readily accessible clinical samples (10). In a mouse model of asthma, we were able to detect elevated miR-21 in the serum of mice with severe airway inflammation (7). In this study, we set out to determine if we could detect increased miR-21 in the serum of human asthma patients. We also sought to better understand the relationship between miR-21 and human allergic disease by analyzing miR-21 levels in serum from pediatric EoE patients. We further tested whether miR-21 was generally associated with atopy, as defined by serum IgE levels. We have found that serum miR-21 is significantly elevated in both asthma and EoE patients.

Materials and Methods

Patient Groups and Clinical samples: The collection and analysis of control, EoE patient and asthma patient samples were approved by the Institutional Review Board of Indiana University and required parental consent for samples from infants and children, following Declaration of Helsinki principles. Clinical samples were taken from two sets of patient cohorts described previously for asthma (11) and for EoE (12). Asthma samples and EoE samples each had separate study-specific controls. Asthma and control patients were enrolled into the study as infants for eczema presentation, and then were followed longitudinally for development of allergic disease. EoE and control patients were enrolled into the study at various ages, after referral to the gastroenterology clinic and subsequent esophagogastroduodenoscopy (EGD). Patient information for the two sets of patient samples is described in Table 1 for asthma and Table 2 for EoE.

MicroRNA analysis: MicroRNAs from esophageal biopsies and human serum were isolated using the miRNeasy isolation kit (Qiagen). Esophageal biopsy and serum samples were obtained as frozen specimens. Biopsy samples were immediately lysed while frozen, whereas serum was thawed prior to miR isolation. Generally, microRNA was isolated from 0.5 ml of serum. QPCR was performed using TaqMan microRNA assays (Applied Biosystems), according to the manufacturer's protocol, and roughly 20% of the sample was used for the preparation of each specific miR cDNA. Normalization of miR-21 and miR-22 was performed using U6 RNA as control. Quantitative real-time PCR was performed by assaying each sample in triplicates, including no-template controls, on a Stratagene Mx3000P real-time PCR system. Relative expression was calculated using the delta-delta CT (ddCT) method.

IgE quantitation: Serum IgE values were taken from a previous study (11).

Results

Increased miR-21 was previously observed in the serum of mice with severe airway inflammation (7), thus we set out to test if circulating miR-21 was elevated in human asthma. We analyzed serum miR-21 levels in pediatric patients recruited into a study on the development of asthma. The patients were recruited as infants, based on a diagnosis of eczema, and subsequently characterized for atopic status and the development of asthma (11). We assessed serum levels of miR-21 in a set of patients that had been analyzed at age 5 for asthma status. We thus tested samples from 8 control patients without asthma and 8 patients with asthma. As shown in Figure 1, we found that serum miR-21 increased in the asthma patients by 2.7-fold, with a *p* value of 0.018. We also analyzed the expression of miR-22, which we found previously to be increased in the lungs of mice with severe airway inflammation (unpublished data). In contrast to miR-21, miR-22 was not increased in the serum of patients with asthma (Figure 1). In our selected patients, 8 out of 16 patients were atopic, as defined by the presence of IgE for specific antigen (11). However, the rate of atopy was similar between asthmatic and non-asthmatic patients (Table 1). Asthmatic patients had on average a two-fold increase in serum IgE over non-asthmatic patients (130 ± 26 IU/ml versus 61 ± 25 IU/ml, Table 1), however, the difference in IgE level was not significant ($p = 0.24$), due to the degree of variation. To further determine whether serum miR-21 levels associated with serum IgE levels, we directly compared these two parameters in a curve-fit linear regression analysis. As shown in Figure 2, serum miR-21 levels correlate very poorly with serum IgE levels, and thus miR-21 levels did not associate positively or negatively with atopy. We conclude that miR-21 levels are a novel serum biomarker for asthma, independent of atopic status.

We next wondered if elevated miR-21 was a general marker for allergic inflammatory disease in humans, and obtained samples from EoE and control patients. We initially tested esophageal biopsies from EoE and control patients for miR-21 expression, and found that, similar to previous reports (8, 13) miR-21 expression is very significantly increased in EoE biopsies compared to control biopsies (Figure 3). However, we observed a greater fold elevation in miR-21 in EoE, almost 50-fold on average, than in the two other studies (8, 13), who observed a roughly 4-fold increase in miR-21 in EoE compared to non-EoE. The expression of miR-22 increased roughly two-fold in EoE biopsies (Figure 3), but the difference was not significant. Next, we assessed miR-21 in serum from EoE patients (Figure 4). Compared to controls, we observed an average of 30-fold increase in serum miR-21 levels in EoE patients, with a high degree of statistical significance. MiR-22 was not detected in the serum of EoE or control patients (data not shown).

Our results contrasted with that of Lu *et al* who detected less increase in miR-21 in esophagus in EoE, and little to no levels of circulating miR-21 in EoE (8). One key factor in miR analysis is the control miR used for normalization (8). We therefore analyzed the specific expression of U6 miR that we used for normalization, in our different samples (Figure 5). U6 levels were essentially constant between control and asthma sera, and between control and EoE biopsy. However, we found that U6 levels were significantly lower in EoE sera compared to control sera (represented as higher Ct value by a factor of 3.3). This average decrease in U6 by about 10-fold in EoE sera can account for the relative increase in miR-21 we observe in some EoE sera, but sera from 11/18 EoE patients have increases in miR-21 that cannot be accounted for the decreased U6 (data not shown). Further, the U6 values in control and EoE sera were remarkably consistent, with a low standard deviation. The miR-21 values however, were much more variable and may reflect active more active inflammation in EoE patients than in other patients. However, there was no association between eosinophil counts in esophageal biopsies from EoE patients and miR-21 levels (data not shown). Serum levels of miR-21 may therefore reflect other aspects of the inflammatory disease than just the degree of eosinophil infiltration into the esophagus in EoE.

Discussion

Overall, our results indicate that elevated circulating miR-21 can potentially serve as a biomarker for allergic inflammatory diseases such as asthma and EoE. Overall, we find that EoE patients have a much greater increase in serum miR-21 than asthmatic patients, however this difference may relate to the severity of disease at the time of sample collection, rather than a difference in disease type. Further research will be required to determine if serum miR-21 levels are a useful predictor of the development of asthma, EoE or other allergic inflammatory diseases.

Our miR-21 results contrast with that of Lu *et al* (8) who detected little to no levels of circulating miR-21 in EoE, as well as a lower level of miR-21 in esophageal biopsy samples. Some of the possible explanations for our ability to detect elevated miR-21 in EoE sera and greater increases of miR-21 in biopsies compared to Lu *et al* are differences in 1) disease severity between patients in the two studies, 2) control patients between the two studies and 3) miR-21 assay sensitivity. As judged by eosinophil infiltration into the esophagus, the level of disease in our EoE population is not more severe than in the Lu *et al* study; in fact, our study used a slightly lower cut-off for eosinophil infiltration than Lu *et al* (15 per high powered field versus 24 per high powered field). In terms of control groups, for the serum analysis, Lu *et al* specifically compared circulating miR-21 in EoE patients to healthy atopic patients. Our controls in the EoE study were patients who had undergone esophagogastroduodenoscopy (EGD) for a variety of non-specific reasons but had normal histology on biopsies of the upper gastro-intestinal tract. However, patient history (Table 2) indicates that our control patients had similar levels of allergy and asthma as the EoE patients. Furthermore, we did not observe an association between the level of miR-21 expression and allergy or atopy, either in the asthma group or the EoE group. Thus, we conclude that the best explanation for our results is that our miR-21 assay was more sensitive, even though the reagents we used were very similar to the reagents used by Lu *et al*. One major difference with our results on serum miR-21 in EoE is the control miR used to normalize miR-21 levels. Lu *et al* used miR-16 as a control for the circulating miRs in EoE, whereas we used U6. We tested miR-16 in our EoE sera but found that it gave much lower signal than the U6 (data not shown). We therefore decided that U6 was a more robust control for the EoE sera. Although still easily detectable in our assay, we did note that U6 levels were consistently decreased in EoE sera (Figure 5). Altered U6 expression in serum has been previously shown in breast cancer (14), and altered serum levels of U6 miR may be a biomarker for chronic disease. Interestingly, in breast

cancer, serum U6 levels were elevated (14), whereas we find in EoE that serum U6 levels were decreased. This suggests there may be disease-specific regulation of U6 miR levels in the serum.

Possibly the most important different between the Lu *et al* study and ours, is that they used "plasma", whereas we used serum. The major difference between plasma and serum is the presence of clotting factors, and conceivably clotting factors could impact miR stability or isolation. Another possibility that is difficult to rule out is cellular contamination of plasma and serum with small numbers of cells or lysed blood cells (15), and possibly our EoE results may be explained by contamination of the sera with cellular debris. However, the key point is that we observe highly significant differences between control and EoE and between control and asthma samples. This may indicate that blood cells from patients with allergic inflammatory disease have a greater propensity to undergo lysis during sample collection. Patients with the most severe ongoing inflammation may be especially prone to this phenomenon. This does not mitigate the possibility of using a miR-21-based assay to diagnose allergic inflammatory disease in human patients.

One likely source of the elevated serum miR-21 we observe in EoE and asthma is eosinophils, since eosinophils are strongly increased in both diseases and they express high levels of miR-21 (16). The eosinophils in EoE and asthma may secrete miR-21 via exosomes, or miR-21 is released from eosinophils when they die in the course of the disease. A possible source of serum miR-21 in specifically EoE is release from inflamed esophagus epithelium, since normal esophagus can expresses miR-21 (17). MiR-21 is increased during Th2 inflammation in EoE and in airway inflammation (5, 18), and we observed striking levels of miR-21 expression in lung epithelium in Th2-type airway inflammatory disease (7). MiR-21 is also up-regulated in Th2 cells (9), and could be released from these cells also by exosomes or after their death.

MiR-21 is most well-known for being an "oncomiR" favorable to cancer cell growth, and as one of the most commonly up-regulated microRNAs in cancer (2, 19, 20). The cytokine-activated transcription factor Stat3 is positive regulator of miR-21 transcription, and may be a common pathway for increased miR-21 expression both in cancer and in inflammation (21). We have shown that miR-21 is activated in Th2 cells by Stat3 (9), and therefore Stat3 activity appears to link much of the aberrant miR-21 expression associated with disease.

Conclusion

We have shown here, for the first time that miR-21 is elevated in the sera of both asthma and EoE patients, whereas we find no relation between serum miR-21 levels and atopy. Our results thus suggest miR-21 can be used a novel and sensitive biomarker for severe allergic inflammatory diseases in patients. Our results warrant a further study into miR-21-based assays for testing different allergic diseases, using a variety of conditions.

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Figure Legends

Figure 1. Serum miR-21 is increased in asthma. Quantitative PCR (QPCR) analysis of miR-21 (A) and miR-22 (B) expression in serum samples prepared from 5 year old patients presenting at 1 year of age with dermatitis and then monitored for allergy symptoms and the development of asthma. Relative miR expression is shown, with average dC_T values from control patient samples set as 1, and fold-change calculated by the ddC_T method.

Figure 2. Serum miR-21 does not correlate with serum IgE levels. Pearson correlation analysis between IgE levels (in IU) and miR21 levels (fold-change from average non-asthmatic control). N = 16 total patients, 8 with asthma and 8 without asthma. *$p<0.05$ (two-tailed Student's t-test) (error bars, s.e.m.)

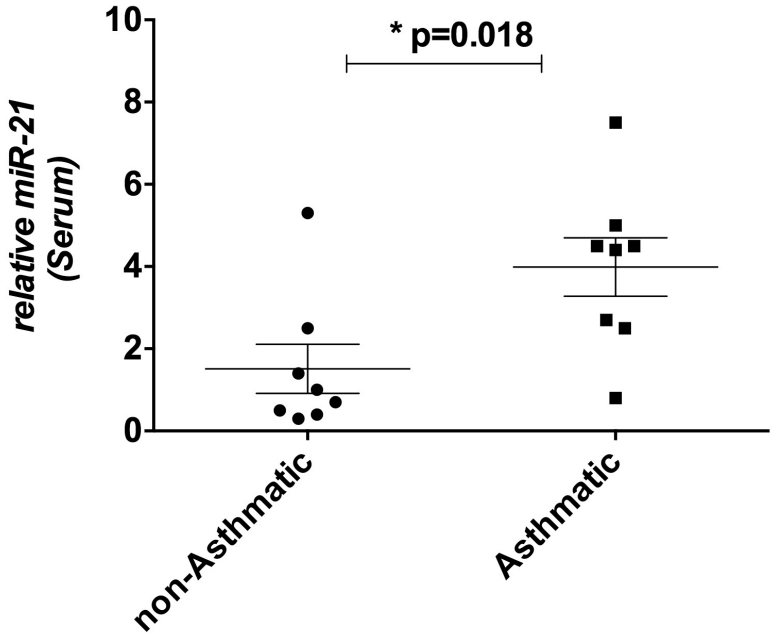
Figure 3. MicroRNA-21 is greatly elevated in the esophagus of patients with eosinophilic esophagitis. Quantitative PCR (QPCR) analysis of miR-21 (A) and miR-22 (B) expression in esophageal biopsy RNA. Relative miR expression was calculated as described in Figure 1.

Figure 4. MicroRNA-21 is significantly elevated in the serum of patients with eosinophilic esophagitis. Quantitative PCR (QPCR) analysis of miR-21 in serum from non-EoE controls and eosinophilic esophagitis (EoE) patients, normalized using U6 as control. Relative miR expression was calculated as described in Figure 1. MiR-22 was not detected at significant levels in serum from either EoE or non-EoE patients. N = 18subjects per group. ***$p<0.001$ (two-tailed Student's t-test) (error bars, s.e.m.)

Figure 5. U6 microRNA expression is significantly decreased in the serum of patients with eosinophilic esophagitis. Quantitative PCR (QPCR) analysis of U6 microRNA in serum from all samples tested in this study. Data shown is raw QPCR Ct values, which correspond to the amplification cycle where the target is first detected. Higher Ct values mean less RNA target in the sample. EoE sera have on average a Ct value of 35.5 compared to a 32.2 Ct value for controls.

Figure 1.

A.


* p=0.018
relative miR-21 (Serum)
10
8
6
4
2
0
non-Asthmatic
Asthmatic

B.

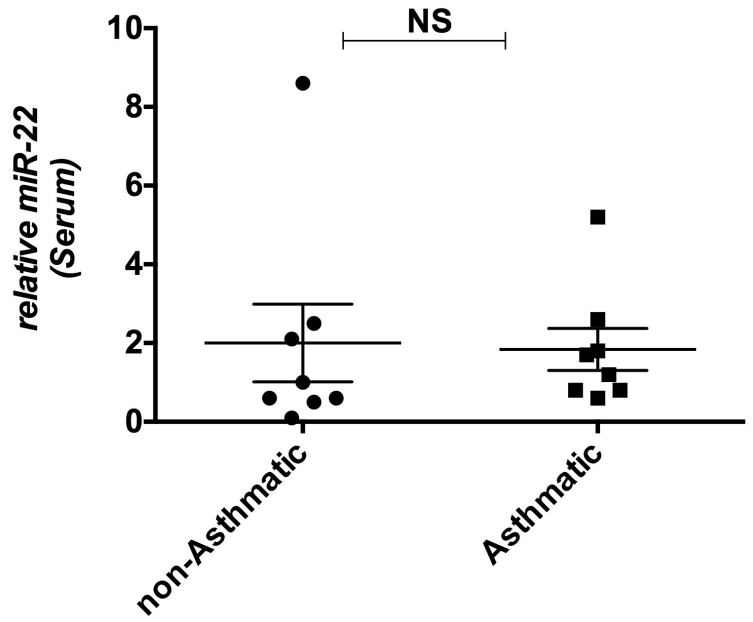

NS
relative miR-22 (Serum)
10
8
6
4
2
0
non-Asthmatic
Asthmatic

Figure 2.

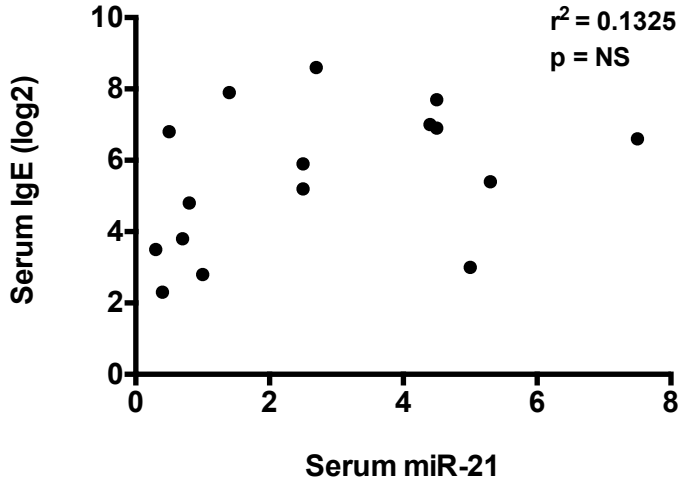
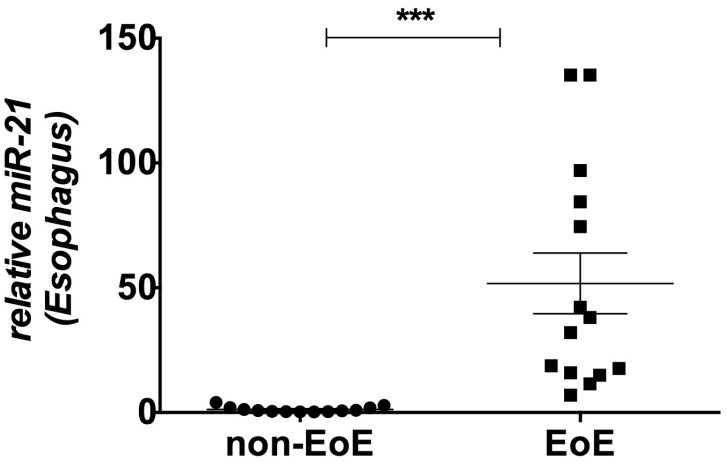


Figure 3.

A.


***
150
100
50
0
relative miR-21
(Esophagus)
non-EoE
EoE

B.

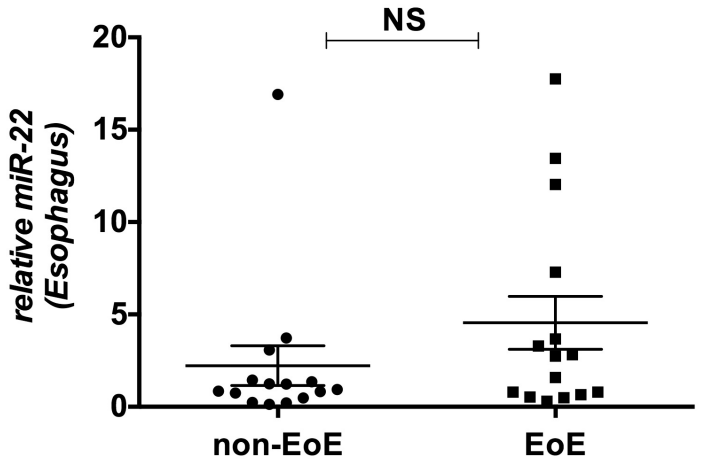

NS
20
15
10
5
0
relative miR-22
(Esophagus)
non-EoE
EoE

Figure 4.

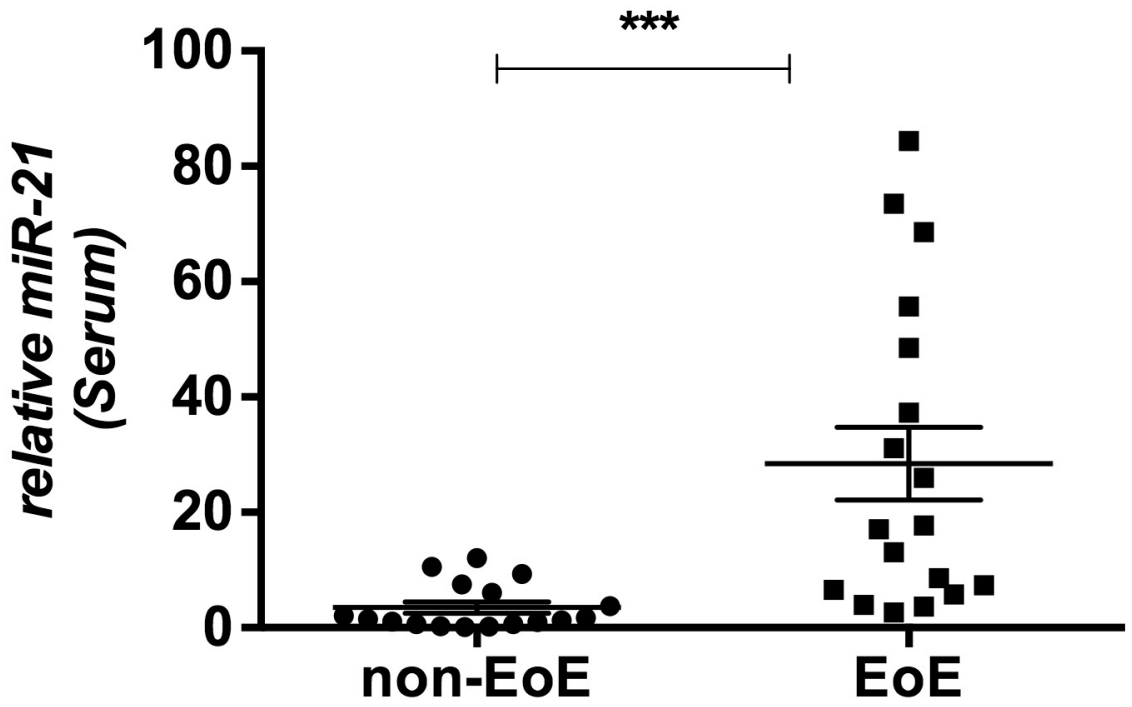


Figure 5

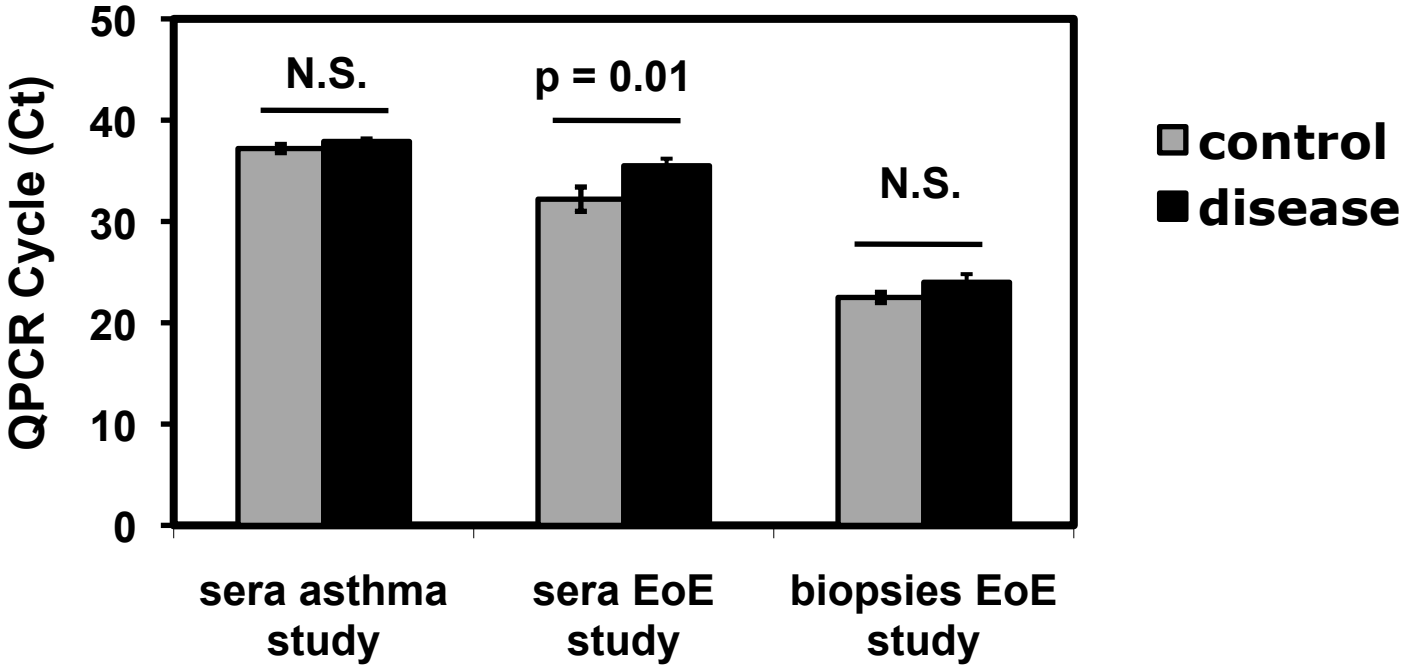

50
40
30
20
10
0
QPCR Cycle (Ct)
N.S.
p = 0.01
N.S.
control
disease
sera asthma study
sera EoE study
biopsies EoE study

Table 1. Asthma study patient information

	Non-asthma (n = 8)	Asthma (n = 8)
Age (years)	5 ± 0.2	5 ± 0.2
SCORAD index	3 ± 1.7	6 ± 1.7
IgE specific for egg or milk	0%	25%
IgE specific for any known antigen	50%	63%
IgE (IU/ml)	61 ± 25	130 ± 26

Values are average ± SEM. None of the parameters were significantly different between the patient groups.

Table 2. EoE study patient information

	Non-EoE (n = 8)	EoE (n = 7)
Age (years)	7 ± 2	7 ± 1
Asthma diagnosis	25%	17%
Known allergy	63%	83%
Eosinophil count in esophageal biopsy (#/hpf)	<15	49 ± 12

Values are average ± SEM. The only significant difference between the patient groups was eosinophil count ($p < 0.01$).