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Basophil degranulation in response to IgE ligation is controlled by a distinctive circadian clock in asthma

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Abstract

Background: Several factors may contribute to the circadian variability of clinical manifestations in asthma and allergy. Basophils play a pivotal role in allergic inflammation. However, evidence for a functional clock governing the effector function of these cells is sparse and contradictory. We have systematically sampled the 24-hour response of basophils to IgE- and non-IgE-dependent ligands in asthma to understand their possible contribution to the diurnal variations of allergic symptoms.

Methods: Leukocytes were collected every 4 hours for 24 hours from 10 patients with moderate, persistent asthma and 10 matched, nonallergic controls, and then incubated with concentrations of anti-IgE, formyl-methionyl-leucylphenylalanine (fMLP), or the Ca²⁺ ionophore, A23187. Histamine release (HR) was tested for time-of-day- or disease-related variability by conventional statistics and for 24-hour rhythmicity by the cosinor method.

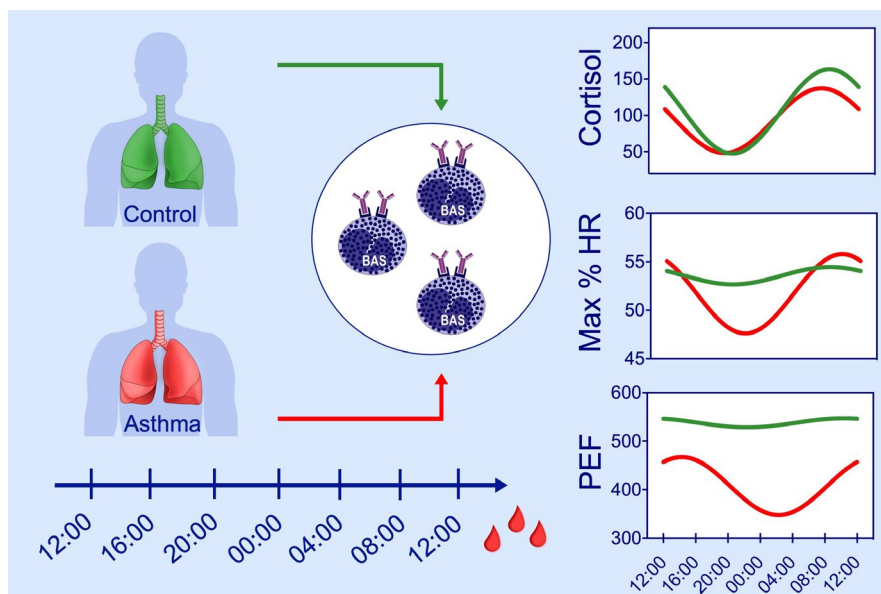
Results: HR induced by anti-IgE was significantly increased at 08:00 vs. 20:00 in basophils from asthmatics but not controls. No significant differences were seen at any time in the response to A23187, while the response to fMLP was significantly higher at 08:00 vs. 20:00 in controls but not asthmatics. The basophil response to anti-IgE, but not fMLP or A23187, varied significantly across the 24 hours in asthma, and its amplitude, percent rhythm, and acrophase were comparable to those of peak expiratory flow or serum cortisol.

Conclusion: Using an integrated statistical approach, we show that basophil responsiveness undergoes significant circadian variability and that distinct patterns of rhythmicity can be recognized depending on the signal delivered, the activation parameters assessed, and the disease status.

KEYWORDS

allergy, asthma, basophils, clock, histamine

Abbreviations: BAT, basophil activation test; fMLP, formyl-methionyl-leucylphenylalanine; FPR, formyl peptide receptor; HR, histamine release; HR_{AUC}, area under the curve of histamine release; HR_{MAX}, maximal percent histamine release; HR_{SENS}, threshold sensitivity of histamine release; MESOR, midline estimating statistic of rhythm; PEF, peak expiratory flow; PR, percent rhythm.



GRAPHICAL ABSTRACT

Basophils were collected serially for 24 hours from asthmatics or controls and activated with IgE- or non-IgE-mediated secretagogues. Histamine release induced by anti-IgE exhibited significant circadian rhythmicity in asthma but not controls, in parallel with asynchronous changes in cortisol and PEF levels. Circadian variability of IgE-dependent basophil responsiveness may contribute to diurnal variability in respiratory function and symptoms in asthma.

Abbreviations: Max % HR, Maximal percent histamine release; PEF, Peak expiratory flow

1 | INTRODUCTION

Several factors are thought to contribute to the diurnal variability of clinical manifestations in patients with allergic disease (reviewed in¹). In particular, abnormal circadian rhythms of lung function and airway inflammatory markers can be observed in patients with asthma, especially so in poorly controlled asthma associated with night-time or early morning symptoms.² The existence of a functional circadian clock in effector cells of allergic inflammation—that is, eosinophils, mast cells, and basophils—is implied in a number of human and animal studies.^{3–8} A recent study reported significant 24-hour variations in peripheral blood or sputum eosinophil numbers and CCL11 concentrations in patients with mild/moderate asthma.³ Expression of the circadian gene, *Clock*, and of *Clock*-controlled genes, *Period* (*Per*) 1, *Per*2, and *brain and muscle ARNT-like 1* was found to follow a circadian pattern in peripheral human or mouse eosinophils, in parallel with asynchronous changes in the 24-hour expression of eosinophil effector genes.⁴ Baseline plasma histamine levels have long been known to undergo consistent time-of-day-related changes,⁹ which was recently shown to depend on integrity of the *Clock* gene in mast cells.⁵ In line with the idea that circadian rhythmicity reflects regulation by an intrinsic clock, expression of mast cell circadian and effector genes, and the IgE-dependent release of histamine and leukotrienes, underwent significant time-of-day-related rhythmicity *in vitro* cultures of cells isolated from human or mouse gut specimens.⁴

In contrast to studies of mast cell or eosinophil biology, evidence for circadian regulation of human basophil effector functions is thus far sparse and inconclusive. Basophils play a central role in allergic

inflammation, immune regulation, as well as lung development and function.^{10–13} Increased expression of *Per*1 and *Per*3, and maximal induction of the basophil activation marker, CD203c—a measure of basophil reactivity—were seen in blood samples collected from allergic rhinitis patients at 07:00 relative to samples collected 12 hours later, challenged *in vitro* with cedar pollen allergen or a crosslinking anti-IgE antibody.⁶ *Vice versa*, basophil half-maximal response, referred to as basophil sensitivity, but not basophil reactivity, was increased at 16:00 vs. 04:00 in a study by Baumann *et al.* looking at anti-IgE-induced expression of another basophil activation marker, CD63, on blood samples from nonallergic donors.⁷ Intriguingly, though, neither parameter of basophil activation appeared to vary significantly in a more recent study from the same investigators, measuring CD63 and CD203c induction by birch allergen in samples collected at six time points from patients with seasonal rhinoconjunctivitis.⁸

Taken together, these findings underscore the intricacies of current *in vitro* models for basophil activation studies. Studies to date have tested the *in vitro* expression of inducible surface markers in relatively homogeneous subject groups sampled at discrete time points. However, as aptly commented in the most recent such study,⁸ “an association with circadian rhythm must still be ascertained”. In other words, previous work has not allowed the generation and analysis of individual around-the-clock curves, or *chronograms*, of human basophil responses to IgE- or non-IgE-dependent ligands. Moreover, possible differences in the responsiveness and function of basophils obtained from allergic or nonallergic subjects, *that is*, basophil releasability, have not been taken into account.^{14,15} We used herein an established

chronobiological approach to analyze the diurnal changes in baseline or induced histamine release (HR) in basophils from asthmatic patients and matched controls. Our data show that basophil activation and effector function follow a traceable circadian rhythm. Distinct patterns of rhythmicity can be recognized depending on the signal delivered and the activation parameters assessed, which are somewhat subverted in basophils from asthmatics relative to nonallergic individuals.

2 | METHODS

2.1 | Subjects

The study was approved by the Ethics Committee of the University of Naples Federico II, School of Medicine, and informed consent was obtained from all participants. These included 10 patients with moderate, persistent, dust mite-sensitive asthma (5/10 females, aged 33.30 ± 3.08 years) and 10 age- and gender-matched, nonallergic controls (5/10 females, aged 31.80 ± 4.27 years). Basophil preparations from prospective donors were preliminarily tested for releasing *in vitro* at least 10% of their histamine content in response to optimal concentrations of an IgE-dependent ligand as outlined below.¹⁴ None of the patients or control subjects had significant comorbidities, for example, obstructive sleep apnea or obesity, or suffered recent upper or lower respiratory infections. None of them had used any topical or systemic glucocorticoids or other medications, besides inhaled albuterol *prn*, for at least 2 weeks prior to and throughout the study. Patient and control pairs were hospitalized for 3 days in a controlled environment within the inpatient facility of the Department of Translational Medicine of the University of Naples Federico II. On the second day, serial 20-mL samples of uncoagulated venous blood were collected in parallel and promptly processed for leukocyte separation every 4 hours for 24 hours starting at 12:00, then 4, 8, 12, 16, 20, and 24 hours thereafter. At the same time, peak expiratory flow (PEF) values were determined using portable meters and additional 2- to 3-mL blood aliquots collected and stored at -20°C for subsequent determinations of serum IgE and cortisol levels.

2.2 | Basophil activation

Whole blood samples were processed immediately after collection to obtain leukocyte-enriched preparations by sedimentation (90 minutes at 22°C) in 8 mmol/L EDTA and 1.1% dextran 70 as described.¹⁴ Basophils, identified by staining with Alcian blue, amounted to 0.5% to 1.5% leukocytes in preparations from different donors, and, in line with earlier evidence, their numbers were relatively constant ($<10\%$ variation) in serial samples from each donor.^{16,17} Duplicate leukocyte aliquots were promptly incubated 45 minutes at 37°C in 25 mmol/L 1,4-piperazinediethanesulfonic acid (PIPES), pH 7.4, 110 mmol/L NaCl, 5 mmol/L KCl, 2.0 mmol/L CaCl_2 , and 0.1% D-glucose, with increasing concentrations of rabbit anti-human IgE myeloma (patient PS; anti-IgE; kindly donated by Dr Donald W. MacGlashan, Jr, The Johns Hopkins University, School of Medicine, Baltimore, MD),

the formyl peptide receptor (FPR) ligand, formyl-methionyl-leucyl-phenylalanine (fMLP), or the Ca^{2+} ionophore, A23187 (both from Merck S.p.A., Milan, Italy). Cell-free supernatants were collected and stored at -20°C for subsequent assay of histamine content using an automated fluorometric technique.¹⁴ Histamine release (HR) was expressed as percent of the total content assessed in parallel samples lysed by addition of 2% HClO_4 , minus the basal, or *spontaneous* release (SR).¹⁴ Individual HR values were the means of duplicate determinations, replicates differing from each other by $<5\%$. Log concentration-response curves of HR induced by anti-IgE, fMLP, and A23187 were fitted to a 4-parameter regression in Prism v7.00 (GraphPad, La Jolla, CA). Basophil reactivity, *that is*, the maximal percent HR (HR_{MAX}), threshold sensitivity (HR_{SENS}), *that is*, 100x the inverse of the secretagogue concentration inducing half-maximal HR (EC_{50}), and the areas under the concentration-response HR curves (HR_{AUC}), were calculated in Prism as described.^{14,18-20}

2.3 | Statistical analysis

The means $\pm$ SEM of values collected at each time point are shown. Discrete time-of-day- or disease-related differences were analyzed by conventional nonparametric statistics in Prism v7.00. The significance of intra-individual differences at 12-hour intervals was analyzed by the Wilcoxon signed-rank test, and differences between groups were analyzed using the Mann-Whitney *U* test. Time series were evaluated for 24-hour variability using the Friedman one-way ANOVA, corrected for multiple comparisons by the Dunn's *post hoc* test. Circadian rhythmicity was analyzed by the cosinor method, which involves fitting chronograms to a cosine function using the least squares procedure.^{21,22} Rhythm parameters were determined with the packages cosinor v1.1/shiny v1.3.1 and CATkit v3.3.3 in R v3.5.0 (The R Foundation, Vienna, Austria). These included the percent rhythm (PR), *that is*, the percent of the variance accounted for by the fitted cosine model, a function of the regression coefficient (r^2) and measure of rhythm prominence; the Midline Estimating Statistic of Rhythm (MESOR), *that is*, the 24-hour rhythm-adjusted mean of the cosine curve fitted to individual values; the amplitude, *that is*, one half the difference between maximum and minimum values in the 24 hours; and the acrophase, *that is*, the time point at which the highest value for each variable was seen.²¹ Single and population mean acrophase values were further elaborated for circular statistics and graphic representation using the Oriana v4 application (Kovach Computing Services, Anglesey, UK). All values and differences were considered significant at a $P < .05$.

3 | RESULTS

3.1 | Comparisons of HR concentration-response curves at distinct time points

Basophils obtained *ex vivo* from each subject at 4-hour intervals for 24 hours were challenged *in vitro* with increasing concentrations of anti-IgE (10^1 - 10^3 ng/mL), fMLP (10^2 - 10^4 nmol/L) or A23187 (3 x

10^1 – 10^3 ng/mL) to construct comprehensive concentration-response curves at each time point. We initially compared the concentration-response curves of HR induced *in vitro* by anti-IgE, fMLP, and A23187 in basophil preparations collected 12-hour apart from the two groups of subjects to try and relate our findings to analogous earlier work by other investigators.^{6–8} As shown in Figure 1A, overlapping HR values were seen in response to anti-IgE concentrations in basophils from controls at 00:00 vs. 12:00 and 04:00 vs. 16:00, while a trend toward increased HR was seen at all concentrations

at 08:00 vs. 20:00. In contrast, we detected significantly decreased HR at the three highest anti-IgE concentrations at 00:00 vs. 12:00 and markedly, significantly increased HR at all anti-IgE concentrations at 08:00 vs. 20:00 in basophils from asthmatics (Figure 1A). Interestingly, the basophil response to fMLP, a non-IgE-dependent signal mediated by FPR innate receptors not explored in similar studies conducted to date, appeared to follow a distinct pattern (Figure 1B). In particular, significantly increased HR was seen at the uppermost fMLP concentration at 08:00 vs. 20:00 in controls only,

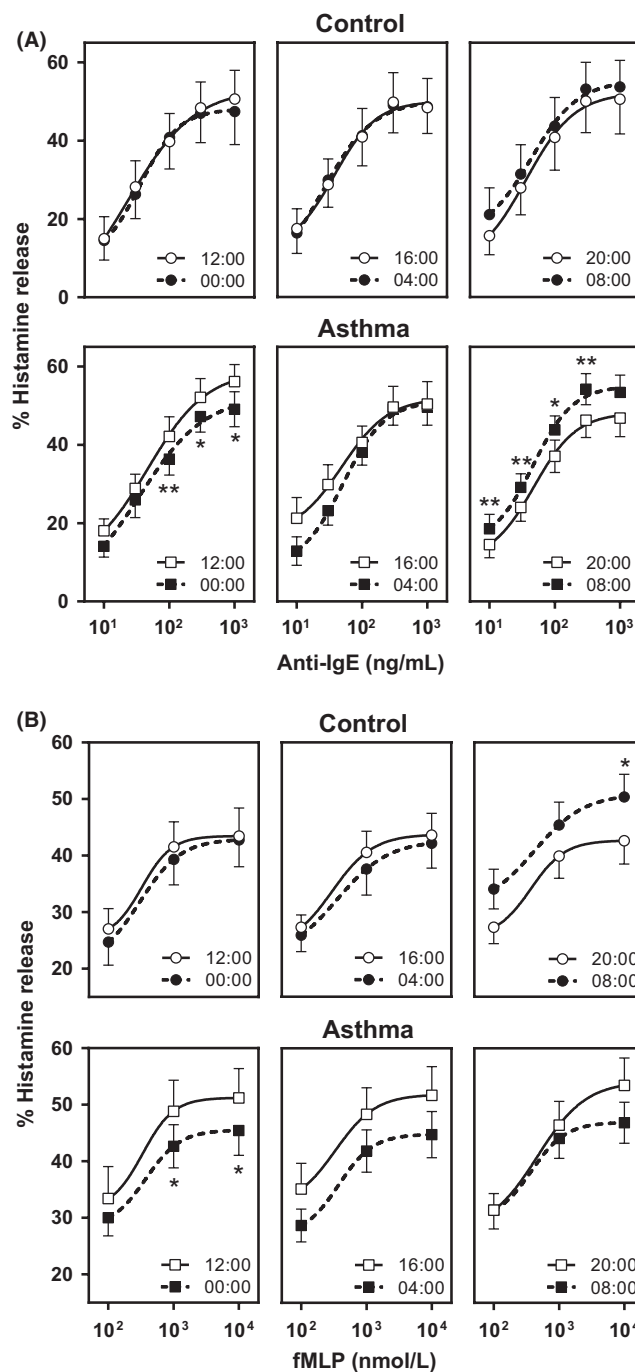


FIGURE 1 Mean (±SEM) HR induced by the indicated concentrations of (A) anti-IgE, (B) fMLP, and (C) A23187 in basophils from 10 nonallergic controls (upper panels) and 10 patients with asthma (lower panels). Significant differences between HR values at the indicated times in response to individual anti-IgE, fMLP, or A23187 concentrations are shown. * $P < .05$ and ** $P < .005$ (Wilcoxon signed-rank test)

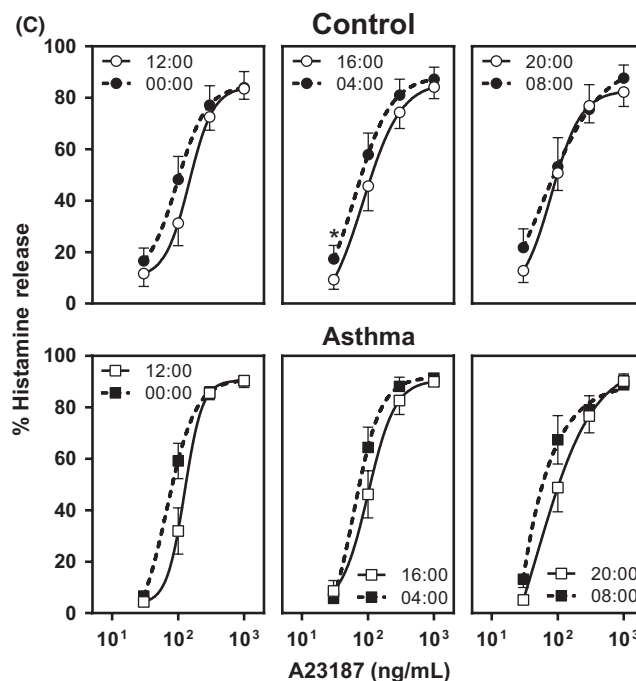


FIGURE 1 (Continued)

while an overall trend toward decreased HR values was apparent in basophils from asthmatics in response to fMLP at 00:00 through 08:00, which reached significance at the two highest fMLP concentrations at 00:00 vs. 12:00 (Figure 1B). Interestingly, no major changes in the basophil response to A23187, a Ca^{2+} -dependent signal bypassing early receptor-associated events,²³ were detected at almost all time points examined in either subject group (Figure 1C).

3.2 | Pairwise comparisons of basophil reactivities and sensitivities at distinct time points

Taken together, these data reveal substantial, significant changes in the response to an IgE-dependent ligand exclusively in asthmatic patients, while significant variability in the FPR-dependent response is detectable in both groups, albeit at distinct time points, and no significant changes are appreciated in either group in the response to a receptor-independent signal. Consistent with these observations, no significant changes were identified at the times tested in all parameters of the response to anti-IgE in basophils from controls (Figure 2A), while basophil reactivity (HR_{MAX}), and the area under the curve (HR_{AUC}) of the basophil response to anti-IgE were significantly higher at 08:00 vs. 20:00 and significantly lower at 00:00 vs. 12:00 in asthmatics (Figure 2B). No significant changes in basophil sensitivity (HR_{SENS}) to anti-IgE were seen in either group (Figure 2). These data are in substantial agreement with earlier work, showing increased HR_{MAX} to anti-IgE or allergen in basophils sampled at 07:00 vs. 19:00 from patients with seasonal rhinitis.⁶ Conversely, we did not confirm earlier findings showing decreased HR_{SENS} to anti-IgE at 04:00 vs. 16:00 in basophils from nonallergic donors,⁷ which were eventually dismissed in a more recent study in

allergic donors from the same group.⁸ As shown in Figure S1, the responsiveness to fMLP was characterized by a distinct disease-related pattern, whereby both the basophil HR_{MAX} and HR_{SENS} were significantly higher at 08:00 vs. 20:00 in controls and the HR_{MAX} lower at 00:00 vs. 12:00 in asthmatics. In agreement with earlier work,⁶ no significant differences were seen at any time in any of the parameters tested in response to A23187 (Figure S2).

3.3 | Overall 24-hour variability of the basophil parameters of response

The findings hitherto described result from pairwise analysis of data collected at discrete time points picked arbitrarily out of a time series, in line with similar strategies from previous work.⁶⁻⁸ This approach, however, relies upon the assumption that most significant differences are detectable at 12-hour intervals and does not sufficiently define the overall variability of these parameters across the 24-hour period. Using a nonparametric ANOVA for repeated measures, we show in Figure 3A that plasma cortisol levels varied significantly over this period in both study groups. Consistent with earlier literature,²⁴ this was mainly accounted for by a steep rise in cortisol levels at 08:00 (the "cortisol awakening response"), which by *post hoc* analysis resulted significantly higher than the levels determined at 16:00 through 00:00 ($P < .0005$). As reported, no statistically or clinically relevant differences were detected between the two groups with the exception of a significant, yet modest difference at 16:00 (Figure 3A).²⁵ In contrast, patients with asthma but not controls exhibited significant PEF variability across the 7 time points examined (Figure 3A). PEF values declined after 16:00 in asthmatics and, relative to this time-of-day or the corresponding values in

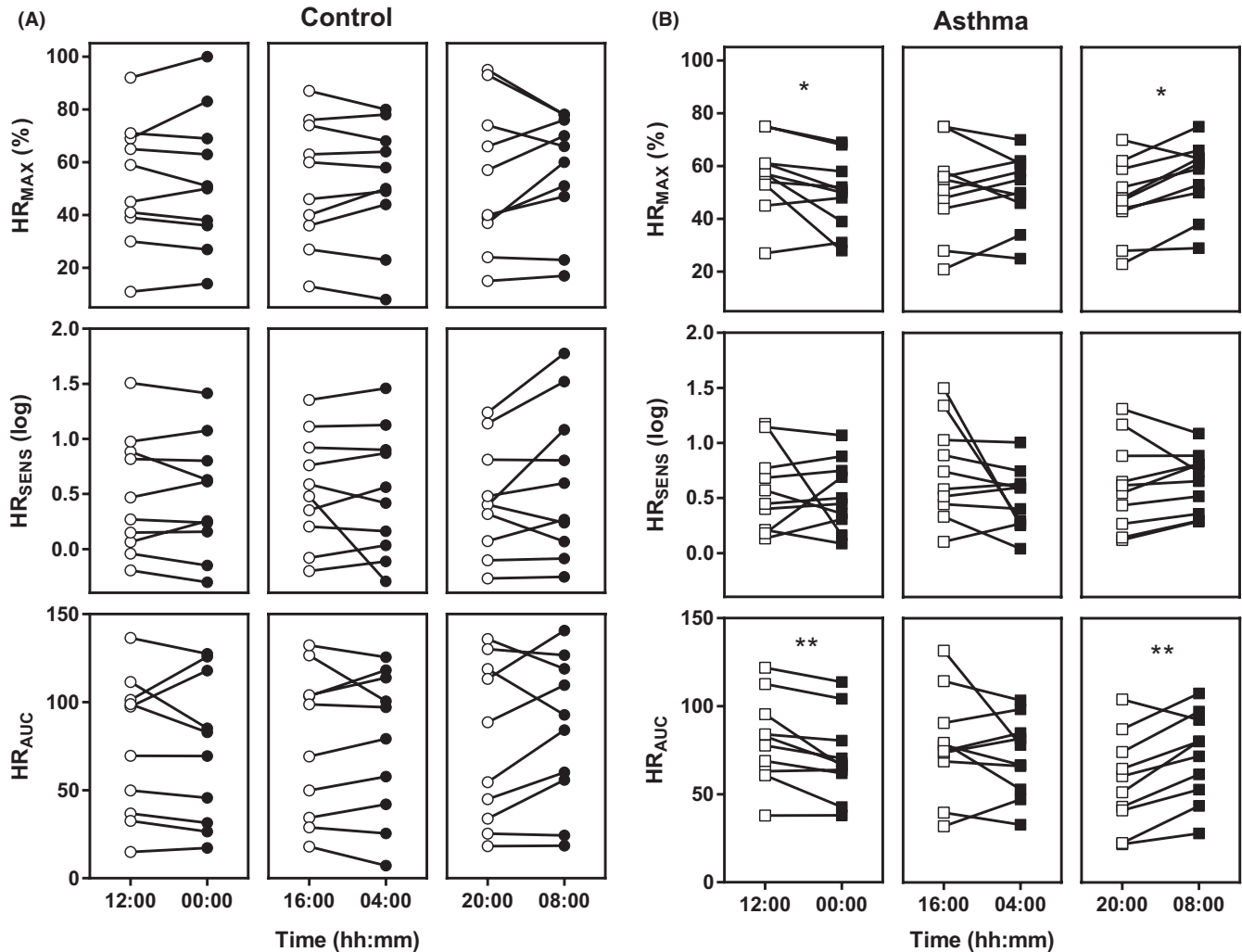


FIGURE 2 Individual reactivities (HR_{MAX}), sensitivities (HR_{SENS}), and areas under the concentration-response curves (HR_{AUC}) in basophils from (A) 10 controls and (B) 10 asthmatics challenged with anti-IgE (10^{-1} - 10^3 ng/mL). Significant differences between the three parameters of response at the indicated times are shown. * $P < .05$ and ** $P < .005$ (Wilcoxon)

controls, were significantly lower at 00:00 and 04:00 (Figure 3A). This is consistent with previous studies showing that, regardless of nocturnal symptoms, lung function declines overnight in patients with respiratory allergy.^{2,3,26} As shown in Figures 3B and S3A, basophils from asthmatics and controls exhibited similarly diverging patterns of diurnal variability in response to anti-IgE. Specifically, basophil HR_{MAX} and HR_{AUC} to anti-IgE varied significantly in asthmatics but not controls, which was mainly accounted for by a significant increase at 08:00 relative to the earlier time points. Conversely, basophil HR_{SENS} to anti-IgE did not vary significantly in either group (Figure S3B). In contrast, basophil HR_{AUC} to fMLP only exhibited significant diurnal variability in controls (Figure 3B), while the other parameters of response to the agonist did not vary significantly in either group (Figure S3). Finally, the overall response to A23187 did not change significantly across the 24 hours in controls or asthmatics (Figure 3B and S3). Likewise, no significant changes were appreciated in parallel determinations of total IgE levels and spontaneous HR in either group (not shown).

3.4 | Determination of the basophil HR response rhythmic parameters

To understand whether the observed diurnal variability resulted from a rhythmic pattern, we tested the 24-hour chronograms of all data for cosine curve fitting.^{21,22} As shown in Figure 4A, cortisol levels in both subject groups underwent a consistent circadian pattern, characterized by significant PR values, significant amplitudes, and similar acrophases ($08:22 \pm 00:10$ and $07:23 \pm 00:13$, respectively). In contrast, the 24-hour PEF variation exhibited significant rhythmicity only in asthmatics, in whom we detected a significant PR, comparable to that of cortisol, a significantly lower MESOR relative to controls, significantly higher amplitude relative to controls or zero-amplitude state, and acrophase at $14:16 \pm 00:10$ (Figure 4A). Likewise, basophil HR_{MAX} and HR_{AUC} in response to anti-IgE exhibited a significantly higher amplitude and a PR almost as prominent as that of cortisol or PEF in asthmatics but not controls (Figure 4B). Acrophases for both parameters were similarly clustered at $09:42 \pm 00:13$ and

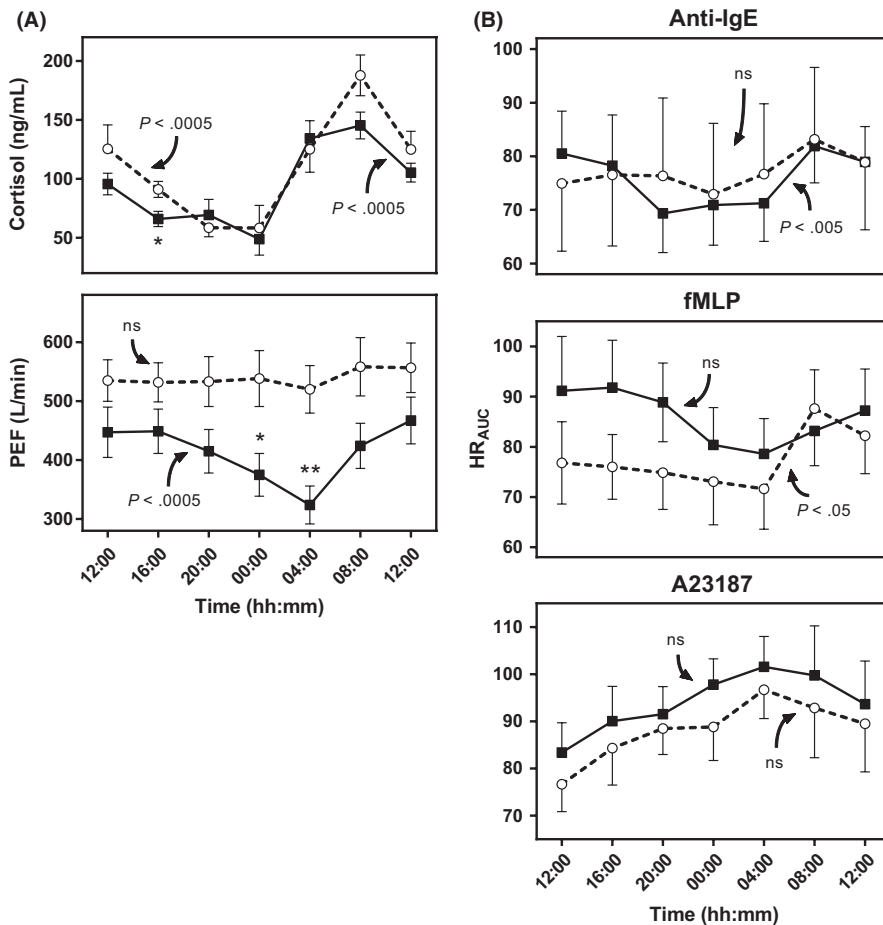


FIGURE 3 (A) Mean ($\pm$ SEM) plasma cortisol and PEF values, and (B) basophil HR_{AUC} in response to anti-IgE, fMLP, or A23187 in 10 controls (open circles) and 10 asthmatics (closed squares) at the indicated times. Significant 24-h variability of each parameter is shown (Friedman's ANOVA for repeated measures). * $P < .05$ and ** $P < .005$ relative to controls (Mann-Whitney U test)

10:58 $\pm$ 00:15, respectively (Figure 4B). Conversely, basophil HR_{SENS} to anti-IgE did not display a significant rhythmic pattern in controls or asthmatics, its variation across time exhibiting a similar amplitude in the two groups, but significantly diverging acrophases (Figure 4B). As shown in Figure S4, the basophil response to fMLP or A23187 was not associated with significant overall rhythmicity, with the exception of the HR_{MAX} to fMLP in asthmatics and the HR_{SENS} to A23187 in controls. Missing a clear connection with the observed 24-hour variability for these parameters (Figure 3 and S3), the overall significance of these findings is questionable. Taken together, these findings show that only the response to anti-IgE displays a consistent, disease-related level of circadian variability, and rhythmicity comparable to more established parameters.

4 | DISCUSSION

The present study is to our knowledge the first to systematically compare time-of-day-related basophil responses to IgE- or non-IgE-dependent agonists in allergic vs. nonallergic subjects. In fact, similar earlier studies have focused on the *in vitro* response to allergen in basophils obtained from allergic patients^{6,8} or the response to anti-IgE in nonallergic subjects.⁷ Furthermore, earlier work has looked for differences in basophil responsiveness at specific time points, set arbitrarily 12-hour apart, rather than analyzing its overall variability

across the 24-hour time series. This approach presents both methodological and conceptual limitations. In fact, we show here that maximal or almost maximal changes in the variables tested may occur at time intervals as short as 4 hours (Figure 3) and that, conversely, differences in parameters tested in pairwise fashion, for example, the HR_{MAX} or HR_{SENS} to fMLP, are no longer significant when looked at in the context of 24-hour time series (compare Figures S2 and S3). On the other hand, a more inclusive approach is exploitable in statistical models aimed at defining the rhythmic nature and robustness of the observed changes where seen.^{21,22}

Our results are in agreement with the view that basophil *releasability*, as inferred in earlier studies looking at this and other parameters of allergic inflammation,^{3,5-8} may be governed by a circadian clock. The study design described here, integrating conventional statistics and chronobiological models, has allowed us to determine that a basophil clock may be subject to disease- and signal-related variables. In fact, we have identified significant differences in the 24-hour patterns of the basophil response to IgE- or non-IgE-mediated signals, which are in turn modified significantly by the disease status. Consistent with earlier work in allergic rhinitis patients,⁶ a clear-cut rise in IgE-dependent basophil reactivity, but not sensitivity, is observed selectively in asthmatic patients at 00:00 through 08:00, concomitant to declining lung function and increased susceptibility to nocturnal/early morning exacerbations. Threshold sensitivity and maximal activation, or reactivity, are independent parameters of the basophil response to

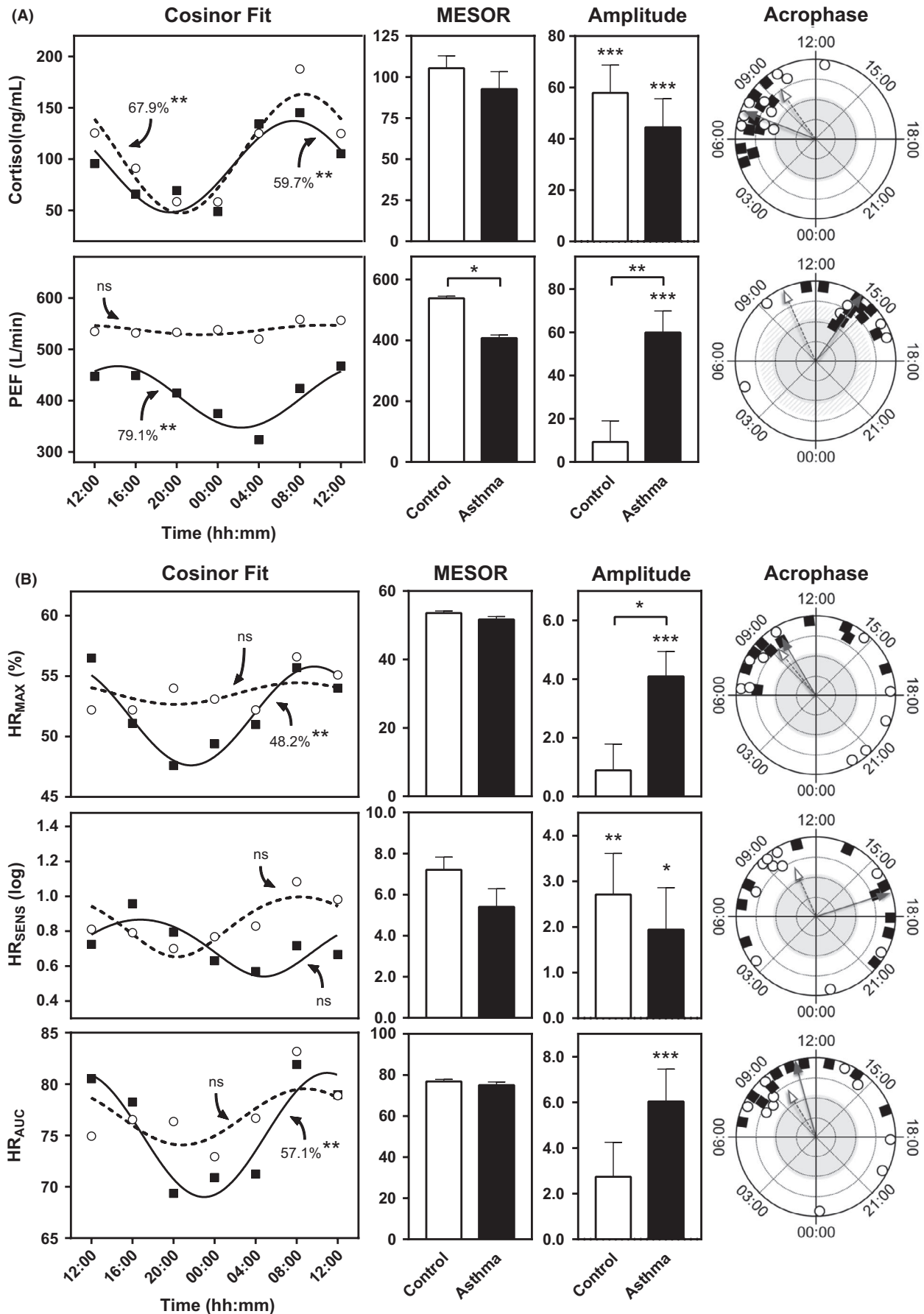


FIGURE 4 Best-fit 24-h chronograms and their MESOR, amplitude, and acrophase, of (A) plasma cortisol and PEF and (B) the basophil response to anti-IgE in 10 controls (open symbols and bars) and 10 asthmatics (closed symbols and bars). Shown are, respectively, significant PR values, significant amplitudes, and significant differences in MESOR and amplitude values between groups (see text). Shown in the circular plots to the right are the individual acrophase values and the associated group vectors in 10 controls (open symbols and arrows) and 10 asthmatics (closed symbols and arrows). The shaded circles inside the plots represent the significance threshold (Rayleigh critical value = .05). * $P < .05$, ** $P < .005$, or *** $P < .0005$ relative to controls or zero amplitude as indicated (F test)

IgE-mediated agonists and are possibly governed by distinct signaling pathways.²⁷ As we show here, only the latter, and in asthmatics only, undergoes rhythmic changes of comparable robustness and amplitude to parallel determinations of plasma cortisol and PEF rates. It can be hypothesized that basophil reactivity to anti-IgE is more exquisitely sensitive to concurrent variations in cortisol levels, and more so in asthma than in nonallergic subjects. However, basophils from asthma, especially severe asthma, have been shown to be less responsive to *in vitro* or *in vivo* glucocorticoid administration.^{28,29} In fact, echoing earlier studies of pulmonary function,²⁶ we failed to detect a significant correlation between the observed changes in PEF or basophil reactivity and time-matched or up to 12-hour earlier changes in cortisol levels (not shown). Moreover, both variables appeared not to change at all in parallel 24-hour determinations in nonallergic controls, in the face of circadian changes in cortisol levels of similar amplitude and acrophase (Figure 4). Of note, a recent study examining blood eosinophil numbers, a glucocorticoid-dependent variable, showed superimposing, consistent rhythmic patterns in asthmatic and nonasthmatic subjects.³ In contrast, the numbers of circulating basophils do not appear to undergo significant diurnal variations in different study groups.¹⁷ Together, these observations suggest that, unlike eosinophils, basophils appear to be controlled by a glucocorticoid-independent, disease-dependent, possibly intrinsic clock. Comprehensive gene expression studies in isolated basophils challenged at selected time points will be necessary to at least preliminarily define the factors involved in the circadian control of their effector phenotype in health and disease.

In substantial support of this view, the basophil response to the FPR ligand, fMLP, which, differently from IgE-dependent stimulation, is not affected by glucocorticoids *in vitro*,³⁰ does not appear to follow a consistent circadian pattern in asthmatics or controls (Figure S4). FPR ligands, including specific components of the local microbial communities, have been involved in the control of mucosal inflammation (reviewed in³¹). Engagement of these innate receptors may play a protective role in animal models of antigen-induced airway inflammation.³² We show here that the basophil response to fMLP, a variable not previously tested in similar studies, is significantly more pronounced at 08:00 relative to other times of day in nonasthmatic subjects, while a somewhat opposite trend is detectable in asthma (Figures S1, S3 and S4). While intriguing in light of the above considerations, this finding cannot be fully explained in models of cosine-based rhythmometry presumably due to excessive variability in the sample examined. Another possible limitation in our study, making use of unfractionated leukocyte preparations, does not allow us to exclude that time- and disease-related changes in trafficking and activation patterns in other FPR-bearing cells, for example,

eosinophils and neutrophils, as well as the relative FPR density on these cells, may contribute to explaining the observed differences between asthmatics and controls. Earlier studies have documented a comparable rhythmic pattern in the expression of clock genes and activation markers in purified, fMLP-stimulated eosinophil preparations from allergic and nonallergic donors.⁴ Analogous studies in homogeneous basophil preparations might help elucidate these points.

In recent years, the *in vitro* measurement of histamine concentrations in basophil supernatants has been increasingly replaced by more convenient, less costly flow cytometric protocols for determination of the basophil responses to allergens or other ligands.^{33,34} This has enabled the adoption of this *in vitro* protocol, commonly referred to as *basophil activation test* (BAT), as a second-line diagnostic tool and a promising replacement for *in vivo* challenge in the workup of allergic patients.^{18,20} While the potential clinical and diagnostic values of HR and flow cytometric determinations are comparable, it cannot be excluded that their endpoints, possibly representing different facets of the same biologic process, may be controlled at least in part by distinct signals.³⁴⁻³⁶ It can be argued that the reported differences in basophil responsiveness in these *in vitro* procedures might partly result from differential manipulation, as flow cytometric determinations are typically conducted in freshly collected, whole blood samples as opposed to enriched leukocyte preparations. While more research along this line will help elucidate these points, our findings are not at substantial variance with earlier work in unfractionated blood cells.⁶ In any instance, the appreciation of a distinctive rhythmic pattern of IgE-dependent basophil activation in allergic patients should possibly be taken into account in the setup of diagnostic BAT protocols, as time-of-day-related fluctuations might be of sufficient amplitude to affect their interpretation. On the other hand, it is of interest that PEF levels, an established parameter of respiratory function closely related to clinical symptoms, undergo fluctuations of similar amplitude and acrophase (Figure 4). The existence of biologically and clinically meaningful circadian changes in basophil multifaceted functions would pinpoint the possible contribution of these cells to the diurnal variations of symptoms and respiratory function in patients with asthma and related conditions, further stressing the critical role of these cells in allergic inflammation. It is hoped that future work in this direction will help identify new targets in the chronotherapy of asthma and other immune conditions.³⁷

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors were involved in the study. GM and VC designed the study. GS, CS, and VC developed the methodology. GS characterized and recruited the study subjects. GS, CS, and VC collected the data. GG and VC analyzed the data. VC prepared the manuscript. GG, CS, and GM reviewed the manuscript. All of the authors approved the final version for submission.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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(A)

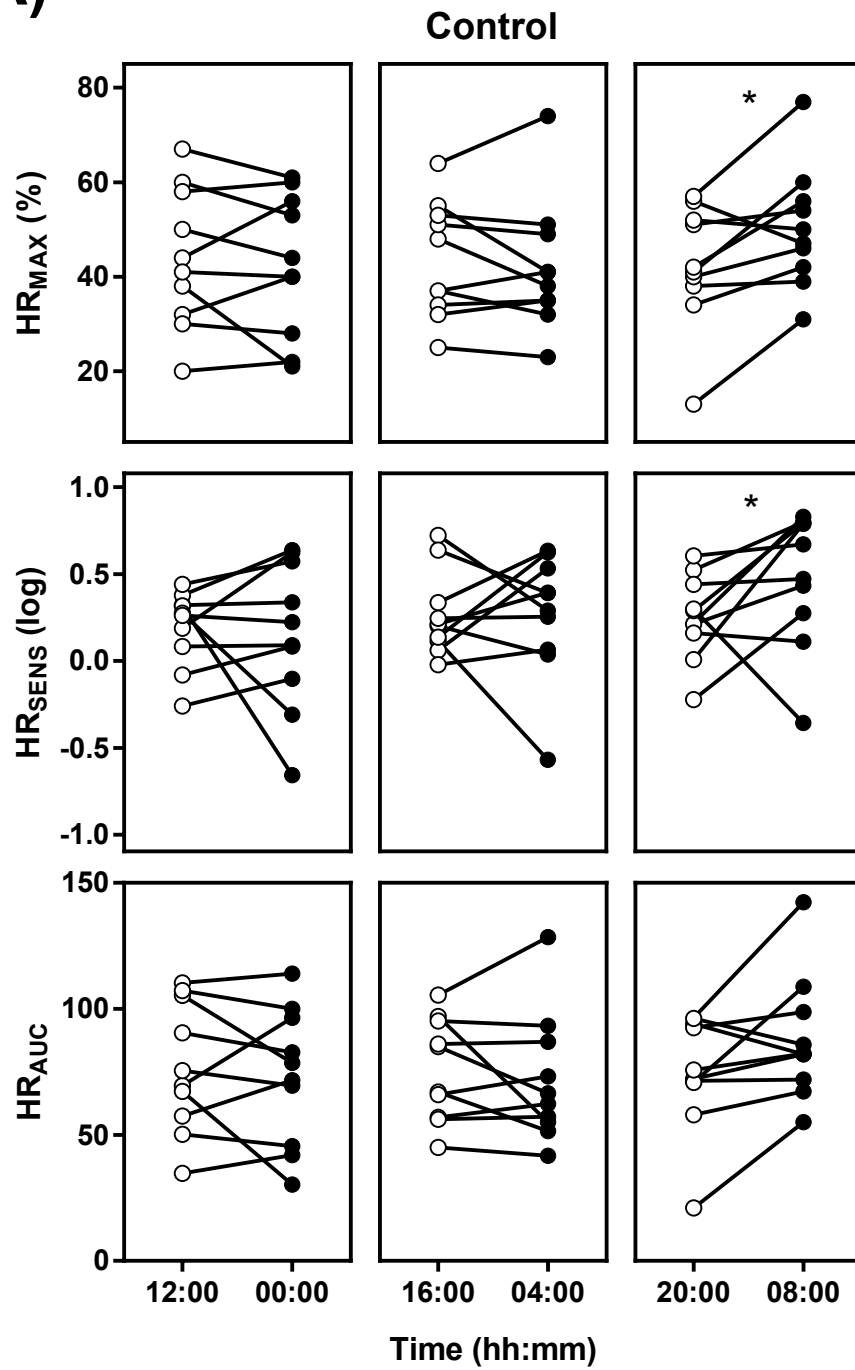


FIGURE S1

(B)

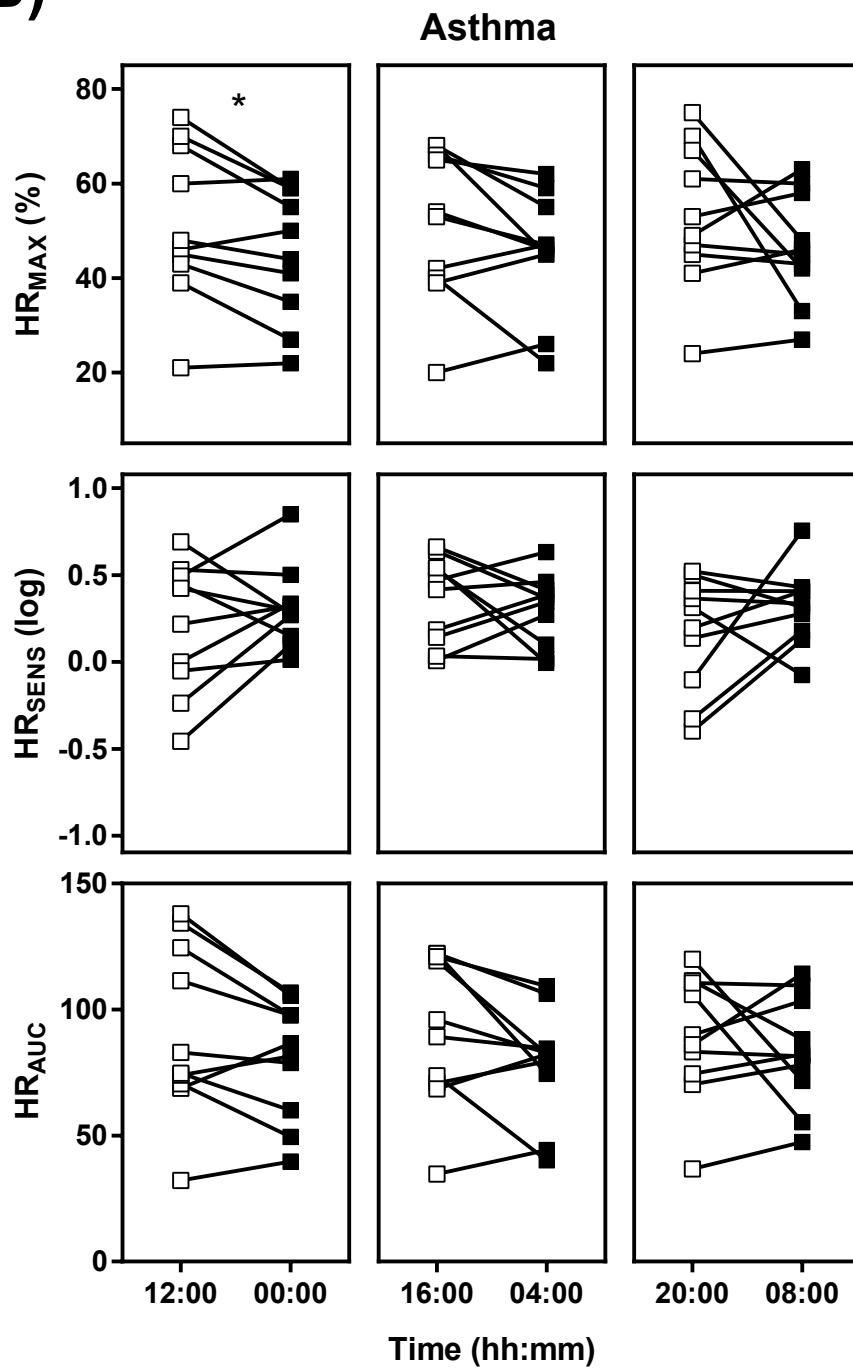


FIGURE S1

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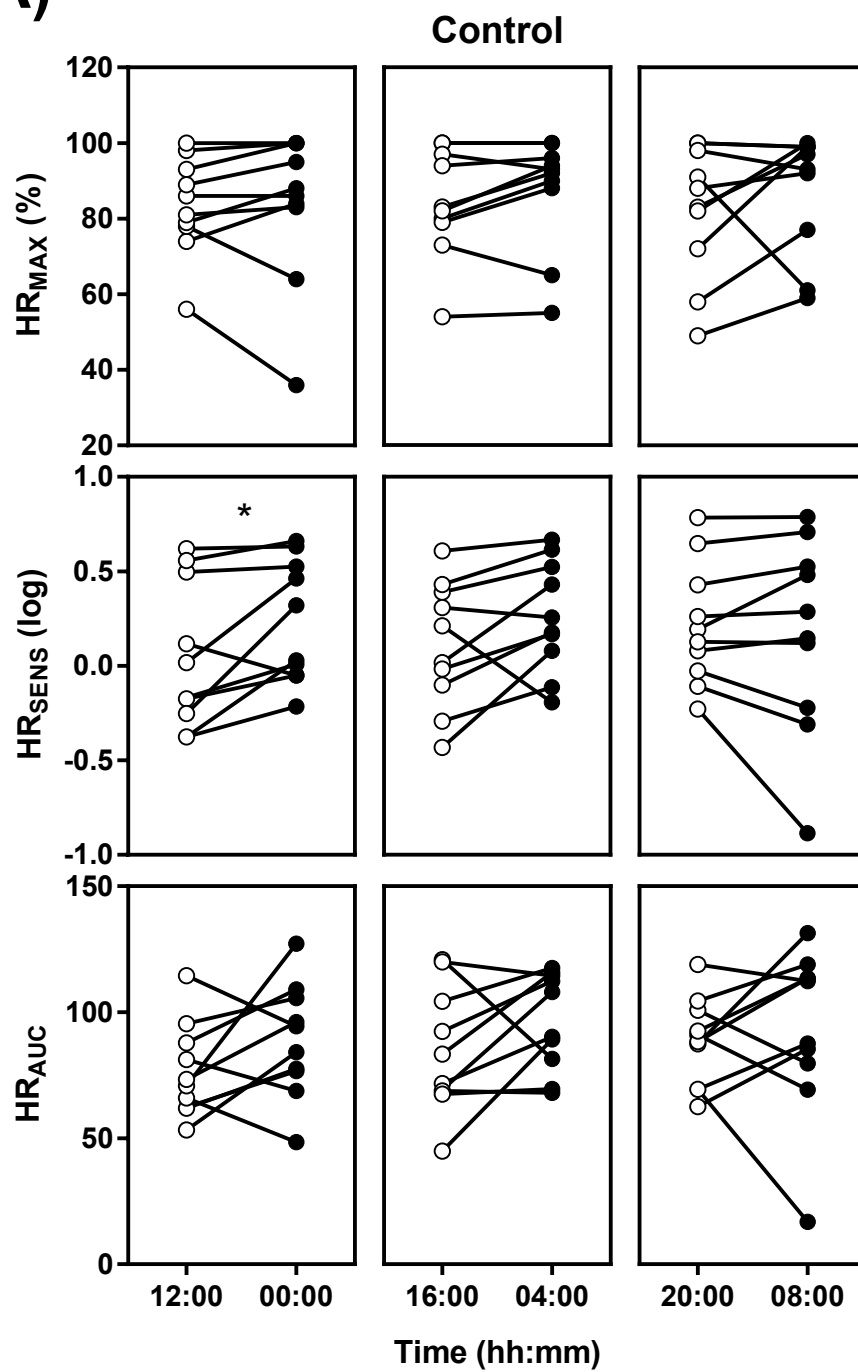


FIGURE S2

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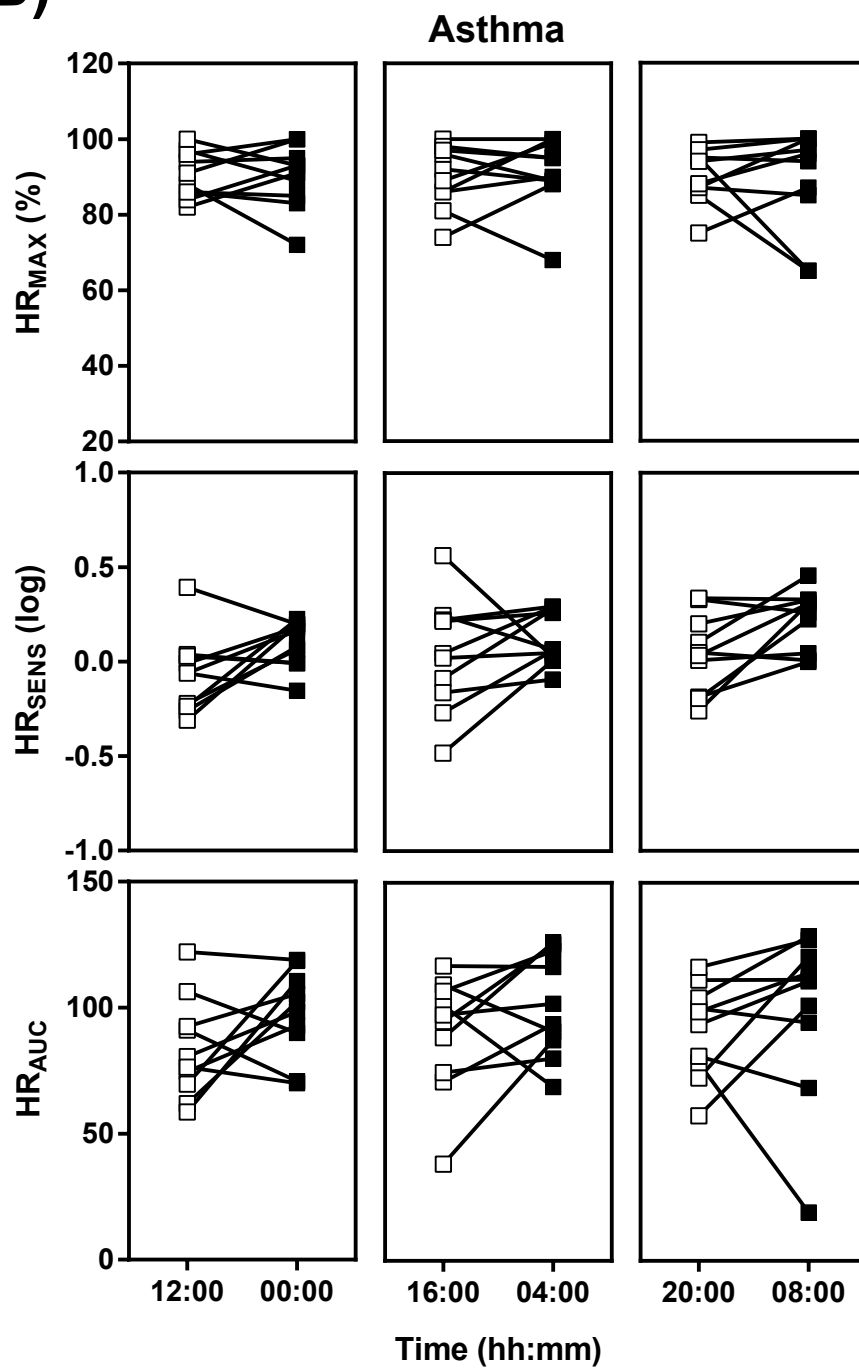


FIGURE S2

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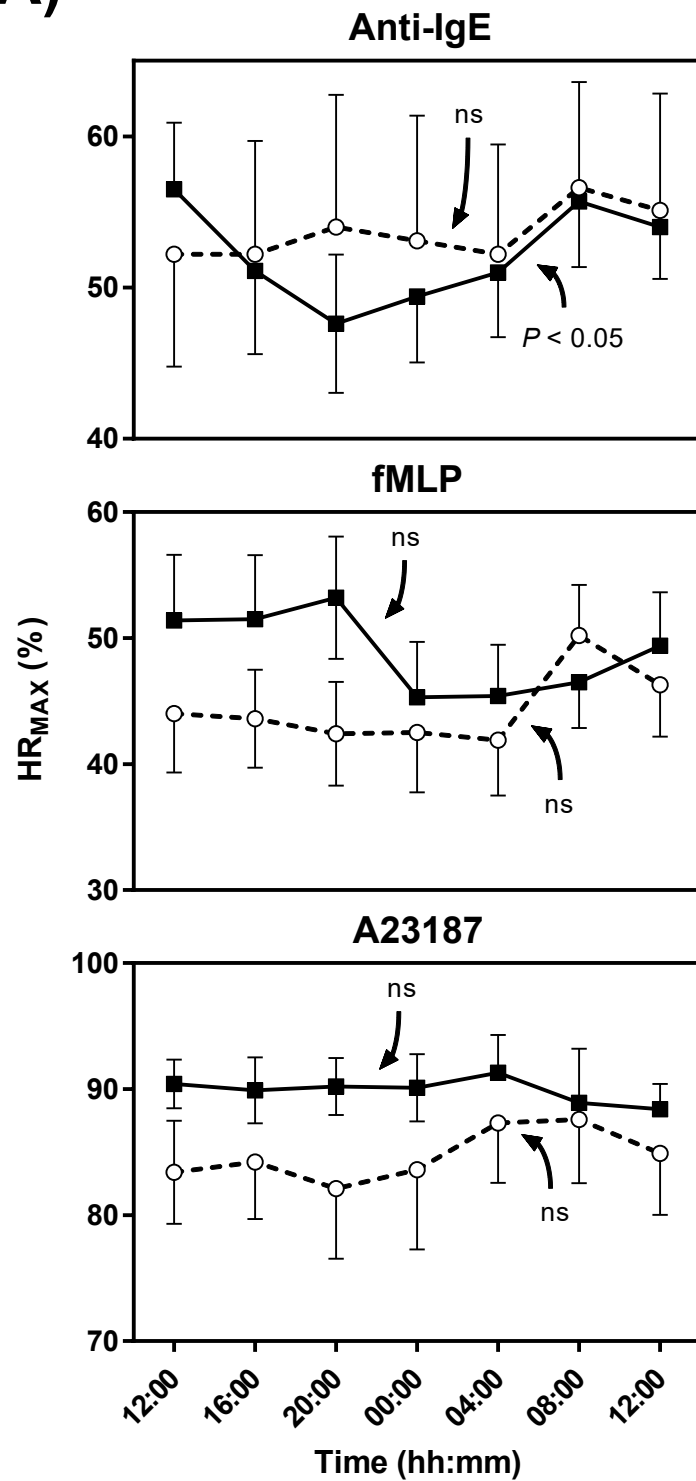


FIGURE S3

(B)

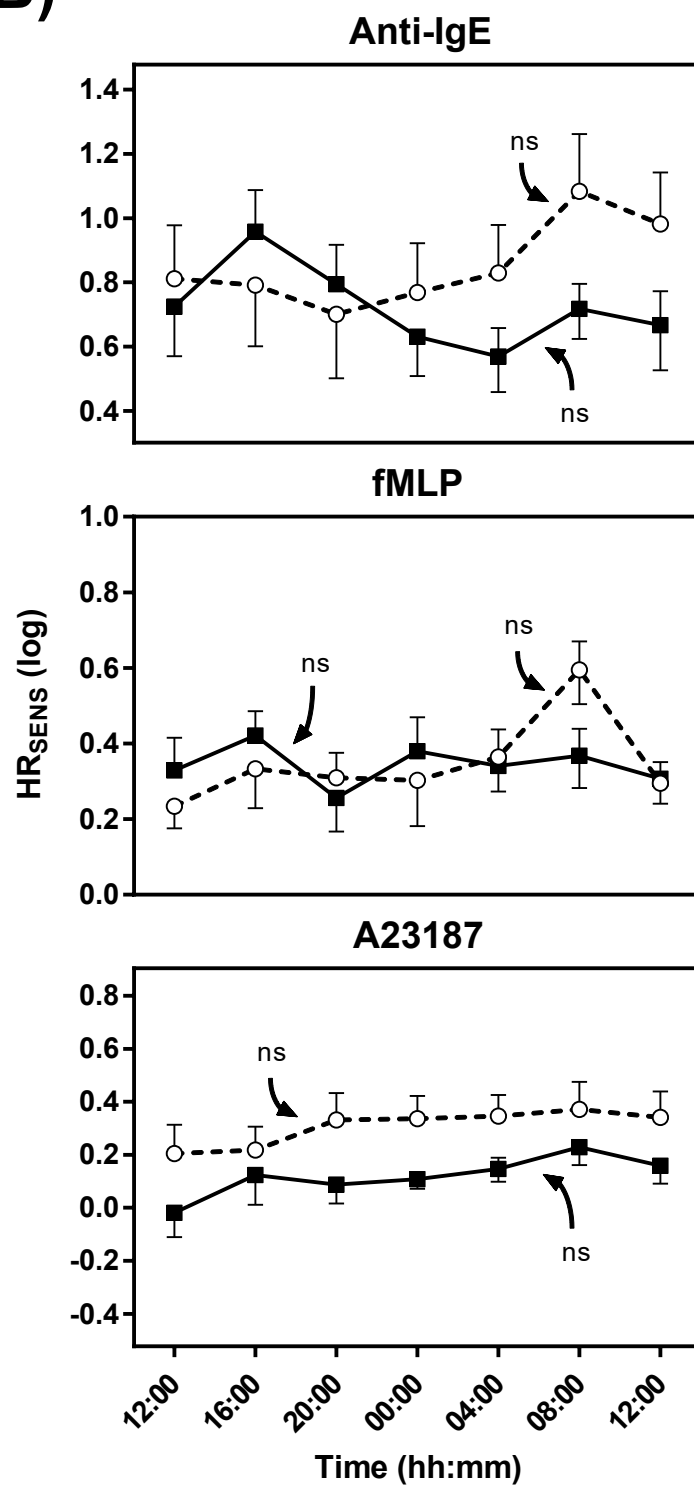


FIGURE S3

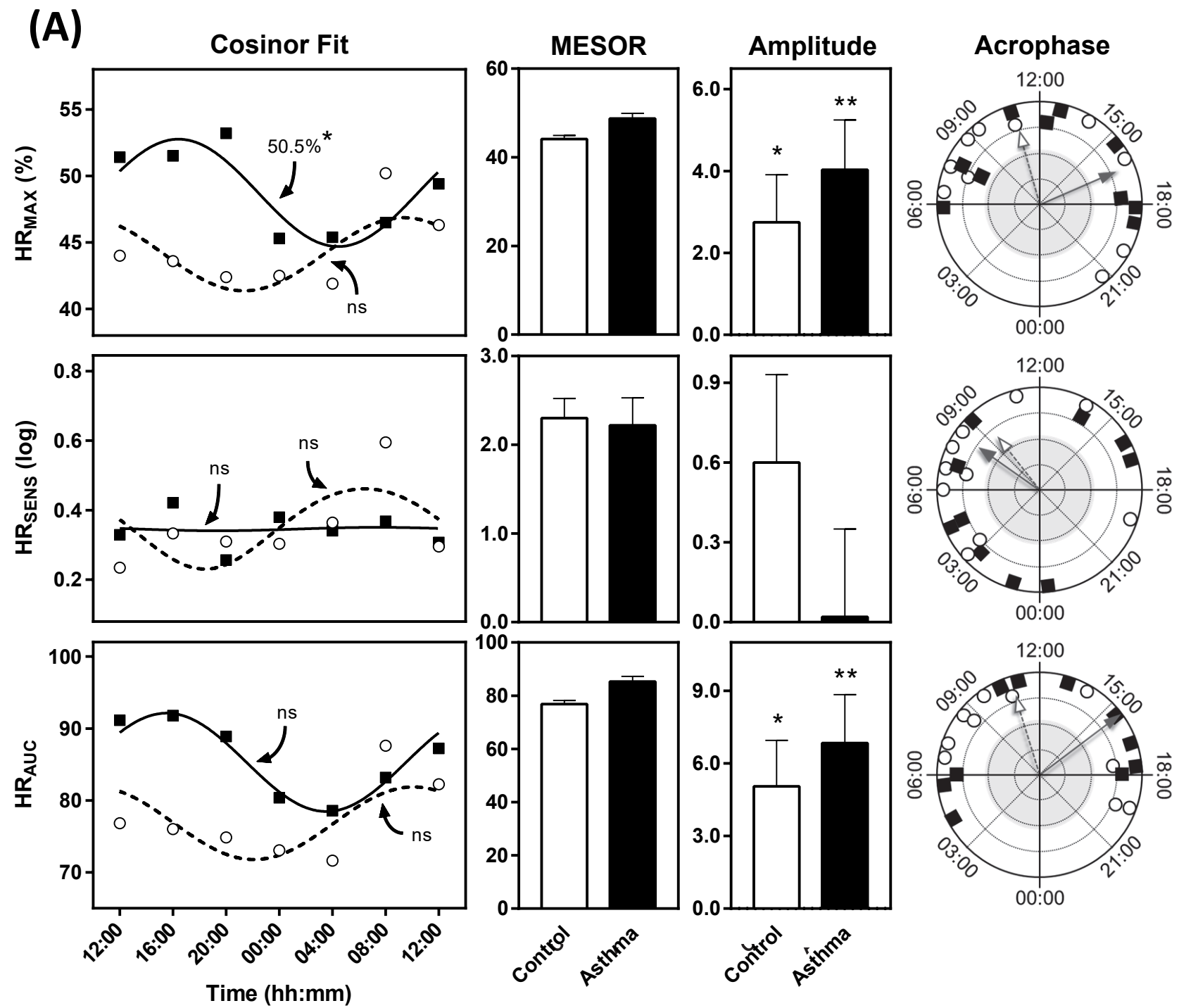


FIGURE S4

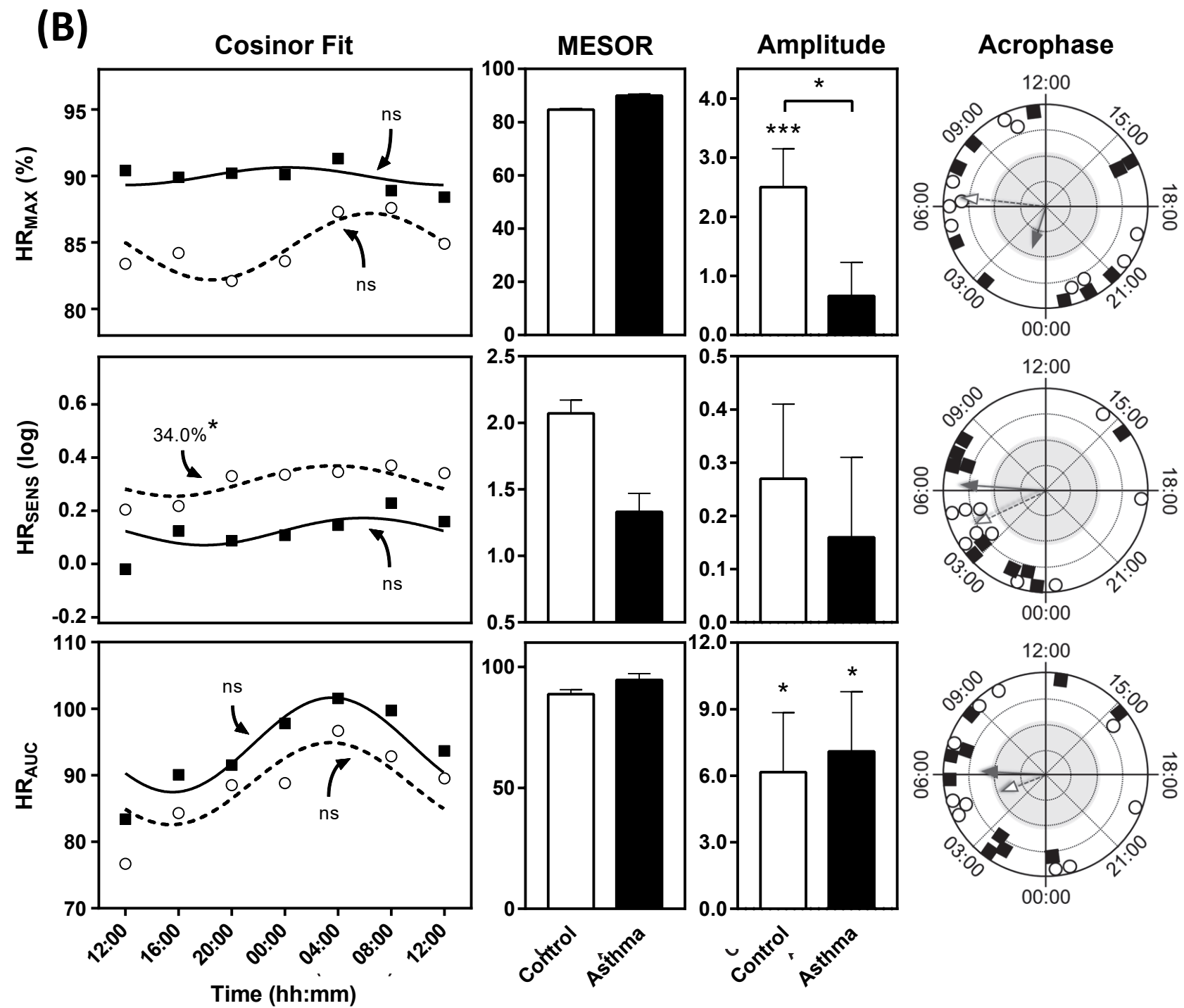


FIGURE S4

SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1 Individual reactivities (HR_{MAX}), sensitivities (HR_{SENS}), and areas under the concentration-response curves (HR_{AUC}) in basophils from (A) 10 controls and (B) 10 asthmatics challenged with fMLP (10^2 - 10^4 nmol/L). Significant differences between the three parameters of response at the indicated times are shown. * $P < 0.05$ (Wilcoxon)

FIGURE S2 Individual reactivities (HR_{MAX}), sensitivities (HR_{SENS}), and areas under the concentration-response curves (HR_{AUC}) in basophils from (A) 10 controls and (B) 10 asthmatics challenged with A23187 (3×10^1 - 10^3 ng/mL). Significant differences between the three parameters of response at the indicated times are shown. * $P < 0.05$ (Wilcoxon)

FIGURE S3 Mean ($\pm$ SEM) basophil (A) HR_{MAX} and (B) HR_{SENS} in response to anti-IgE, fMLP, or A23187 in controls (open circles) and asthmatics (closed squares) at the indicated times. Significant 24-hour variability of each parameter is shown (Friedman's ANOVA)

FIGURE S4 Best-fit 24-hour chronograms and their MESOR, amplitude, and acrophase, of the basophil response to (A) fMLP and (B) A23187 in 10 controls (open symbols and bars) and 10 asthmatics (closed symbols and bars). Shown are, respectively, significant percent rhythm (PR) values, significant amplitudes, and significant differences in MESOR and amplitude values between groups (see text). Shown in the circular plots to the right are the individual acrophase values and the associated group vectors in 10 controls (open symbols and arrows) and 10

asthmatics (closed symbols and arrows). The shaded circles inside the plots represent the significance threshold (Rayleigh critical value = 0.05). * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$