Altered circadian gene expression in primary human airway epithelial cells in asthma

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To the Editor:

Cellular circadian rhythms regulate multiple processes and have been hypothesised to influence epithelial function in asthma [1]. Clinical studies demonstrate increased risk for asthma exacerbations, asthma-related deaths and asthma symptoms at night, supporting the hypothesis that asthma is circadian regulated [1–3]. Airway resistance has circadian variability in patients with asthma and single timepoint measurements of adults with severe asthma demonstrate differences in gene expression of core circadian genes in blood [4, 5]. *PERIOD3* has increased rhythmicity in blood from patients with asthma, coinciding with altered rhythms of serum ceramides in asthma [6]. We utilised primary airway epithelial cells (AECs) differentiated at an air–liquid interface (ALI) as an *ex vivo* model for molecular characterisation [7, 8]. The airway epithelium is a critical mediator of inflammation in asthma and primary AEC cultures are an important pre-clinical model for investigation of biological processes in asthma. AECs cultured at an ALI form an organotypic model that recapitulates the major cell types of human airway epithelia and demonstrate rhythmicity of core circadian genes after temperature synchronisation [7].

AECs from healthy children (n=6, five female/one male; age 7–15 years; no history of allergic rhinitis or atopic dermatitis) and children with physician-diagnosed allergic asthma (n=6, one female/five male; age 9–16 years; all atopic with history of allergic rhinitis or atopic dermatitis) underwent differentiation followed by temperature synchronisation using a temperature cycled incubator as previously described [7]. AECs were obtained from subjects using blind bronchial brushes while under general anaesthesia for elective procedures as previously described [7, 8]. Diagnostic criteria for allergic asthma included a 1-year history of physician-diagnosed asthma, daily use of inhaled corticosteroid or leukotriene receptor antagonist, use of short-acting β-agonist two or more times a month, and the presence of one or more of: positive skin prick test or serum-specific IgE for an aeroallergen, serum total IgE above the normal limit, or a prior physician diagnosis of allergic rhinitis or atopic dermatitis. AECs were obtained under studies #12490 or #1596 approved by the Seattle Children's Hospital Institutional Review Board with investigations conducted following the rules of the Declaration of Helsinki of 1975. Two independent cultures from each donor were harvested for RNA every 4 h over 48 h under constant temperature conditions. RNA was sequenced after poly-A library preparation using 150-bp end-reads at 20–30 million reads per sample by Novogene. Reads were aligned using STAR after adapter trimming to the Ensembl version of the human genome (GRCh38, Ensembl 91) [9]. Quality of reads and alignment were assessed using PICARD (version 1.134), FASTQC (version 0.11.3) and Samtools (version 1.2). Protein-coding genes were filtered for expressed genes (>0.1 counts per million in ⩾40 samples) for analysis. Counts were normalised using the trimmed mean of M and transformed to $\log_2$ counts per million mapped reads with observation-level weights by using *voomWithQualityWeights*) [10]. CompareRhythms using the cosinor function was used to assess rhythmicity with a false-discovery rate (FDR) adjusted p-value of 0.05, minimum amplitude 0.2, period length of 24 and differential rhythmicity FDR cutoff of 0.2. CircaPower sample size calculation estimated that 144 samples across evenly spaced timepoints using an intrinsic effect size of 0.4 at an α of 0.05 would provide 86% power to detect rhythmicity with intrinsic effect size estimated by publicly available human *post mortem* data. Our study design provided 24 samples across evenly spaced timepoints, from six human donors per group based on consensus recommendations [11, 12]. Normalised counts and CompareRhythms classification output are available at https://doi.org/10.5061/dryad.1jwstqk2g [13]. CompareRhythms classification of same, change, gain or loss was assessed using EnrichR with background set genes defined as expressed, protein-coding genes [14]. Protein from

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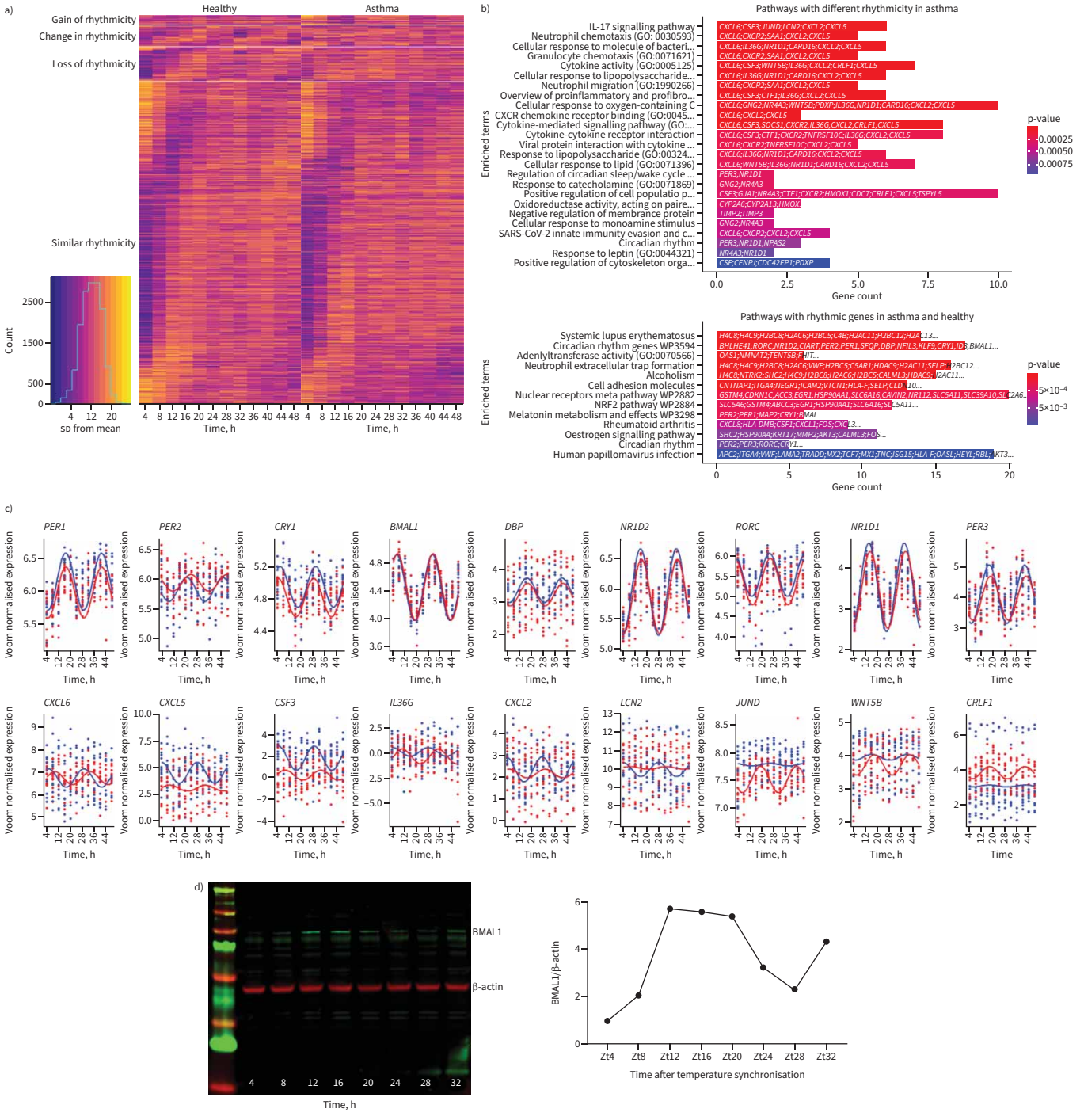

FIGURE 1 a) Heatmap of gene expression in airway epithelial cells (AECs) from healthy (left) and asthma (right) donors for 110 genes with differential rhythmicity (top) and 536 genes with similar rhythmicity (bottom) in asthma and healthy donors ranked by phase. n=6 healthy and n=6 asthma with two biological replicates per donor line. Time 0 h corresponded to final media change and return to constant temperature conditions. b) EnrichR analysis of 536 rhythmic genes in AECs from healthy and asthma donors (top) and 110 genes with altered rhythmicity in AECs from asthma as compared to healthy donors (bottom). c) Individual voom-normalised gene expression in counts per million of circadian rhythm genes, cytokine signaling pathway genes, and neutrophil chemotaxis pathway genes from healthy (red) and asthma (blue) donors with line fit parameters from CompareRhythms. d) BMAL1 western blot in a healthy donor AECs showing BMAL1 protein level at Zt4–Zt32 h after temperature synchronisation with total fluorescence quantification normalised to β-actin and to the Zt4-h timepoint. Time is presented in hours after return to constant temperature. IL: interleukin.

cell lysate was extracted using RIPA buffer and western blot performed using anti-BMAL1 antibody (Abcam ab93806) and anti-β-actin for loading control (Invitrogen 15G5A11/E2), with secondary detection with IRDye 800CW goat anti-rabbit and IRDye 680RD donkey anti-mouse.

Analysis of core circadian genes demonstrated rhythmic expression in *PER1*, *PER2*, *PER3*, *CRY1*, *BMAL1*, *NR1D2*, *NR1D1* and *DBP*. Comparing rhythmic expression between AEC cultures from healthy and asthma donors revealed 36 genes that had a change in rhythmicity, 16 genes that gained rhythmicity in asthma and 58 genes that lost rhythmicity, whereas 536 genes had no significant differences in rhythmicity between healthy and asthma donors (figure 1a). Larger amplitude differences were more apparent in the first 24 h following temperature cycling with decay in the second 24 h under constant conditions consistent with a prior study in mice AEC-ALIs [15].

Genes with rhythmicity in both asthma and healthy AEC cultures were enriched for circadian rhythm genes, nuclear receptor genes and the neutrophil extracellular trap formation pathway. Due to the small number of genes with gain or loss of rhythmicity, gene set analysis was performed on the 110 genes with differential rhythmicity in asthma. Genes with differential rhythmicity in asthma (combined set of gain, loss or altered amplitude or phase) were enriched for genes related to interleukin (IL)-17 signalling, neutrophil chemotaxis, cytokine mediated signalling and receptor interaction, and viral protein interactions with cytokine receptors (figure 1b). Genes identified as rhythmic in the IL-17 signalling pathway were *CXCL6*, *CSF3*, *JUND*, *LCN2*, *CXCL2* and *CXCL5*, with *CXCL2*, *CXCR2*, *CXCL5*, *CXCL6*, *CRLF1* and *IL36G* in the cytokine-mediated signalling pathway, and CXCR chemokine receptor binding gene ontologies. *CXCL2* gained rhythmicity and C*XCL5*, *LCN2* and *CSF4* had altered rhythmic expression in AECs from asthma donors as compared to healthy. In contrast, *JUND*, *WNT5B*, *LCN2* and *CRLF1* lost rhythmicity in AECs from asthma donors as compared to healthy (figure 1c). By western blotting, BMAL1 also had 24-h rhythmic levels in AECs (figure 1d).

We found that a majority of circadian clock genes had similar rhythms of expression in healthy and asthma airway epithelium, but that circadian genes *PER3* and *NR1D1* had altered rhythmicity due to a gain in amplitude in asthma as compared to healthy. Our finding that *PER3* had altered rhythmicity in AEC cultures from children with asthma parallels a finding in blood sampled over the day by Krakowiak *et al* [6] that *PER3* had increased amplitude of rhythmicity of expression in patients with asthma. Such a change in amplitude of expression in asthma could explain the findings in single-timepoint studies of decreased circadian gene expression and highlights the need for serial sampling when assaying circadian gene expression [4, 16]. Our finding of altered *PER3* amplitude in an organotypic epithelial cell model independent of a systemic or immune cell-mediated signalling suggests that alterations of circadian rhythms of gene expression are intrinsic to the airway epithelium. Our finding that *CXCL5* expression has increased rhythmicity in asthma may be due to alteration of clock gene outputs, as in mice, *Cxcl5* expression increases with loss of *Bmal1* in bronchiole airways, indicating that *CXCL5* is regulated by the core circadian gene *BMAL1* [17]. The loss of *Bmal1* also led to a steroid-independent regulation of the *Cxcl5* locus with gain of rhythmicity in *Cxcl5*, which is hypothesised to explain time-of-day therapeutic effects on steroid administration [17]. Future work is needed to elucidate what genetic and epigenetic changes mediate the changes in clock output genes found here, and to investigate protein and post-translational modification rhythmicity. The impact of allergen exposure and specific cytokines within the airway microenvironment on rhythmic gene expression in future studies could provide insight into the origin of the rhythmic changes we observe here.

A limitation of this study is the small sample size (six healthy children and six children with asthma) and absence of *in vivo* circadian measures such as chronotype or core body temperature rhythms. Furthermore, our model only assessed rhythmicity in AECs, without accounting for the tissue environment of the lung or other environmental factors, which may also influence rhythms *in vivo*. Future studies will need to investigate whether phenotype and endotype subsets of asthma (*e.g.* type-2 high *versus* type-2 low asthma) exhibit differential rhythmicity, and the impact of medications, to allow for the development of chronotherapeutic strategies targeting a personalised approach to circadian medicine, and to investigate the interplay of epithelial circadian rhythms and rhythms in immune cells, such as Th17 cells. Our study highlights the need to consider time when investigating diseases such as asthma so that, ultimately, therapies can be targeted to the right patient at the right time of day.

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