To gain insight into what biological role the methylation differences might contribute, the methylation data were functionally annotated, using Gene Ontology and the Human Protein Reference Database (www.hprd.org; Figure 2C), with subsequent Ingenuity Pathway Analysis to determine the biological pathways represented in the 75 unique hypermethylated genes and the 202 unique hypomethylated genes. The top pathway represented in the Ingenuity Pathway Analysis of the 75 hypermethylated genes was "cellular growth and proliferation," with 11 molecules represented (BMP7, GRM3, IGFBP7, NPY, RPTOR, MAPK10, HEY1, MGP, PRKCE, MEIS1, and PRDM16; $P < 3 \times 10^{-4}$ to 5×10^{-2}).

In summary, these data provide *ex vivo* evidence that SAE basal cells from COPD smokers, and to a lesser extent SAE basal cells from "healthy" smokers, are limited in their ability to regenerate a fully differentiated epithelium. In addition, the methylation studies show that the DNA methylation profile of SAE basal cells that do not survive to Day 28 on ALI culture is significantly different than the profile of SAE basal cells that are able to successfully differentiate, suggesting that changes in DNA methylation acquired by basal cells of COPD smokers and healthy smokers *in vivo* may be one mechanism for the differences observed. We hypothesize that the lung microenvironment in individuals with COPD provide signals necessary to maintain the "COPD" epigenetic code within SAE basal cells, continuously supporting the disease state by altering the capacity of these stem/progenitor cells to maintain normally differentiated SAE. Therefore, even though the process of DNA methylation is reversible (15), the COPD lung environment might continuously stimulate the COPD DNA methylation profile that may determine abnormal SAE maintenance and regeneration. Thus, it is possible that studies focusing on modulating methylation profiles of SAE basal cells relevant to their capacity to regenerate normally differentiated mucociliary airway epithelium may lead to novel therapeutics to treat or prevent COPD. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Acknowledgment: The authors thank J. Barkas, M. Elnashar, and A. Rogalski for help with the study and N. Mohamed for help in preparing this manuscript.

Michelle R. Staudt, Ph.D.*
Lauren J. Buro-Auriemma, Ph.D.*
Matthew S. Walters, Ph.D.
Jacqueline Salit, M.S.
Thomas Vincent, Ph.D.
Renat Shaykhiev, M.D., Ph.D.
Jason G. Mezey, Ph.D.
Ann E. Tilley, M.D.
Robert J. Kaner, M.D.
Weill Cornell Medical College
New York, New York

Melisa W. Y. Ho, M.D.‡
Hoffmann-La Roche
Nutley, New Jersey

Ronald G. Crystal, M.D.
Weill Cornell Medical College
New York, New York

*These authors contributed equally to this study.
‡Present address: GlaxoSmithKline, King of Prussia, Pennsylvania.

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Early Intermittent Hypoxia Induces Proatherogenic Changes in Aortic Wall Macrophages in a Murine Model of Obstructive Sleep Apnea

To the Editor:

Obstructive sleep apnea (OSA) is a highly prevalent condition throughout the lifespan, affecting 2–10% of the general population at any given age. It is associated with an extensive array of cognitive, behavioral, metabolic, and cardiovascular morbidities

(1). In recent years, OSA has emerged as an independent risk factor for cardiovascular disease (2) and has been causally associated with a high prevalence of hypertension, atrial fibrillation, congestive heart failure, stroke, and more specifically, coronary heart disease (3). OSA is associated with activation of multiple inflammatory pathways, disruption of lipid metabolism, and endothelial dysfunction through oxidative stress mechanisms (4), all of which predispose to atherogenesis. Long-term chronic intermittent hypoxia during sleep (IH), a prototypic constitutive element of OSA, induces atherosclerosis in murine models and plays a critical role in OSA-associated cardiovascular morbidities (5). However, the molecular mechanisms underlying OSA-induced atherosclerosis are not well understood.

Atherosclerosis is currently viewed as a chronic inflammatory process, in which macrophages play a key pathophysiologic role (6). Monocyte recruitment to the circulation, their entrapment in the vascular wall, and their subsequent differentiation into lipid-laden macrophages are fundamental processes involved in atheromatous plaque formation (7, 8). Shifts across the spectrum of heterogeneous macrophage populations encompassing a continuum between major macrophage phenotypes have been described as a fundamental process in atherogenesis. Generally speaking, proinflammatory macrophages are implicated in foam cell generation, lipid loading, plaque formation, and plaque rupture, whereas anti-inflammatory macrophages play an atheroprotective role (9). In addition to changes in macrophage polarity within the vascular wall, macrophage proliferation has also emerged as a critical determining factor in atherogenesis, although the origin of the expanding macrophage population (i.e., tissue-resident vs. bone-marrow derived) is still controversial (10, 11).

We hypothesized that IH during the sleep period will induce shifts toward a proatherogenic state in the spectrum of macrophages within the vascular wall. To examine our hypothesis, we exposed 8-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) to IH with alternating 90-second cycles (21% $F_{I_{O_2}}$ followed by 6% $F_{I_{O_2}}$, 20 cycles/h) for 12 hours/day or to room air for 6 weeks. All mice were kept on a regular low-fat chow diet (n = 12–20/group). After exposures, mice were asphyxiated using CO_2 and were exsanguinated via cardiac puncture. The vasculature was perfused with PBS containing 20 U/ml heparin. Full-length aortas were dissected, cleaned, and enzymatically digested. Single-cell suspensions were prepared from the digested tissues by cycles of washing and resuspending. Cells were incubated with Fc blocker for 30 minutes to reduce autofluorescence and were subsequently fixed with 1% paraformaldehyde. After two cycles of washing, pelleted cells were incubated with antibodies for macrophage markers previously implicated in atherogenesis and metabolic dysregulation: CD11b-PB, F4/80-PE/Cy7, Ly6c-APC-Cy7, CD36-FITC, and CD64-PE (Biolegend, San Jose, CA). Subsequently, cells were washed and analyzed using BD FACS CANTO II (BD Biosciences, San Jose, CA). Results were analyzed using FlowJo software. Data are expressed as mean ± standard error of the mean. Room air and IH conditions were compared with Student's *t* tests or nonparametric testing as appropriate. Statistical significance was assumed at $P < 0.05$.

Macrophages were defined as CD11b and F4/80 double-positive cells (Figure 1). Significant increases in the percentage of macrophages out of total cell number emerged in IH-exposed mice (6.4% ± 0.3% vs. 8.1% ± 0.3%; $P = 0.003$). In addition, a trend toward increase in the absolute macrophage number was observed (cells/mg tissue: 247 ± 28 vs. 414 ± 93; $P = 0.18$). Furthermore, IH also induced shifts in the macrophage population toward a proinflammatory phenotype. Specifically, the IH group showed significantly higher expression (measured by mean fluorescence intensity) of Ly6c (522 ± 23 vs. 699 ± 43; $P = 0.004$) and increased the percentage of Ly6c(hi) cells (9.2% ± 0.5% vs. 11.7% ± 0.9%; $P = 0.03$). In contrast, tissue-resident marker CD64 expression in the aortic macrophages was downregulated after IH exposures (mean fluorescence intensity: 3,207 ± 453 vs. 2,967 ± 483; $P = 0.03$), as well as the percentage of CD64-positive cells (46.4% ± 2.4% vs. 36.9% ± 2.1%; $P = 0.006$). CD64 expression was threefold higher in Ly6c(lo) compared with Ly6c(hi) cells, thus reinforcing the notion of two distinct macrophage populations. Finally, scavenger receptor CD36 expression was increased in the IH group (mean fluorescence intensity: 1,694 ± 56 vs. 2,129 ± 209; $P = 0.005$), as was the percentage of $CD36^+$ macrophages (46.7% ± 1.7% vs. 57.3% ± 3.3%; $P = 0.03$). Of note, despite the decrease in the number of $CD64^+$ cells, the proportion of $CD36^+$ cells out of $CD64^+$ cells was increased in the IH group (14.1% ± 0.9% vs. 20.8% ± 2.2%; $P = 0.01$).

Current findings show, for the first time, an increase in the number of macrophages in the aortic wall after IH exposures. Macrophages have been extensively studied in the context of atherosclerosis, and the presence of CD11b cells is crucial to the initial steps of atherogenesis (6, 8). The expansion of this population at this early stage in the course of IH exposures is suggestive of this being a key step in IH-induced atherosclerotic plaque formation. Furthermore, IH induced several interesting changes in the phenotype of aortic wall macrophages. First, the Ly6c(hi) population was increased, as was the total expression of Ly6c on the aortic wall macrophages. Ly6c is a well-established marker of proinflammatory macrophages recruited from circulating bone marrow-derived monocytes into the arterial wall in the process of atherogenesis (6). Second, increases in Ly6c were accompanied by reciprocal decreases in CD64, a marker of tissue-resident macrophages (12). These findings add to the current debate regarding the source of the expanding macrophage population in the vascular wall during atherogenesis. Indeed, Robbins and colleagues (10) showed that resident macrophages predominate in apolipoprotein E −/− and low-density lipoprotein receptor −/− mice on a high-fat diet. Although our experiments were not designed to resolve this debate (which would require more complex techniques, such as bone marrow depletion and parabiosis), our findings point to the bone marrow as the source of

D.G. is supported by National Institutes of Health grants HL-65270, HL-086662, and HL-107160.

Author Contributions: A.G.-H. participated in the conceptual framework of the project, performed experiments, analyzed data, and drafted components of the manuscript. I.A., A.K., and S.X.Z. performed experiments. Y.W. served as blinded observer and provided critical input. D.G. conceptualized the project, provided critical input in all phases of the experiments, analyzed data, drafted the ulterior versions of the manuscript, and is responsible for the financial support of the project and the manuscript content. All authors have reviewed and approved the final version of the manuscript.

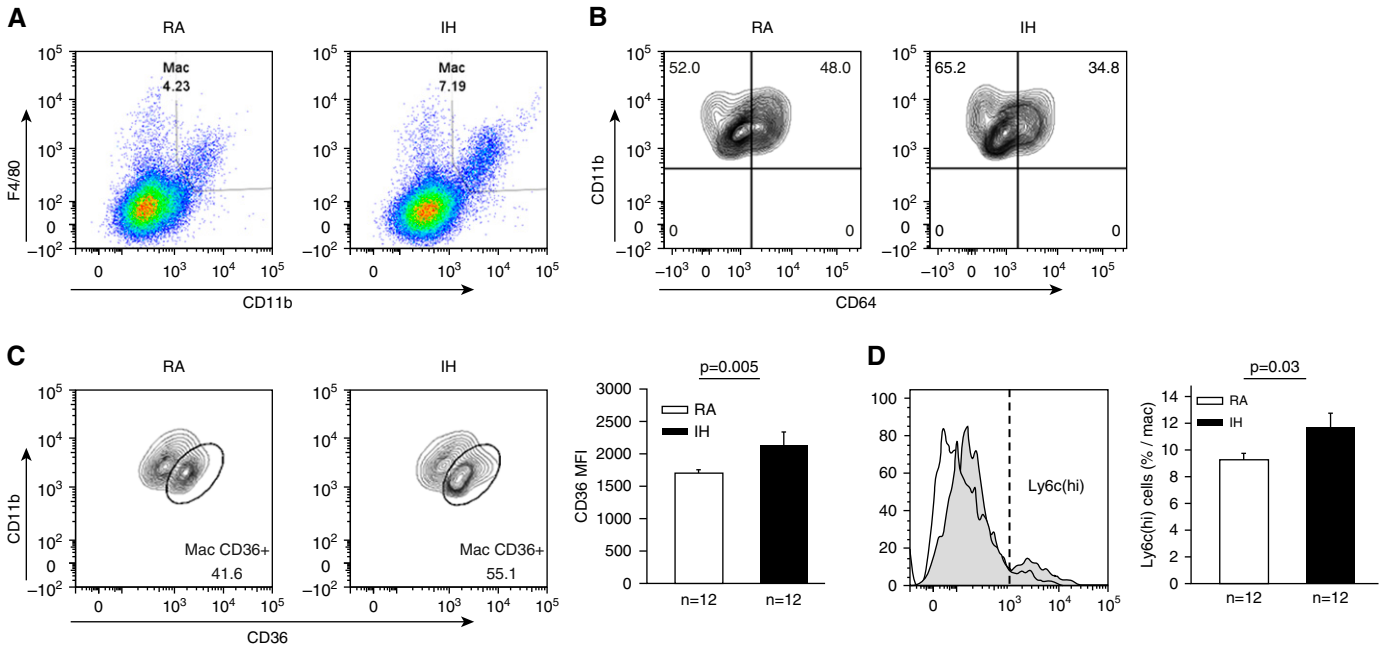

Figure 1. Changes in macrophage populations in the aortic wall of mice exposed to intermittent hypoxia (IH) or room air (RA) during sleep for 6 weeks. (*A*) Representative example of fluorescence-activated cell sorter analysis of macrophages (CD11b and F4/80 positively labeled cells) and the increase in such population with IH. (*B*) Representative example of fluorescence-activated cell sorter analysis for $CD64^{+}$ cells (resident macrophages) illustrating the reduction in resident macrophages after IH exposures. (*C*) IH is associated with significantly increased expression of CD36 in CD11b and F4/80 positively labeled cells indicating metabolic activation. (*D*) IH induced significant increases in Ly6C(hi) cell expression, indicating shifts toward proinflammatory macrophages recruited from the bone marrow. MFI = mean fluorescence intensity.

the expanding macrophage population in IH, rather than to resident macrophages, as seen in high-fat diets. Aligned with this interpretation, OSA has been previously shown by our group and others to target and activate specifically the myeloid population in other disease models, such as obesity and cancer (13).

IH also induced increased expression of CD36 surface marker within the macrophage population. CD36 is a membrane glycoprotein participating in oxidized low-density lipoprotein uptake and foam cell formation, the initial critical stage of atherosclerosis (14). Blood monocytes and their lesional progeny also have the potential to emigrate from the plaque (6), and CD36 pathway activation has been shown to inhibit this migration process, which might in turn cause macrophage entrapment in atherosclerotic lesions. Because of its critical role in atherosclerosis, CD36 has also been suggested as a treatment target for atherosclerosis (14). In addition, in line with the proinflammatory interpretation of the changes in our study, CD36 expression has been recently described as identifying a novel metabolically activated macrophage phenotype, which expresses proinflammatory cytokines, but not the classic proinflammatory macrophage surface markers (15).

In summary, IH during the sleep period for 6 weeks leads to expansion of the macrophage population in the aortic wall. In addition to modestly increased cellularity, IH induces changes in macrophage population composition with a shift toward a proinflammatory, metabolically activated phenotype and recruitment of bone marrow-derived macrophages. These changes occur in a relatively resistant animal model for development of atherosclerosis; that is, in the absence of a high-fat diet or of high atherogenic risk predisposing gene knockout mice, thus adding to their potential biological significance. Plans for future studies include the effect of longer IH exposures on macrophage populations within the aortic wall and elucidation of the functional properties associated with the phenotypic switch induced by IH. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Alex Gileles-Hillel, M.D.
Isaac Almendros, Ph.D.
Abdelnaby Khalyfa, Ph.D.
Shelley X. Zhang, M.D., M.Sc.
Yang Wang, M.D., Ph.D.
David Gozal, M.D.
University of Chicago
Chicago, Illinois

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Cumulative Radiation Exposure to Abdominal Organs in Patients with Cystic Fibrosis Should Not Be Forgotten

To the Editor:

We read with interest the recent excellent article by Kuo and colleagues regarding radiation risk from monitoring cystic fibrosis with computed tomography (CT) (1). We wish to compliment the authors on such an informative article. A number of important points were raised, some of which we wish to comment on.

The authors summarize the low radiation-related risk from CT imaging in patients with cystic fibrosis (PWCF). It should be highlighted, however, that a recent study from our group unexpectedly found that the cumulative effective dose (CED) from abdominal imaging is substantial, emphasizing a relatively silent iatrogenic source of exposure. A more than fivefold increased use of CT imaging was observed in PWCF during the last 2 decades (2), and abdominal imaging, predominantly CT, accounted for 43% of total CED in PWCF. This increased usage of abdominal CT is explained by the undisputed pivotal role that CT plays in accurate and expeditious detection and characterization of abdominal complications in PWCF, which are now more frequently being encountered (3).

In addition, CF cancer standardized incidence ratio (SIR) from any site is reported to be between 3.2 (95% confidence interval [CI], 2.1–4.6) (4) and 1.1 (95% CI, 1.0–1.3) (5). Both of these studies, however, agree that PWCF are particularly at increased risk for gastrointestinal malignancy, with reported gastrointestinal SIRs of 5.6 (95% CI, 1.5–14.4) (4) and 3.5 (95% CI, 2.6–4.7) (5), with a preponderance for esophagogastric, small bowel, colorectal, pancreatic, and biliary neoplasms. In the posttransplant CF population, a cancer SIR of 2.7 (95% CI, 1.8–3.9) and a substantial gastrointestinal SIR of 17.3 (95% CI, 10.7–26.5) (5) are reported.

With ever-increasing life expectancy resulting from improved therapies, the phrase "life-limiting condition" may be less frequently used in the future (3). Median expected lifespan is anticipated to be more than 50 years of age for those born since 2000 (6). It is worth noting that these figures predate the emergence of CFTR modulators, such as ivacaftor, which may further improve survival in subpopulations.

Kuo and colleagues conclude that the risk for lifetime radiation-induced cancer mortality in PWCF is low. We wholly agree that the true level of risk associated with radiation exposure in the diagnostic range is controversial and is not fully determined at this time. We argue, however, that the (likely) small but not insignificant radiation-related risk from abdominal imaging may be an important issue in PWCF in the future, as a result of increasing lifespan, increasing frequency of abdominal problems in an aging CF population, significant underlying gastrointestinal cancer risk, and increasing use of abdominal imaging, as detailed earlier. In the clinical context of suspected abdominal complications, the appropriate use of abdominal CT has undisputed benefits that far outweigh any risks associated with radiation exposure. In spite of the undoubted benefits of CT, however, the use of CT scanning requires serious consideration and audit, and practice must be governed by the principles of justification, optimization, and dose limitation, with a substitution of magnetic resonance imaging or ultrasound where possible.

There is no question that CT represents the major contributor to CED in patients with CF and other patient groups (e.g., patients with Crohn's disease) with chronic diseases. Reducing radiation exposure associated with CT scanning should be a major priority in these patient groups, requiring multidisciplinary input from all who care for these patients. There are major new opportunities for substantial reductions in radiation exposure associated with CT scanning, with new developments such as iterative reconstruction techniques, which are substantially reducing CT radiation dose in PWCF (7). These efforts are being further strengthened by major developments in dose management and patient dose-tracking software, which will be particularly beneficial in patient groups at risk for higher lifetime CED, such as patients with CF. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Kevin P. Murphy, M.B.
Cork University Hospital
Cork, Ireland
and
University College Cork
Cork, Ireland