Nicotine alters mucin rheological properties

Eric Y. Chen,1,2,3 Albert Sun,1 Chi-Shuo Chen,1 Alexander J. Mintz,1 and Wei-Chun Chin1

1Bioengineering, University of California at Merced, Merced, California; 2Center for Biomedical Engineering, Chang Gung University, Taoyuan, Taiwan; and 3MicroBase Technology Corporation, Taoyuan, Taiwan

Submitted 30 November 2012; accepted in final form 14 May 2014

Chen EY, Sun A, Chen CS, Mintz AJ, Chin WC. Nicotine alters mucin rheological properties. Am J Physiol Lung Cell Mol Physiol 307: L149–L157, 2014. First published May 16, 2014; doi:10.1152/ajplung.00396.2012.—Tobacco smoke exposure, the major cause of chronic obstructive pulmonary disease (COPD), instigates a dysfunctional clearance of thick obstructive mucus. However, the mechanism underlying the formation of abnormally viscous mucus remains elusive. We investigated whether nicotine can directly alter the rheological properties of mucin by examining its physico-chemical interactions with human airway mucin gels secreted from A549 lung epithelial cells. Swelling kinetics and multiple particle tracking were utilized to assess mucin gel viscosity change when exposed to nicotine. Herein we show that nicotine (∼50 nM) significantly hindered postexocytotic swelling and hydration of released mucins, leading to higher viscosity, possibly by electrostatic and hydrophobic interactions. Moreover, the close association of nicotine and mucins allows airway mucus to function as a reservoir for prolonged nicotine release, leading to correlated pathogenic effects. Our results provide a novel explanation for the maltransport of poorly hydrated mucus in smokers. More importantly, this study further indicates that even low-concentration nicotine can profoundly increase mucus viscosity and thus highlights the health risks of second-hand smoke exposure.

mucin swelling kinetics; mucociliary clearance; viscosity; tobacco smoke; environmental tobacco smoke; chronic obstructive pulmonary disease

EXPOSURE TO CIGARETTE SMOKING OR ENVIRONMENTAL TOBACCO SMOKE (ETS) IS THE PREDOMINANT RISK FACTOR IN DEVELOPING CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) (12, 57). Conventional pathogenic theory considers smoking-induced chronic airway inflammation to be a vital component in the etiology of COPD (3, 9). Supporting clinical research data have indeed found a close association between the presence of inflammatory cells (2, 23), and abnormally high oxidative stress (9, 32), with disease progression. However, the assumption that airway inflammation accounts for all important pathological aspects risks oversimplification of the multifaceted nature of this disease and potentially limits the progress of COPD therapies (3, 12). Small airway occlusion with viscous mucus is one of the major clinical manifestations that is closely associated with accelerated decline in lung function, morbidity, and mortality in COPD patients (23–24, 55). Various models such as submucosal gland hypertrophy, hyperplasia, and metaplasia have been proposed that converge on mucin hypersecretory response, underhydration of airway surfaces, and an impaired mucociliary clearance (10, 15, 17, 29, 32, 42, 56). Hypersecretion of MUC5AC and 5B mucins has been shown to critically contribute toward COPD (28, 29). Excessive mucus built up in the airway surface layer may further disturb the periciliary liquid volume and ciliary beat frequency thus contributes to mucociliary clearance dysfunction (29, 38). All these results suggest that airway mucus plays a defining role in COPD pathophysiology (17, 38).

Aberrant mucin concentration due to the hypersecretion may lead to deviation from optimal mucus viscoelasticity and amplification of COPD-associated viscous obstructive mucus production (38, 56). Supporting this premise, macrorheological analysis of COPD sputum unveiled greater inherent viscosity (45). Although an increased release of the low-charge MUC5B in COPD patients has been postulated to form less-expanded networks that may be inefficiently transported (17, 28), it remains elusive how mucin oversecretion affects mucus rheology and disease development. More importantly, whether cigarette smoking can directly affect rheological properties of airway mucus is unknown. Nicotine, one of the major addictive constituents in tobacco, has been shown to elicit mucus hypersecretion (31), but the possibility that nicotine could directly alter the rheological properties of mucus by hampering hydration and increasing mucus viscosity has not, heretofore, been considered.

Mucus network has a characteristic tangled topology, the rheological properties of which are governed mainly by the density of mucin polymers that decreases with the square of the volume of the mucin matrix (54). Therefore, the degree of hydration primarily dictates mucus rheological properties (54). We utilized polymer-swelling kinetics to calculate the diffusivity of mucin matrices, which is closely related to mucin viscosity (13, 41, 49). The slow hydration rate of a mucin matrix (low mucin gel diffusivity) is associated with more viscous, poorly hydrated, and less-transportable mucus that characterizes the thick, viscous occlusion commonly found in COPD (13, 38).

Our research reports for the first time that nicotine can directly modulate the rheological properties of mucins released from airway cells through interacting with mucin matrices. This study provides much needed understanding of the interactions between nicotine and mucins that will, in turn, shed light on the pathogenesis of COPD and may facilitate the development of possible therapeutic interventions.

MATERIALS AND METHODS

A549 cell culture. The human airway A549 was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in F-12 medium (Invitrogen, Carlsbad, CA) supplemented with l-glutamine, penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum (13). Cultures were incubated in a humidified incubator at 37°C/5% CO2.

Porcine primary airway epithelial cells. The isolation and culture protocol of porcine primary airway epithelial cells was adapted from our previously published protocol (34). In brief, pig tracheas were obtained from a local slaughterhouse, washed quickly with ice-cold
PBSS, and transferred to the laboratory on ice in the HBSS buffer. Epithelial tissue was carefully removed on ice-cold HBSS used immediately for primary epithelial cell isolation.

**Secretory granule labeling.** The presence of secretory granules of A549 cells was identified by staining with quinacrine (10 μM; Sigma-Aldrich, St. Louis, MO) (13). The nucleus was counterstained with Hoechst (1:1,000; Sigma-Aldrich) (13). Expression of MUC5AC in A549 cells was confirmed by immunostaining (data not shown).

**Swelling kinetics and A549 cell preparation.** The experimental procedures followed the protocols published previously (13). In brief, A549 cells were detached and resuspended with HBSS (Invitrogen) in glass-bottom dishes (MatTek, Ashland, MA) and briefly equilibrated in 37°C incubator prior to adding nicotine (5 nM, 10 nM, 50 nM, 100 nM, and 1 μM; Sigma-Aldrich). Both HBSS and nicotine were buffered with Tris·HCl/MES (Sigma-Aldrich) at pH 7.3 throughout the experiments (13). Degranulation of A549 cells was induced by 1 μM ionomycin (VWR, Brussels, CA) (1, 13). To assess the swelling kinetics of nonpreloaded cells, A549 cells were bathed in HBSS containing 5 nM–1 μM nicotine throughout measurement. The nicotine-preloaded cells were evaluated by incubating with 10 nM–1 μM nicotine in HBSS for 15 min at 37°C followed by repellicon to the fresh HBSS before stimulating exocytosis. To measure the swelling kinetics of nicotine-preloaded isolated granules, A549 secretory granules were extracted (according to our previous protocol), resuspended in intracellular buffer (maintained at pH 7.3 with Tris·HCl/MES), and allowed to settle on poly-L-lysine-coated dishes with 100 nM and 1 μM nicotine for 15 min at 37°C (34). Isolated granules were then replenished with fresh intracellular buffer before stimulation of mucin matrix release with 10 μM monensin (20) and 20 μM valinomycin (Sigma-Aldrich) (37). Video recordings of mucin network swelling were captured at 30 frames/s with a Nikon Eclipse TE2000U inverted microscope (Technical Instrument, Burlingame, CA), and all dishes were mounted on the 37°C thermostated stage.

**Mucin viscosity determination by multiple particle tracking.** Particle transport rates were measured by analyzing trajectories of redfluorescent carboxylate-modified microspheres (500 nm; Invitrogen) by using Auto Video Spot Tracker software (University of North Carolina, Chapel Hill, NC) and recorded by using a fast and highly sensitive Q-Imaging scientific camera (RETIGA-SRV, Technical Instrument, Burlingame, CA) and magnified by 1000× using a Nikon Eclipse TE2000U microscope. The results were considered significantly different when P < 0.05.

**RESULTS**

Nicotine reduces mucin matrix hydration and increases mucin viscosity. To study the effect of nicotine on mucin gel hydration, we first measured the swelling kinetics of mucin matrices when exposed to nicotine of both intact and isolated granules of cultured A549 cells, a model human respiratory epithelium expressing representative mucins (13, 39, 58). Fluorescent quinacrine images (green) confirmed the presence of secretory granules in A549 cells (Fig. 1A). Figure 1B, inset shows a representative plot of mucin gel swelling from which we derived an expression for characteristic relaxation time that is proportional to the second power of the mucin matrix radius expansion (Fig. 1B). This correlation yields the hydrated mucin network diffusivity (D) (cm²/s), which is used to evaluate the impact of the surrounding solution’s properties such as ionic species, organic polycations, and hydrophobic molecules on mucin swelling (Fig. 1B) (13, 54). Several phase-contrast images illustrating the process of mucin matrix gel expansion were also presented (Fig. 1C). We then

L150 NICOTINE DIRECTLY RETARDS MUCIN HYDRATION
Conducted swelling kinetics experiments on mucin secreted from three separate A549 samples: nonpreloaded cells, preloaded cells, and preloaded isolated granules with nicotine. The resultant values of D in three experimental conditions are summarized in Table 1. The magnitude of D in nicotine preloaded cells and preloaded-isolated granules decreased by ~70% compared with the control (HBSS) and intracellular buffer only correspondingly (Fig. 1D). Similarly, nonpreloaded cells exposed to 1 μM nicotine in HBSS resulted in a diminished D that is about 65% lower than in the control (Fig. 1D). We further investigated the effect of nicotine on mucin diffusivity from isolated primary porcine airway epithelial cells (nonpreloaded cells and preloaded cells). Data from Fig. 1E show that exposure to 100 nM nicotine in HBSS significantly decreased porcine mucin D by ~45–50% in contrast to the control.

Experiments using high-resolution multiple particle tracking (MPT) showed a drastic decrease in particle transport in mucin solutions containing nicotine that parallels an elevation in viscosity (Fig. 2, A and B). The elevation in mucin solution viscosity starts to plateau between 0.1 and 50 μM. Moreover, the trends shown in Fig. 2, A and B correspond to the drop in mucin matrix diffusivity illustrated in Fig. 1. These results suggest that at low nicotine concentrations (100 nM) mucin hydration and transportability are also likely to be significantly compromised in smokers.

Nicotine promotes airway mucin self-aggregation. Since nicotine and mucin polymers contain both hydrophobic and complementary ionic moieties (at physiological pH, mucins are largely negative charged and nicotine is positive charged), we tested whether nicotine can directly interact with the mucin network by forming cross-links to aggregate mucins. To verify the biochemical interaction, we measured the effect of nicotine on the spontaneous self-assembly properties of diluted human airway mucin secreted from A549 cells (expressing both MUC5AC and 5B) (13, 58). Our results show that mucins (~70 μg/l) can self-assemble to form small gels (500–600 nm) (Fig. 3). Although 50 μM nicotine dramatically increased the equilibrium size of the assembled mucin gels by ~1.85 fold (1.1 μm), removing Ca^{2+}-cross-linkers with EGTA diminished the gel size (~700 nm). Shielding mucin charges (high salt) yielded similar gel size (~700 nm). Under a Ca^{2+}-free control (EGTA chelated) without nicotine, the mucin aggregate dimension (~220 nm) was similar to sizes reported for human respiratory mucus (6, 11). Our data demonstrated that nicotine promotes mucin aggregation and can function as a potential cross-linker for mucin gel aggregates.

Nicotine accumulation in intracellular mucin granules. Prior to exocytosis, nicotine can infiltrate cytosolic granules, associate with matrices, and be sequestered in vesicles (35, 36). To demonstrate this possibility we tested the storage capacity of nicotine in secretory granules. Both intact cells and isolated secretory granules were used. Equilibration of A549 cells in HBSS containing 1 or 10 μM nicotine leads to rapid nicotine accumulation in intact secretory granules (Fig. 4A). Nicotine partition kinetics in isolated granules produced even more accumulation (Fig. 4B). These results indicate that nicotine, like other tertiary amines, can permeate and concentrate in granules against substantial concentration gradients that is consistent with other studies (16, 36, 40).

The fact that nicotine interacts with mucin networks suggests that it not only can be stored in mucin matrices but can also be gradually released into the airway. We examined whether mucin gels could modulate nicotine release. Figure 5 shows that nicotine released from 1 and 2% mucin was correspondingly ~35 and ~50% lower than the mucin-free control at the 30-min period. After 24 h, the ratio of nicotine released from mucin containing nicotine samples when compared with the control remained similar (data not shown). The data suggest strongly that higher mucin content enables more nicotine-mucin association, modulating the amount of free nicotine available for sustained release.

**DISCUSSION**

Airway mucin gels serve myriad roles in maintaining pulmonary integrity (17, 51). These multifaceted functions rely immensely on the harmonious orchestration of optimal mucus rheology, hydration, and mucociliary action, which is fundamentally regulated by Donnan equilibrium (38, 51, 54, 56). Poor mucus hydration leads to improper dispersion and luminal obstruction as cardinaly found in COPD (38, 51). We are the first to demonstrate that nicotine can directly alter mucin rheological properties by hindering hydration and increasing viscosity. This intriguing discovery is a distinct departure from the conventional model of hypersecretion-induced viscous mucus formation (29, 38, 56) and necessitates alternative therapeutic strategies for treating COPD.

Network hydration fundamentally determines mucus rheological properties (13, 38, 54). Measurements of mucin matrix diffusivity provide direct assessment of deviation in mucin viscosity released from cells under both physiological and pathological conditions (13, 54). Like many pulmonary disorders, particularly in COPD, cigarette smoking has been shown to induce mucin hypersecretion; however, whether it directly affects mucin rheological properties remains elusive. Although cigarette smoke extract has been commonly utilized in respiratory disease related studies (4, 22), the complexity of its composition (more than 4,500 molecules) prevents us from identifying a clear mechanism (47). Nicotine, on other hand, is a common major addictive compound in cigarette smoke that has well-established cellular and molecular mechanisms relating to pulmonary mucus hypersecretion disorder (31). In this study, that nicotine can directly influence mucus rheological properties via physicochemical processes is to be investigated. We tested whether nicotine could limit mucin matrix hydration at typical inhalation concentrations. First, our data showed that, at nicotine concentrations equivalent to environmental exposure (≈50 nM) (5), mucin network swelling rates were reduced by ~2- and ~1.5-fold as shown by human and porcine airway epithelial cells, correspondingly (Fig. 1, D and E). At just 100 nM, the decrease in diffusivity reached a maximum for nonpreloaded cells, preloaded cells and preloaded isolated granules (Fig. 1D) in human airway respiratory cells. The same nicotine concentration also engendered a similar maximal reduction of D in both nonpreloaded and preloaded primary porcine airway cells (Fig. 1E). Both data suggested that the nicotine-induced mucin swelling hindrance may be conserved throughout different species, as a result of comparable mucin molecules and architecture (46, 50) and further underlined the hypersensitivity of mucin rheological properties to nicotine.
exposure. In addition to the data generated from human A549 cells and primary porcine airway epithelium, our results can be strengthened with the usages of primary human airway epithelium and to compare mucin network hydration rates between normal subjects and COPD patients. Outcomes from Fig. 1, D and E further indicated that the blood nicotine concentration (~300–500 nM) obtained during direct cigarette smoking is highly likely to hinder mucin hydration (31). Modified mucus hydration may potentially be a critical concern for nicotine replacement therapy (NRT) users. A popular option of NRT is

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)

![Diagram D](image4)

![Diagram E](image5)
Table 1. Summary of mucin matrix diffusivities when exposed to varying concentrations of nicotine

<table>
<thead>
<tr>
<th>Nicotine preloaded</th>
<th>Preloading Buffer for 15 Min</th>
<th>Bathing Buffer for Swelling Kinetics Measurement</th>
<th>Diffusivity, Cm²/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpreloaded cells</td>
<td>Not preloaded</td>
<td>HBSS buffer</td>
<td>1. [Nicotine] = 0 nM</td>
</tr>
<tr>
<td>Nicotine preloaded cells</td>
<td>HBSS buffer</td>
<td>[Nicotine] = 0 μM</td>
<td>1.44 ± 0.26 μm²/s (n = 10)</td>
</tr>
<tr>
<td>Nicotine preloaded isolated granules</td>
<td>Intracellular buffer</td>
<td>Intracellular buffer</td>
<td>1. [Nicotine] = 0 μM</td>
</tr>
<tr>
<td></td>
<td>[Nicotine] = 100 nM</td>
<td>2. [Nicotine] = 1 μM</td>
<td></td>
</tr>
</tbody>
</table>

Diffusivities of means±s.d. are provided for each nicotine concentration. *Significantly different from control (nicotine-free) (P < 0.0001; Student’s t-test); n represents the number of cells or granules investigated in the experiments.

Electronic cigarette (E-cigarette). A brief delivery of nicotine by electronic cigarette through inhalation has been shown to increase respiratory impedance and peripheral airflow resistance in healthy adult smokers when compared with a control group (53). At the same time, ceasing its usage has also revealed significant improvement of cough, sputum production, and breathlessness (25). Therefore, it is a reasonable concern that the exposure of nicotine to airway via such route may equally hinder proper mucin hydration and alter rheological properties. Similar undesirable effects are implicated in other NRT including nicotine nasal spray, vapor inhaler, and nebulizer (8, 44). Safety assessment and monitoring the non-conventional adverse effects are very much needed for various NRT products. Our laboratory results might be limited by themselves; for example mucus extrusion might become more frequent in vivo. However, animal experimental data indicated that expelled mucus remained adherent at the secretory site, and, when dislodged, the mucins mass had slower transport rate than the control (smoke-free) (31). These data further suggested that mucous extrusion in cigarette smoke-exposed rodents is hindered. Moreover, in vitro and in vivo evidences have shown that exposure to cigarette smoke could possibly generate stagnant mucus and impair mucociliary clearance by inducing airway surface liquid dehydration (15). These studies supported the notion of cigarette smoke-induced improper gel hydration and altered rheological characteristics in smokers (26).

Since polymer diffusivity and viscosity are inversely proportional to each other (13), we examined whether nicotine could increase mucin viscosity. A representative porcine gastric mucin was used since it is a widely acceptable model to investigate the viscoelastic natures and biological applications of mucins (33, 48). Nicotine at 100 nM drastically elevates mucin solution viscosity (Fig. 2, A and B). Our data suggest that a very low level of sustained or transient exposures to nicotine can potentially hamper normal ciliary beat frequency, disrupting mucociliary clearance in both active smokers and populations exposed to ETS. Aligning with published reports, smoke-exposed rats showed zones of inactive cilia that have been associated with stagnant mucus (26). Impaired mucus clearance also leads to bacterial persistence, associated inflammation, and tissue damage (24, 38). Furthermore, a momentary exposure to secondhand smoke caused acute decrement in lung functions and can exert a substantive role in eliciting chronic respiratory diseases (18). Both nicotine and mucins are amphiphilic structures containing complementary features amenable to physicochemical
interactions (35, 36). It is highly plausible that at neutral pH the polyanionic carbohydrate side chains and the nonglycosylated mucin core (6, 17, 54) can bind with monoprotonated and hydrophobic moieties of nicotine (35). It is also likely that nicotine can interact with the nodes of tightly bound protein domains in MUC5B mucins (27). Through these mechanisms, nicotine can limit mucin hydration at both the intra- and extracellular levels. We used dynamic laser scattering to authenticate that nicotine affects airway mucin self-assembly properties by enlarging the equilibrated mucin gel size (~1.85-fold). This result is corroborated by the presence of mucus flakes in tobacco smoke-exposed rat airways (26). Data from EGTA-treated nicotine-containing mucin samples when compared with EGTA-treated control indicates that nicotine can replace conventional Ca$^{2+}$ to function as cross-linkers for mucin networks (Fig. 3). Therefore, nicotine at concentrations found in smokers’ airways (~50 µM) (14) may readily link mucins together to form sizable aggregates. Differences between charge-shielded (high salt-nicotine sample; ~710 nm) and nonsheilded mucin aggregate sizes demonstrate that electrostatic interaction strongly potentiates hydrophobic binding among mucin and nicotine. Both these interactions probably contribute to retarding normal mucin hydration, yielding the thick mucus found in smokers. Moreover, the gel-on-brush model further complemented our data, suggesting that nicotine may interact with membrane-spanning mucins and large mucopolysaccharides tethered to cilia and epithelial surface to destabilize periciliary layer, possibly impeding mucus clearance in smokers (7).

To further prove that nicotine hinders network hydration through direct interaction with intracellular mucin matrices, Fig. 4 has validated nicotine concentrating in cytosolic vesicles (35, 43). This phenomenon can primarily be explained by pH partitioning. That is, chemically similar tertiary amine drugs (e.g., disobutamide, a cardiac antiarrhythmic drug) or weak alkaline compounds such as quinacrine (Atabrine, an antipro-

---

**Fig. 2.** Measurements of fluorescent particle displacement and microviscosity changes. A: multiple particle tracking was used to calculate mean-square displacements needed to derive diffusivity of 500-nm-diameter carboxylated polystyrene particles in 1% porcine gastric mucin solution with increasing nicotine concentration (0–50 µM) in PBS. The mucin content used in this experiment is consistent with the range found in cervical, nasal, and lung mucus (1–3% of wet weight). B: diffusivity was then used to derive the values for mucin solution viscosity. The mucin solution viscosity values increased significantly when compared with the mucin-only control (n = 32). Data were presented as means ± SE; n represents the number of independent experimental samples investigated.

**Fig. 3.** Nicotine changes the sizes of mucin self-assembly gels. Nicotine increased the equilibrium mucin gel particle sizes. After addition of 50 µM nicotine to 1% m-filtered human airway mucin (70 µg/ml) prepared in HBSS (Tris·HCl/ MES, pH 7.3) for 24, 48, 72, and 96, the sizes of mucin gels (●, P < 0.0001; n ≥ 14; 1-way ANOVA) were significantly larger than the control (mucin-only; ○, n = 14). The nicotine-treated mucin gel sizes (●, P < 0.0001; n ≥ 14; 1-way ANOVA) were also markedly larger than mucin gels prepared in 0.6 M NaCl HBSS with 50 µM nicotine (Tris·HCl/MES, pH 7.3) (◆; n = 10) after 24 h of incubation. Hydrodynamic mucin gel sizes of EGTA-nicotine-treated (○; P < 0.01; n ≥ 8; 1-way ANOVA) and EGTA-high-salt-nicotine-treated (●; P < 0.01; n ≥ 12; 1-way ANOVA) samples were larger than that of EGTA-treated mucin control (◆; n ≥ 14) after 24 h of EGTA treatment. Data were presented as means ± SD; n represents the number of independent experimental samples investigated.
tozoal; Fig. 1A) or acridine orange are sequestered into acidic organelles and protonated, thus becoming trapped owing to granular pH gradients and the chemical pKa (35, 36, 40). Hence, it seems evident that nicotine at levels equivalent to ETS or cigarette smoking can result in significant accumulation in mucin granules of airway goblet cells.

Furthermore, our results indicate that mucin gels serve not only as a reservoir but also as a control-release modulator for nicotine (Fig. 5). Consequently, the body may experience prolonged exposure due to a sustained nicotine release from airway mucus beyond the period of active smoking and ETS exposures. Our data are supported by the lack of COPD decline among formerly heavy smokers (19). That chronic inflammation persisted after smoking cessation can be elucidated by the persistent nicotine released from mucus that attracts and activates neutrophils (32, 52). In addition, gradual release and retention of tobacco carcinogens and nicotine from mucus may be an additional mechanism linking lung carcinogenesis and COPD susceptibility (2).

This study has provided evidence supporting that nicotine directly retards mucin hydration through interactions with mucins possibly via cross-linking. The fact that low levels of tobacco smoke exposure may lead to nicotine accumulation in secretory granules highlights the hazardous potential of ETS for nonsmokers. Furthermore, airway mucus potentially sustains harmful blood nicotine concentration for extended time after tobacco inhalation. In addition, nicotine is likely to cause significant systemic effects by inducing widespread changes in the release kinetics of other secretory matrices (54) that may contribute to the complex pathology found in tobacco smokers or in individuals chronically exposed to ETS.

ACKNOWLEDGMENTS

We thank Paul M. Quinton and John M. Mintz for critical suggestions and thorough editing.

GRANTS

This study was supported in part by National Science Foundation (CBET-0932404), National Institutes of Health (1R15HL095039), and Linkou Chang Gung Memorial Hospital (CMRPD1C0031).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


REFERENCES