Patient-specific, re-engineered cardiomyocyte model confirms the circumstance-dependent arrhythmia risk associated with the African-specific common SCN5A polymorphism p.S1103Y: Implications for the increased sudden deaths observed in black individuals during the COVID-19 pandemic

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BACKGROUND During the early stages of the coronavirus disease 2019 (COVID-19) pandemic, a marked increase in sudden cardiac death (SCD) was observed. The p.S1103Y-SCN5A common variant, which is present in ~8% of individuals of African descent, may be a circumstance-dependent, SCD-predisposing, proarrhythmic polymorphism in the setting of hypoxia-induced acidosis or QT-prolonging drug use.

OBJECTIVE The purpose of this study was to ascertain the effects of acidosis and hydroxychloroquine (HCQ) on the action potential duration (APD) in a patient-specific induced pluripotent stem cell–derived cardiomyocyte (iPSC-CM) model of p.S1103Y-SCN5A.

METHODS iPSC-CMs were generated from a 14-year-old p.S1103Y-SCN5A-positive African American male. The patient’s variant-corrected iPSC-CMs (isogenic control [IC]) were generated using CRISPR/Cas9 technology. FluoVolt voltage-sensitive dye was used to assess APD90 values in p.S1103Y-SCN5A iPSC-CMs compared to IC before and after an acidotic state (pH 6.9) or 24 hours of treatment with 10 mM HCQ.

RESULTS Under baseline conditions (pH 7.4), there was no difference in APD90 values of p.S1103Y-SCN5A vs IC iPSC-CMs (P = NS). In the setting of acidosis (pH 6.9), there was a significant increase in fold-change of APD90 in p.S1103Y-SCN5A iPSC-CMs compared to IC iPSC-CMs (P < .0001). Similarly, with 24-hour 10 μM HCQ treatment, the fold-change of APD90 was significantly higher in p.S1103Y-SCN5A iPSC-CMs compared to IC iPSC-CMs (P < .0001).

CONCLUSION Although the African-specific p.S1103Y-SCN5A common variant had no effect on APD90 under baseline conditions, the physiological stress of either acidosis or HCQ treatment significantly prolonged APD90 in patient-specific, re-engineered heart cells.

KEYWORDS Drug-induced long QT syndrome; Induced pluripotent stem cell–derived cardiomyocyte; Long QT syndrome; p.Ser1103-Tyr-SCN5A

Introduction
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for coronavirus disease 2019 (COVID-19), has caused more than 4.5 million deaths worldwide according to the World Health Organization COVID-19 dashboard (as of August 30, 2021).1 Early in the COVID-19 pandemic, the repurposing of hydroxychloroquine (HCQ), a disease-modifying antirheumatic and
antimalarial drug, to fight COVID-19 was explored due to its antiviral properties in vitro. The Food and Drug Administration (FDA) granted Emergency Use Authorization on March 28, 2020. However, HCQ results in heart rate-corrected QT interval (QTc) prolongation and therefore has the potential to cause drug-induced QT prolongation, drug-induced torsades de pointes, and even drug-induced sudden cardiac death (SCD) in some individuals. In fact, on April 24, 2020, the FDA cautioned against the use of HCQ for COVID-19 treatment outside of a hospital setting or a clinical trial due to these risks of heart rhythm problems. HCQ has since been proven to be an ineffective treatment of COVID-19 in the RECOVERY (Randomised Evaluation of COVID-19 Therapy) trial.4

The p.Ser1103Tyr-SCN5A (p.S1103Y-SCN5A) common genetic variant found in the SCN5A-encoded Na,1.5 sodium channel is present in 8%–10% of individuals of African descent and may be a circumstance-dependent, SCD-predisposing, proarrhythmic polymorphism in the setting of an acidotic state akin to persistent late sodium current phenotype in the setting of an acidic state akin to SCN5A-mediated long QT syndrome (LQTS) type 3 (LQT3). Acidosis can be caused by a severe hypoxic state, such as is seen in some COVID-19 patients. This common, ethnic-specific genetic variant potentially exacerbates health disparities already caused by the pandemic. Severe hypoxic states are also seen in many other conditions and infections, and the genetic variant is potentially contributory to other long-standing pre-existing health disparities and perhaps many future ones.

In light of the FDA’s past Emergency Use Authorization of HCQ, we performed this study to ascertain the effects of acidosis and HCQ on the cardiac action potential duration (APD) in a patient-specific induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) model of p.S1103Y-SCN5A.

Methods
Case description and generation of patient-specific and isogenic control iPSC lines

The heterozygous p.S1103Y-SCN5A missense variant was identified incidentally in a 14-year-old black male without QT prolongation (QTc = 394 ms). Blood samples were obtained from the patient after obtaining informed consent in accordance with this Mayo Clinic Institutional Review Board (09-006465) approved study.

Peripheral blood mononuclear cells (PBMCs) were reprogrammed by episome. Blood media was used to grow PBMCs for 3 to 7 days in a 5% CO2, 37°C, humidified incubator. In brief, Blood media is composed of Iscove’s modified Dulbecco’s medium (IMDM; Gibco, 12440053), 20% KnockOut Serum Replacement (Gibco, Thermo Fisher Scientific, Waltham, MA, 10828010), insulin-like growth factor (IGF-1; Peprotech US, Cranbury, NJ, 100-11), erythropoietin (EPO; Peprotech, 100-64), interleukin-3 (IL-3; Peprotech, 200-03), and human stem cell factor (hSCF; Peprotech, 300-07). DNA for transfection was prepared with pCXLE-hOCT3/4-shp53-F (OCT3/4) (Addgene, Watertown, MA, 27077), pCXLE-hSK (SOX2/KLF4) (Addgene, 27078), pCXLE-hUL (L-MYC) (Addgene, 27080), and PCXWB-EBNA1 (EBNA-1) (Addgene, 37624). The P3 Primary Cell 4D-Nucleofector X Kit (Lonza, Lonza Group Ltd, Basel, Switzerland, V4XP-3024) and the Lonza 4D Nucleofector machine were used to reprogram the PBMCs. Afterward, the PBMCs were placed in a 37°C, 5% CO2, humidified incubator. Media was changed to 50:50 of Blood media and ES media on day 2 after transfection. ES media is composed of KnockOut Dulbecco’s modified Eagle medium (DMEM; Gibco, 10829018) with 20% KnockOut Serum Replacement. On day 4, the media was replenished with ES media only. On day 7, media was changed to mTeSR1 (Stemcell Technologies, 85850) only with 1% antibiotic-antimycotic (Gibco, 15240-062). mTeSR1 media was changed every other day until visible iPSC colonies were seen. Colonies were picked at around 21 days post-transfection with the Yamanaka factors.

Patient-derived p.S1103Y-SCN5A variant iPSCs were “gene-corrected” using CRISPR/Cas9 technology to generate an isogenic control (IC) line by Applied StemCell (Milpitas, CA).

CM differentiation
The iPSCs were cultured in mTeSR1 in 6-well plates (Falcon, 353046) with Matrigel (Corning, 356278) coating and incubated at 37°C and 5% CO2. The mTeSR1 media was changed daily. At 80%–90% confluence, iPSCs were disaggregated with ReLeSR (Stemcell Technologies, 05872), seeded into 24-well plates (Corning Incorporated, Corning, NY, 3524), cultured in mTeSR1 with 10 μM ROCK inhibitor (Tocris, Bio-Technne Corporation, Minneapolis, MN, 1254), and allowed to grow for 2–4 days until 80%–90% confluent. For differentiation, the culture medium was changed to RPMI 1640 medium (Gibco, 11875-093) supplemented with B27-minus insulin (Gibco, A18956-01) containing 5 μM CHIR99021 (Sigma, SML1046) for 48 hours (day 2). After 48 hours, the medium was changed to RPMI-B27-minus insulin containing 5 μM IWP2 (Sigma-Aldrich, Millipore Sigma, Burlington, MA, I0536) and incubated until day 4. On day 4, the medium was changed back to RPMI 1640 medium supplemented with B27-minus insulin, and cells were maintained in this media until beating iPSC-CMs appeared, typically around day 10 or 12. One week after the initial observation of beating, the media was changed, and the iPSC-CMs were maintained in RPMI 1640 medium supplemented with B27 (Gibco, 17504-044).

Dissociation of iPSC-CMs
The iPSC-CM aggregate cultures were maintained in RPMI 1640 medium supplemented with B27 in 24-well plates. At
differentiation day 30, iPSC-CMs were subjected to enzymatic dissociation using CM dissociation media (Stemcell Technologies, 05025) to obtain single cell suspensions of CMs. The iPSC-CMs were first washed with 1 mL of phosphate-buffered saline (PBS) for 30 seconds. After aspiration of the PBS, 250 μL of CM dissociation media was added to each well. After 2 minutes of incubation at room temperature (RT), the CM dissociation media was aspirated, and the cells were incubated for 3 minutes in a 5% CO₂ incubator at 37°C. After incubation, 500 μL of DMEM (Corning, 10-013-CV) with 20% fetal bovine serum (FBS; Gibco, 10370-086) was added to each well. The solution was triturated 4–6 times with a 1000-μL pipette tip, and cells were transferred to a Matrigel-coated 35-mm glass bottom dish (MatTek Corporation, Ashland, MA, P35G-1.5-10-C) and cultured at 37°C and 5% CO₂ for 24 hours. The media was then changed to RPMI 1640 medium supplemented with B27 and stored in a 5% CO₂ incubator at 37°C until use.

**Live cell imaging for APD measurement**

The iPSC-CMs were cultured on 35-mm glass bottom dishes that were precoated with Matrigel (Corning, 356278) at 37°C and 5% CO₂. For imaging, cells were incubated at 37°C and 5% CO₂ for 20 minutes in Tyrode’s solution (Alfa Aesar, Thermo Fisher Scientific, Tewksbury, MA, J67607K2) containing the fluorescent voltage-sensitive dye FluoVolt (Invitrogen, Thermo Fisher Scientific, Waltham, MA, F10488). The Tyrode’s/FluoVolt solution contained 0.5 μL FluoVolt dye, 5 μL PowerLoad concentrate (part of FluoVolt kit: Invitrogen, Thermo Fisher Scientific, Waltham, MA), and 4 mL Tyrode’s solution. The cells were then washed 3 times in fresh Tyrode’s solution. During imaging, the dishes were kept in a heated 37°C stage-top environment chamber supplied with 5% CO₂. Imaging of voltage–indicated cellular APD was taken under a 40× water objective using a Nikon Eclipse Ti light microscope (Nikon Instruments Inc., Melville, NY). Time-lapse videos of multiple, individual beating iPSC-CMs, paced at 1 Hz, were recorded at a speed of 20 ms per frame for 20 seconds at 15% LED power. Single regions of interest were selected for every beating iPSC-CM captured in the recordings. The iPSC-CMs were (1) untreated (pH 7.4 without HCQ); (2) in an acidic condition of pH 6.9; or (3) treated with 10 μM HCQ in normal pH 7.4 for 24 hours. The raw data were exported to Excel software (Microsoft, Redmond, WA) and then analyzed with an in-house developed Excel-based program. To calculate APD₉₀ fold-change, each APD₉₀ of a treated iPSC-CM was divided by the average baseline APD₉₀ of each dataset (eg, 1 IC clone 1 iPSC-CM treated with HCQ APD₉₀ divided by IC clone 1 iPSC-CMs average baseline APD₉₀). An APD₉₀ fold-change value close to 1.0 was considered no change.

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde for 10 minutes at RT, followed by washing 3 times with PBS. The cells were then permeabilized/blocked with 0.1% Triton X-100/PBS (PBST)/5% goat serum for 1 hour at RT, and subsequently incubated in primary antibody solution made of PBST/5% goat serum containing 1 μg/mL of Oct-4 (Invitrogen, PAB-27438) and SSEA-4 (ThermoFisher Scientific, Waltham, MA, MA1-021) primary antibodies for iPSCs, or 1 μg/mL of cardiac troponin T (cTnT; Proteintech, 15513-1-AP) or 1 μg/mL of SCN5A (Santa Cruz Biotechnology, Inc., Dallas, TX, sc-271255) primary antibody for iPSC-CMs, at 4°C overnight. The next day, cells were washed 3 times with PBST/5% goat serum at RT before being incubated in PBST/5% goat serum containing 1 μg/mL of Oct-4 (Invitrogen, PAB-27438) and SSEA-4 (ThermoFisher Scientific, Waltham, MA, MA1-021) primary antibodies for iPSCs, or 1 μg/mL of cardiac troponin T (cTnT; Proteintech, 15513-1-AP) or 1 μg/mL of SCN5A (Santa Cruz Biotechnology, Inc., Dallas, TX, sc-271255) primary antibody for iPSC-CMs, at 4°C overnight. The next day, cells were washed 3 times with PBST/5% goat serum at RT before being incubated in PBST/5% goat serum with a 1:200 dilution of Alexa Fluor 568 IgG (H+L) Cross-Adsorbed Goat anti-Mouse (Invitrogen, A-11004) and/or Alexa Fluor 488 IgG (H+L) Cross-Adsorbed Goat anti-Rabbit (Invitrogen, A-11008) secondary antibodies at RT for 1 hour. 4’,6-diamidino-2-phenylindole (DAPI) was added to each secondary antibody solution at dilution of 1 μg/mL. After secondary antibody incubation,
cells were washed 3 times with PBST and finally covered in 500 µL of PBS for imaging. Images were acquired on a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy, LLC, White Plains, NY) in the Mayo Microscopy and Flow Cytometry Cell Analysis Core Facility.

**Statistical analysis**
All datapoints are shown as mean value, and bars represent the standard error of the mean. The Student t test (2-tailed) was performed to determine statistical significance between 2 groups, and 1-way analysis of variance and Tukey post hoc test was performed for comparisons among ≥3 groups. P < .05 was considered significant.

**Results**
Generation of patient-specific iPSCs and CRISPR/Cas9-engineered IC iPSCs

The heterozygous pS1103Y-SCN5A missense variant was identified incidentally in a 14-year-old black male who presented for a second opinion cardiology evaluation for hypertrophic cardiomyopathy (HCM) and ultimately was dismissed with insufficient evidence for a clinical diagnosis for HCM. The patient had a normal electrocardiogram (ECG), with QTc = 394 ms.

After generation of patient-specific (pS1103Y containing) and CRISPR/Cas9 S1103Y variant-corrected IC iPSCs and iPSC-CMs, immunofluorescence (IF) imaging showed that the iPSCs contained the pluripotency markers Oct-4 and SSEA-4, and the iPSC-CMs contained the cardiac marker cTnT (Figure 1A). Sanger sequencing confirmed that the pS1103Y-SCN5A variant was in the patient line and that the IC was free of this variant (Figure 1B). Using quantitative IF imaging, we selected cardiac-specific marker harboring cells and calculated the mean value of fluorescent intensity of SCN5A staining divided by area. IF imaging showed there was no significant difference in SCN5A protein expression levels between pS1103Y-SCN5A iPSC-CMs and IC iPSC-CMs (P = NS) (Figure 2).

**Patient-specific pS1103Y-SCN5A iPSC-CMs are more sensitive to APD-prolonging effects of either acidosis or HCQ**

The fluorescent voltage-sensitive dye FluoVolt was used to assess APD<sub>90</sub> in pS1103Y-SCN5A iPSC-CMs compared to ICs at (1) baseline (pH 7.4); (2) after an acidic state (pH 6.9); or (3) after 24 hours of treatment with 10 µM HCQ at normal pH 7.4. Representative APD<sub>90</sub> tracings are shown in Figures 3A and 3B. Under baseline conditions (pH 7.4), there was no difference in APD<sub>90</sub> values of pS1103Y-SCN5A iPSC-CMs (clone 1: 398 ± 7 ms; n = 21) vs IC (clone 1: 395 ± 6 ms; n = 21; P = NS) (Figure 3C). Baseline APD<sub>90</sub> measurements were also similar between pS1103Y-SCN5A iPSC-CMs (clone 2: 364 ± 3 ms; n = 20) and IC (clone 2: 379 ± 3 ms; n = 20; P = NS) (Figure 3C).

However, at pH 6.9, APD<sub>90</sub> was prolonged significantly in pS1103Y-SCN5A iPSC-CMs (clone 1: 398 ± 7 ms; n = 21; vs 477 ± 6 ms; n = 15; Δ+79 ms; P < .0001) but not in IC iPSC-CMs (clone 1: 395 ± 6 ms; n = 21; vs 377 ± 6 ms; n = 15; Δ−18 ms; P = NS). This was also observed in pS1103Y-SCN5A clone 2 and IC clone 2, for which APD<sub>90</sub> was significantly prolonged in the pS1103Y iPSC-CMs (clone 2: 364 ± 3 ms; n = 20; vs 440 ± 5 ms; n=19; Δ+76 ms; P < .0001) following an acidic state but not in IC (clone 2: 379 ± 3 ms; n = 20; vs 371 ± 2 ms; n = 19; Δ−8 ms; P = NS).

We also calculated APD<sub>90</sub> fold-change. To calculate APD<sub>90</sub> fold-change, each APD<sub>90</sub> of treated iPSC-CMs was divided by the average baseline APD<sub>90</sub> of each dataset (eg, IC clone 1 acidosis APD<sub>90</sub> divided by IC clone 1 average baseline APD<sub>90</sub>). An APD<sub>90</sub> fold-change value close to 1.0 was considered no change. Under the setting of acidosis
(pH 6.9), there was a significant increase in fold-change of APD\textsubscript{90} only in the 2 clones of p.S1103Y-SCN5A (clone 1: 1.199 ± 0.014; clone 2: 1.209 ± 0.013) compared to the 2 IC clone iPSC-CMs (clone 1: 0.956 ± 0.015; clone 2: 0.979 ± 0.006; P < .0001) (Figure 3D).

Similarly, APD\textsubscript{90} was prolonged significantly in p.S1103Y-SCN5A iPSC-CMs (clone 1: 398 ± 7 ms; n = 21; vs 504 ± 9 ms; n = 15; Δ+106 ms; P < .0001; clone 2: 365 ± 3 ms; n = 19; vs 404 ± 4 ms; n = 17; Δ+39 ms; P < .0001) but not in the IC iPSC-CMs (clone 1: 395 ± 6 ms; n = 21; vs 394 ± 6 ms; n = 15; Δ=1 ms; P = NS; clone 2: 422 ± 4 ms; n = 20; vs 422 ± 3 ms; n = 20; Δ0 ms; P < .0001) after 24 hours of treatment with 10 μM HCQ. The fold-change of APD\textsubscript{90} was significantly higher in p.S1103Y-SCN5A clone 1 and clone 2 iPSC-CMs (1.268 ± 0.022 and 1.107 ± 0.011) compared to IC clone 1 and clone 2 iPSC-CMs (0.999 ± 0.015 and 1.001 ± 0.008; P < .0001) (Figure 3E).

Discussion

African Americans who possess the p.S1103Y-SCN5A variant have an increased risk of acquired arrhythmias.\textsuperscript{7} This variant is also associated with an increased risk of sudden unexplained death and sudden infant death syndrome (SIDS) in black individuals.\textsuperscript{6,8,9} Tester et al\textsuperscript{8} found that this variant was overrepresented in an autopsy-negative sudden unexplained death cohort. p.S1103Y-SCN5A was also over-represented in African American SIDS cases reported by Van Norstrand et al.\textsuperscript{9} Similarly, Plant et al\textsuperscript{6} determined that African American infants who are homozygous for p.S1103Y-SCN5A were at a 24-fold increased risk for developing SIDS. Common risk factors for SIDS include apnea and/or respiratory acidosis.\textsuperscript{6}

Although it previously was shown in an HEK cell model that acidosis can affect cells with this p.S1103Y-SCN5A variant,\textsuperscript{6} we confirmed these findings in a patient-specific, re-engineered heart cell model. Our data also established
that the p.S1103Y-SCN5A variant was necessary to produce the significant APD prolongation observed in the patient’s iPSC-CMs after HCQ treatment. Therefore, HCQ is another potential risk factor for individuals having the p.S1103Y-SCN5A variant because it can cause drug-induced arrhythmias and drug-induced LQTS. HCQ is currently being used to treat malaria, systemic lupus erythematosus, and rheumatoid arthritis, for which its cardiac safety profile is substantial.3,10

Given the prevalence of p.S1103Y-SCN5A and the increased risk of drug-induced arrhythmias and potential drug-induced SCD associated with HCQ, genetic testing for p.S1103Y-SCN5A may be warranted before the initiation of HCQ and other QT-prolonging drugs in individuals of African descent. Importantly, a baseline 12-lead ECG alone may not be suitable for screening, as p.S1103Y-SCN5A does not seem to affect APD90 under baseline conditions. In emergency situations in which treatment with any known QT-prolonging drug needs to begin immediately, in-hospital, post-treatment QT monitoring should be performed. With mobile ECG devices capable of accurately assessing QTc in nearly any setting,11 consideration of routine pre- and post-treatment QTc monitoring soon will become practically and financially feasible. Given the circumstance-dependent risk of p.S1103Y-SCN5A, this capability is particularly important for patients of African descent with concomitant QT risk factors or those at risk for metabolic or respiratory acidosis, including those with moderate-to-severe manifestations of COVID-19.

The iPSC-CM model used herein has advantages over the noncardiac HEK cell-based models previously utilized. HEK cell models lack (1) the majority of structural and interacting proteins involved in the cardiac APD; and (2) the personalized, precision medicine nature of iPSC-CM models. iPSC-CMs provide a more physiologically relevant system for patient-specific studies. This specific p.S1103Y-SCN5A iPSC-CM model would be useful for testing the effects of other QT-prolonging drugs, exercise-induced arrhythmias, and/or the cytokine storm phenomenon that can occur during COVID-19 infection. This model could be applied to test potential therapies for these conditions as well.

Limitations

Our study clearly demonstrated the adverse effects of both acidosis and HCQ on the APD of a single patient-derived iPSC-CM model compared to its gene variant corrected isogenic control. Although the use of an isogenic control clearly demonstrates that the S1103Y variant is largely responsible for this effect, this was only tested in setting of a single patient-derived iPSC model. The use of multiple S1103Y positive iPSC lines with varying genetic back-grounds is warranted. While iPSC-CMs provide the opportunity for patient-specific modeling of genetic arrhythmia syndromes, this model system has inherent limitations. For example, the development of mature microstructures such as transverse tubules are lacking in iPSC-CMs in comparison to adult human, rabbit, or mouse ventricular myocytes. The clone-to-clone variability of iPSC-CMs has also been noted. Here, we elected to study multiple iPSC-CM clones to overcome this issue of variability. Nonetheless, the utility of the iPSC-CM platform for modeling arrhythmic and cardiomyopathic conditions has been demonstrated to be highly effective. In this study, FluoVolt voltage-sensitive dye was used to assess APD. Although voltage-sensitive dyes such as Fluovolt have become a powerful and reliable tool for high throughput action potential measurement, the labor-intensive patch-clamp technique is still considered the gold standard.

Conclusion

Although the African-specific p.S1103Y-SCN5A common variant had no effect on APD under baseline conditions, the physiological stress of either acidosis or HCQ treatment significantly prolonged APD in patient-specific, re-engineered heart cells. Further epidemiological studies are needed to determine whether this African-specific p.S1103Y-SCN5A common polymorphism and its circumstance-dependent reduction in repolarization reserve have contributed to the increased rate of SCD and the racial outcome disparities observed during the COVID-19 pandemic.

Acknowledgment

We would like to thank the team in the Mayo Clinic Microscopy and Cell Analysis Core facility in Rochester, Minnesota, for their dedication to quality assistance and care with the equipment.

References