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27. Meidell RS, Gerard RD, Sambrock JF. Molecular biology of thrombolytic agents. In: Roberts R, editor. Molecular Basis of Cardiology. Cambridge, MA: Blackwell Scientific Publications, 1993:295–324.

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23. Cohn PF. Silent Myocardial Ischemia and Infarction. 3rd edition. New York, NY: Marcel Dekker, 1993:33.

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10. Henkel J. Testicular Cancer: Survival High With Early Treatment. FDA Consumer magazine [serial online]. January-February 1996. Available at: http://www.fda.gov/fdac/features/196_test.html. Accessed August 31, 1998.

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Prasugrel Versus Ticagrelor in Patients With CYP2C19 Loss-of-Function Genotypes

Results of a Randomized Pharmacodynamic Study in a Feasibility Investigation of Rapid Genetic Testing

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HIGHLIGHTS

- Our study supports the feasibility of using rapid CYP2C19 genotyping among both patients with stable and acute coronary syndrome undergoing diagnostic coronary angiography, with intent to undergo ad hoc PCI, in real-world clinical practice.
- Rapid bedside genetic testing assay allows for very rapid turnaround times of results, with patients approached the same day of their procedure and availability of CYP2C19 genotypes within 1 h of sampling and before patients undergoing PCI.
- Among carriers of CYP2C19 loss-of-function alleles undergoing PCI there were no differences in levels of platelet inhibition between prasugrel and ticagrelor (loading and maintenance dosing).

From the University of Florida College of Medicine-Jacksonville, Jacksonville, Florida, USA. The present study was funded by an investigator-initiated grant from the Scott R. MacKenzie Foundation for genome research. Spartan Bioscience provided the Spartan RX system and the reagents used free of charge. These entities had no role in study design conception, conduct of the study or decision to publish these results. Dr. Franchi has received payment as an individual for a consulting fee or honorarium from AstraZeneca and Sanofi. Dr. Rollini has received payment as an individual for a consulting fee or honorarium from Chiesi. Dr. Pineda has received payment as an individual for consulting from Edwards Lifesciences; and educational institutional grants from Abbott, Boston Scientific, Medtronic, Edwards Lifesciences, and Gore Medical. Dr. Angiolillo has received consulting fees or honorarium from Abbott, Amgen, Aralix, AstraZeneca, Bayer, Biosensors, Boehringer Ingelheim, Bristol-Myers Squibb, Chiesi,
The feasibility of rapid genetic testing in patients undergoing percutaneous coronary intervention (PCI) and the comparison of the pharmacodynamic effects of prasugrel versus ticagrelor among carriers of cytochrome P450 2C19 loss-of-function alleles treated with PCI has been poorly explored. Rapid genetic testing using the Spartan assay was shown to be feasible and provides results in a timely fashion in a real-world setting of patients undergoing coronary angiography (n = 781). Among patients (n = 223, 28.5%), carriers of at least 1 loss-of-function allele treated with PCI (n = 65), prasugrel, and ticagrelor achieve similar levels of platelet inhibition. (A Pharmacodynamic Study Comparing Prasugrel Versus Ticagrelor in Patients Undergoing PCI With CYP2C19 Loss-of-function [NCT02065479]) (J Am Coll Cardiol Basic Trans Science 2020;5:419-28) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
patients undergoing coronary angiography and to compare the PD effects of prasugrel and ticagrelor selectively among those identified as having a CYP2C19 LOF allele and undergoing PCI.

METHODS

STUDY DESIGN AND PARTICIPANTS. This was a prospective, randomized, parallel design, open-label investigation conducted in patients scheduled to undergo diagnostic coronary angiography with intent to undergo ad hoc PCI (Clinicaltrials.gov identifier: NCT02065479). The study was performed at the University of Florida Health-Jacksonville (Jacksonville, Florida). Patients were screened the same day of the scheduled procedure and before going to the interventional suite. Specific study inclusion and exclusion criteria are provided in the Supplemental Appendix. In brief, patients age 18 to 75 years scheduled for diagnostic coronary angiography with intent to undergo ad hoc PCI and who did not have any contraindications to treatment with prasugrel or ticagrelor were considered for CYP2C19 genetic testing. Patients could have been on aspirin monotherapy (81 mg every day) or on DAPT with aspirin (81 mg every day) and clopidogrel (75 mg every day); patients who were not on any antiplatelet medication were treated with aspirin 325 mg the morning of the procedure. Patients with stable ischemic heart disease and patients with non-ST-segment elevation acute coronary syndrome (ACS) were eligible. Only patients undergoing urgent/emergent coronary angiography that would not allow for genetic testing results to be available at the time of PCI were excluded (e.g., patients undergoing primary PCI, cardiogenic shock). Patients meeting study entry criteria underwent rapid genetic testing using the Spartan RX assay (Spartan Bioscience, Ottawa, Ontario, Canada). The Spartan RX assay identifies the following CYP2C19 alleles: *1, *2, *3, and *17. The most common LOF alleles are *2 and *3. Therefore, carriers of *2 or *3 LOF carrier status (homozygotes [*2/*2, *3/*3], or *2/*3) or heterozygotes (*1/*2, *1/*3, *2/*17, *3/*17) were considered eligible for randomization if they proceeded with PCI. Patients who were noncarriers of LOF alleles (*1/*1, *1/*17, or *17/*17) were not eligible for randomization and considered as screen failures and treated per standard of care; similarly, patients with CYP2C19 LOF alleles who did not undergo PCI were considered as screen failures and treated per standard of care.

Patients identified to be CYP2C19 LOF allele carriers and undergoing PCI were randomly assigned 1:1 using a computer-based randomization system to either prasugrel (60 mg loading dose to 10 mg daily maintenance dose) or ticagrelor (180 mg loading dose to 90 mg twice a day maintenance dose). Randomization was stratified according to baseline antiplatelet therapy (aspirin alone vs. DAPT with aspirin and clopidogrel). Loading dose administration was given immediately after PCI as per local standard of care. Randomized patients underwent PD testing at 5 time points: 1) baseline (before initiating the PCI procedure and loading dose administration of antiplatelet therapy); 2) 30 min after loading dose administration; 3) 2 h after loading dose administration; 4) 24 h after loading dose administration or at hospital discharge (whichever came first); 5) during routine clinical follow-up 1 to 4 weeks after PCI while on maintenance dose antiplatelet therapy. At 24 h and at follow-up, blood was collected before the morning dose of prasugrel or ticagrelor to measure trough levels of platelet inhibition. Laboratory personnel were blinded to treatment assignments. Compliance to randomized treatment was assessed by pill count and patient interview. After completing the study, the choice and length of DAPT were left at the discretion of the treating cardiologist. The study complied with the Declaration of Helsinki, was approved by the University of Florida Institutional Review Board, and all patients gave their written informed consent. A flow diagram of the study design is illustrated in Figure 1.

GENETIC AND PD TESTING. Spartan RX rapid genotyping. Spartan RX (Spartan Bioscience Inc.) defines CYP2C19 (*1, *2, *3, *17) allele status within 1 h. This test consists of 4 separate steps intended to be done in <8 min: acquisition of a buccal swab; insertion of the swab into the cartridge; insertion of the reaction solution into the device; and analysis of CYP2C19 genotype triggered by a button on the device. In this study, patients with the *2 or *3 LOF carrier status (homozygotes [*2/*2, *3/*3], or *2/*3) or heterozygotes (*1/*2, *1/*3, *2/*17, *3/*17) were considered eligible for randomization, whereas noncarriers of LOF alleles (*1/*1, *1/*17, or *17/*17) were not (28-30).

VerifyNow point-of-care testing. The VerifyNow System is a turbidimetric-based optical detection system that measures platelet-induced aggregation as an increase in light transmittance (Accriva, San Diego, California), and was used according to the manufacturer’s instructions (29). The assay is based on microbead agglutination and uses specific reagents for the pathways of interest. The instrument measures this change in optical signal and reports results
in P2Y12 reaction units (PRUs). HPR was defined by PRU > 208 (31).

**STUDY ENDPOINTS AND DETERMINATION OF SAMPLE SIZE.** The primary endpoint of the study was the noninferiority in platelet reactivity, measured as PRU, at 24 h or hospital discharge (whichever came first) of prasugrel versus ticagrelor among LOF allele carriers. Under the assumption of 0 difference in mean PRU between treatments and a common standard deviation of 50 PRU, a sample size of 60 patients with valid data would allow for the 95% confidence interval (CI) to stay within 40 PRU with an 85% power and alpha = 0.025 (27,32). Considering up to a 25% rate of invalid results due to hemolysis or drop-out, we planned to randomize up to a total of 80 patients to ensure complete data for analysis. Noninferiority was assessed using a 95% CI of the difference in mean PRU between the 2 groups. The 40 PRU noninferiority margin was defined according to previously published studies (33). Exploratory endpoints included assessment of PD differences between prasugrel and ticagrelor at 30 min, 2 h, and 1 to 4 weeks, and rates of HPR at all study time points.

**STATISTICAL ANALYSIS.** Continuous variables are expressed as a mean ± SD or median (interquartile range) as appropriate. Categorical variables are expressed as frequencies and percentages. Comparisons between continuous variables was performed using Student’s t-test or Wilcoxon rank-sum test. Comparisons between categorical variables were performed using chi-square test or Fisher exact test. Missing data were not imputed. An analysis of variance method with a general linear model, with treatment as the main effect, was used to evaluate the primary noninferiority endpoint as well as all superiority between-group comparisons at each time point. Least squares mean differences in PRU between groups and the corresponding 2-sided 95% CI for the difference was obtained based on the analysis of variance model and used to assess noninferiority. The p values are used to report superiority testing, and a 2-tailed p value of <0.05 is considered to indicate a statistically significant difference for all the analyses performed. The PD population included all patients with PD data and without a major protocol deviation thought to significantly affect the effects of ticagrelor or prasugrel. The PD population was used for analysis of all primary and exploratory PD variables. Statistical analysis was performed by our group using SPSS v24.0 software (SPSS Inc., Chicago, Illinois).

**RESULTS**

Between March 2014 and September 2018, a total of 781 consecutive patients scheduled for left heart catheterization with the intent to undergo PCI were genotyped. Of these, 222 (28.5%) patients were carriers of at least 1 LOF: 9% were homozygotes (*2/*2, n = 20) and 91% were heterozygotes (*1/*2, n = 189; *1/*3, n = 1; *2/*17, n = 32). Of the cohort of CYP2C19 LOF carriers, 157 patients did not meet criteria to be randomized. Thus, a total 65 patients underwent PCI and were randomized to either prasugrel (n = 32) or ticagrelor (n = 33). These patients represented the PD population of the study (Figure 2). Baseline characteristics of the PD population are summarized in Table 1. CYP2C19 LOF carriers who were not randomized were more likely to be female and less likely to have ACS compared with those who were randomized (Supplemental Table 1).

In the PD population, 8 patients (12%) were homozygotes for *2/*2, and 57 (88%) were heterozygotes (*1/*2: 78.5%; *1/*3: 1.5%; *2/*17: 8%). Fifty-one (78.5%) and 14 (21.5%) patients were on aspirin or DAPT before randomization, respectively. No ischemic or Bleeding Academic Research Consortium type 2 to 5 bleeding events were observed; 3 patients (9%) receiving ticagrelor experienced dyspnea, which led to drug discontinuation in 1 patient, versus none.
of those receiving prasugrel; 1 patient receiving prasugrel had a stroke; 2 patients had chest pain during follow-up that did not require any intervention (prasugrel, n = 1; ticagrelor, n = 1).

**PHARMACODYNAMIC RESULTS.** At baseline, PRU levels were similar between groups. A significant reduction in PRU was observed as early as 30 min following loading dose for both prasugrel (p = 0.018) and ticagrelor (p = 0.029). PRU levels continued to markedly reduce to a similar extent with no differences between groups at 2 h and 24 h (or hospital discharge) after loading dose administrations and remained low at follow-up while on maintenance therapy (Figure 3). Median time between study drug loading dose and primary end point sample was 20 h (interquartile range: 7 to 22 h). Mean PRU levels at this time point were 33 ± 56 for prasugrel versus 36 ± 41 for ticagrelor (mean difference = −3; 95% CI: −28 to 22; p = 0.814 for superiority) meeting the primary endpoint of noninferiority. HPR rates also significantly reduced over time, with no differences between groups at any time point (Figure 4). Results were consistent irrespective of baseline antiplatelet treatment regimen (aspirin monotherapy or DAPT; Supplemental Figure 1), with no cohort by treatment group interaction (p for interaction >0.05 for all time points)

**DISCUSSION**

The implementation of genotype-guided selection of P2Y₁₂-inhibiting therapy in patients undergoing PCI has been limited in real-world clinical practice by the availability of assays able to provide results of CYP2C19 genotypes in a timely fashion (16–18). Patients most commonly undergo ad hoc PCI immediately following diagnostic angiography, which further emphasizes the importance of having readily available genotyping results. Earlier small-scale investigations have suggested the clinical utility of genotype-guided selection of oral P2Y₁₂ inhibitors in patients undergoing PCI (34–37). However, some of these were conducted using genotyping approaches that would require several days, making results unavailable until after hospital discharge (34,35). This has important implications. First, switching antiplatelet treatment after hospital discharge is of limited practicality; second, the early post-PCI period is when the risk for thrombotic complication is
highest, underscoring the importance of optimized antiplatelet therapy during this time frame; third, adherence is improved when medications are prescribed before hospital discharge (38,39). The results of our investigation overcome these limitations and support the feasibility of using a rapid CYP2C19 genotyping assay among both patients with stable coronary syndrome and patients with ACS undergoing diagnostic coronary angiography, with intent to undergo ad hoc PCI, in real-world clinical practice. In particular, such rapid bedside genetic testing assay allowed for very rapid turnaround times of results, with patients approached the same day of their procedure and availability of CYP2C19 genotypes within 1 h and before patients undergo PCI. This in contrast to other studies assessing the feasibility of rapid bedside genetic testing, in which results were not available before the start of the PCI procedure (29,30,40). Therefore, similar to other standard laboratory tests (e.g., complete blood count, creatinine, liver function, coagulation panel) performed in these patients, in our study, genotyping results were readily available when the decision on choice of oral P2Y₁₂-inhibiting therapy most commonly occurs (i.e., at the time of PCI).

The use of platelet function or genetic testing to tailor the selection of P2Y₁₂-inhibiting therapy as a strategy to improve outcomes has been a subject of investigation for more than a decade (31). Although earlier studies of platelet function testing, mostly evaluating a strategy of escalation of P2Y₁₂-inhibiting therapy, have failed to show any clinical benefit, the TROPICAL ACS (Testing Responsiveness to Platelet Inhibition on Chronic Antiplatelet Treatment for Acute Coronary Syndromes) trial demonstrated that a strategy of platelet function guided de-escalation was noninferior to maintaining conventional nonguided treatment at 12 months in patients with high-risk ACS undergoing PCI (41). The results of this study led an update in revascularization guidelines that now indicate that platelet function testing can be considered to help guide the decision on choice of antiplatelet therapy in patients with ACS who cannot take the more potent P2Y₁₂ inhibitors (42). However, there are some limitations associated with platelet function guided de-escalation. First, following de-escalation, clopidogrel maintenance therapy needs to be maintained for a certain period for it to achieve its full antiplatelet effects before performing platelet function testing to assess responsiveness, and if patients have inadequate platelet inhibition they would need to be switched back to a more potent P2Y₁₂ inhibitor; second, patients with inadequate response to clopidogrel during this time frame are exposed to an increased risk of thrombotic complications (43). The presence of inadequate clopidogrel response can be further exacerbated by a drug-drug interaction that may occur with de-escalation from ticagrelor to clopidogrel (33). Indeed, using a genotype-guided approach overcomes these shortcomings, as also endorsed by expert consensus recommendations (31). Importantly, the clinical impact of a genotype-guided de-escalation approach was recently demonstrated in the POPular Genetics (Patient Outcome after primary PCI Genetics) study (40). In particular, this study was the first adequately powered randomized study of a CYP2C19 genotype-guided strategy for selection of oral P2Y₁₂ inhibitor (LOF carriers received

| TABLE 1 Baseline Characteristics of the PD Population |
|---------------------------------|---------------------------------|------------|
|                                  | Prasugrel (n = 32)              | Ticagrelor (n = 33) |
| Age, yrs                        | 60 ± 9                          | 58 ± 8 |
| Gender, male                    | 25 (78)                         | 26 (79) |
| BMI, kg/m²                      | 31 ± 5                          | 31 ± 5 |
| Race                            | 21 (66)                         | 23 (70) |
| White                           | 9 (28)                          | 8 (24) |
| Black                           | 2 (6)                           | 2 (6) |
| Genotype                        | 0.398                           |          |
| *1/*2                           | 24 (75)                         | 27 (82) |
| *1/*3                           | 0 (0)                           | 1 (3)   |
| *2/*17                          | 4 (12.5)                        | 1 (3.0) |
| *2/*2                           | 4 (12.5)                        | 4 (12.0) |
| ACS                             | 17 (53)                         | 18 (54) |
| SHHD                            | 15 (47)                         | 15 (46) |
| Aspirin                         | 25 (78)                         | 26 (79) |
| DAPT                            | 7 (22)                          | 7 (21)  |
| Hypertension                    | 28 (87)                         | 29 (88) |
| Dyslipidemia                    | 20 (62)                         | 24 (73) |
| Active smoking                  | 11 (34)                         | 9 (27)  |
| Diabetes mellitus               | 14 (44)                         | 10 (30) |
| Prior MI                        | 10 (31)                         | 5 (15)  |
| Prior PCI                       | 11 (34)                         | 15 (45) |
| Prior CABG                      | 7 (22)                          | 8 (24)  |
| Creatinine, mg/dl               | 1.0 ± 0.3                       | 0.9 ± 0.2 |
| Platelet count, × 10³/μl        | 232 ± 70                        | 213 ± 45 |
| Hematocrit, %                   | 41 ± 5                          | 42 ± 3  |
| Medications*                    |                                 |          |
| Insulin therapy                 | 6 (19)                          | 5 (15)  |
| OAD                             | 8 (25)                          | 9 (27)  |
| Beta-blockers                   | 14 (44)                         | 20 (61) |
| ACE-I/ARB                       | 16 (50)                         | 19 (58) |
| Statins                         | 20 (62.5)                       | 25 (76) |

Values are mean ± SD and or n (%). *Medications at the time of coronary angiography.

ACE-I = angiotensin converting enzyme inhibitor; ACS = acute coronary syndrome; ARB = angiotensin receptor blocker; BMI = body mass index; CABG = coronary artery bypass graft; DAPT = dual antiplatelet therapy; MI = myocardial infarction; OAD = oral antidiabetic drugs; PD = pharmacodynamics; PCI = percutaneous coronary intervention; SHHD = stable ischemic heart disease.
ticagrelor or prasugrel and noncarriers received clopidogrel) and showed this strategy to be noninferior to standard treatment with ticagrelor or prasugrel at 12 months with respect to thrombotic events and resulted in a lower incidence of major or minor bleeding events among patients with ST elevation myocardial infarction undergoing primary PCI (40). Indeed, it may be argued that this and other studies support the feasibility of performing rapid bedside genotyping; however, none of these executed the test routinely before undergoing diagnostic coronary angiography enabling in-laboratory guidance of P2Y12-inhibiting therapy in patients requiring PCI (29,30,40).

Although prior investigations have assessed the comparative PD effects of prasugrel and ticagrelor, yielding conflicting findings (26,27), this is the first study to assess this specifically among CYP2C19 LOF carriers. We observed no differences in platelet reactivity between prasugrel and ticagrelor among CYP2C19 LOF carriers undergoing PCI during the entire study time-course assessing the effects of both the loading and maintenance doses of these agents. Although CYP enzymes are involved in prasugrel metabolism, which could potentially affect its PD effects, the contribution of CYP2C19 is minimal, as also supported by the very high levels of platelet inhibition achieved with prasugrel in our study (44). Moreover, our investigation confirms results of prior studies on the prompt and enhanced platelet inhibitory effects associated with escalation from clopidogrel to prasugrel or ticagrelor, leading to a reduction in HPR rates when a loading dose is used (27,28).

In addition to the POPular Genetics study, in which ticagrelor was the most commonly used potent P2Y12 inhibitor among patients who were CYP2C19 LOF carriers, our study observations also have important clinical implications in light of the recently reported ISAR REACT 5 ( Intracoronary Stenting and Antithrombotic Regimen: Rapid Early Action for Coronary Treatment 5) study (45). ISAR REACT 5 had hypothesized that ticagrelor would reduce ischemic events to a greater extent than prasugrel in patients with ACS undergoing invasive management in part based on the assumption of the enhanced antiplatelet effects of ticagrelor (45). However, ISAR REACT 5 failed to demonstrate its study hypothesis and actually showed significantly reduced ischemic events with prasugrel over ticagrelor at 12 months without differences in bleeding. A number of reasons have been provided to explain such study findings, including compliance issues (once-daily administration of prasugrel vs. twice-daily with ticagrelor), increased rate of nonbleeding side effects with ticagrelor (i.e., dyspnea) leading to drug discontinuation, and differential pharmacologic profile (short half-life, reversibility
of action, and drug interactions with ticagrelor). Our current investigation, showing no differences in PD effects between prasugrel and ticagrelor among CYP2C19 LOF carriers, is in line with prior investigations from our group assessing the comparative PD effects between these agents not stratified according to CYP2C19 genotype (27). Overall, these observations may have practical and cost implications, given that prasugrel is now available in a generic formulation in many countries, making it an attractive treatment option, including among physicians who elect an alternative agent to clopidogrel based on results of genetic testing.

**STUDY LIMITATIONS.** This study was not designed to assess the clinical outcomes of a genotype-guided strategy in patients undergoing PCI, which is the objective of a number of ongoing investigations (23–25). Moreover, it was not designed to compare the safety and efficacy of prasugrel versus ticagrelor. Our study used only the VerifyNow assay for PD assessments. However, the choice to use only this rapid bedside assay was in line with the overall scope of this investigation to consider tests, both PD and genetic, of simple and practical utility, as well as fast turnaround. Ultimately, there are more genetic polymorphisms of CYP2C19 than those assessed by the Spartan RX assay. However, their prevalence is extremely rare and of unclear clinical significance (31).

**CONCLUSIONS**

Rapid CYP2C19 genotyping using the Spartan assay is feasible in providing results in a timely fashion in a real-world clinical practice of patients undergoing coronary angiography. Among patients with CYP2C19 LOF carrier status undergoing ad hoc PCI in this setting, prasugrel and ticagrelor markedly reduce levels of platelet reactivity to a similar extent with no differences between groups. Ongoing large-scale clinical investigations will help define the safety and efficacy of using genetic testing to individualize the choice of oral P2Y12-inhibiting therapy in patients undergoing PCI.

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COMPETENCY IN MEDICAL KNOWLEDGE: The implementation of genotype-guided selection of P2Y₁₂- inhibiting therapy in patients undergoing PCI has been limited in real-world clinical practice by the availability of assays able to provide results of CYP2C19 genotypes in a timely fashion. The results of our investigation overcome these limitations and support the feasibility of using a rapid genotyping assay among both patients with stable coronary syndrome and patients with ACS undergoing diagnostic coronary angiography, with intent to undergo ad hoc PCI, in real-world clinical practice. In our study, genotyping results were readily available when the decision on choice of oral P2Y₁₂-inhibiting therapy most commonly occurs (i.e., at the time of PCI). The potential clinical benefits of a genotype-guided approach have been recently demonstrated. However, none of these performed the test routinely before undergoing diagnostic coronary angiography, enabling in-laboratory guidance of P2Y₁₂-inhibiting therapy in patients requiring PCI. Although CYP enzymes are involved in prasugrel metabolism, which could potentially affect its PD effects, our study showed no differences in platelet reactivity between prasugrel and ticagrelor among CYP2C19 LOF carriers undergoing PCI during the entire study time-course, assessing the effects of both the loading and maintenance doses of these agents. These observations may have practical and cost implications, given that prasugrel is now available in a generic formulation in many countries, making it an attractive treatment option, including among physicians who elect an alternative agent to clopidogrel based on results of genetic testing.

TRANSLATIONAL OUTLOOK: Our current investigation demonstrating no differences in PD effects between prasugrel and ticagrelor in this subset of patients, which is in line with prior investigations from our group assessing the comparative PD effects between these agents not stratified according to CYP2C19 genotype, support that recent trial findings showing significantly reduced ischemic events with prasugrel over ticagrelor cannot be attributed to differential levels of P2Y₁₂ inhibition. However, our study was not designed to assess the clinical outcomes of a rapid genotype-guided strategy in patients undergoing PCI. Moreover, it was not designed to compare the safety and efficacy of prasugrel versus ticagrelor in CYP2C19 LOF carriers, which would also warrant further investigation in large-scale clinical trials.

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**KEY WORDS** genotype testing, percutaneous coronary intervention, pharmacodynamics, prasugrel, ticagrelor

**APPENDIX** For a supplemental table and figure, and other material, please see the online version of this paper.
Tailored Antiplatelet Therapy in Patients Undergoing PCI

Is Rapid Bedside Genetic Testing the Answer?

David P. Faxon, MD

Prolonged dual antiplatelet therapy after stent placement is the standard of care for all patients due to a significant reduction in major cardiovascular events largely due to prevention of early and late stent thrombosis. The combination of the P2Y12 platelet inhibitor clopidogrel and aspirin has been the preferred therapy. However, it has been recognized that 10% to 40% of patients have resistance to clopidogrel, which results in an inadequate reduction in platelet aggregation (high on treatment platelet reactivity [HPR]) and results in significant increase in major adverse cardiac events following percutaneous coronary intervention (PCI) (1). A number of genetic polymorphisms of cytochrome 450 enzyme system, a key modulator of the conversion of the clopidogrel pro-drug into the active compound have been identified. In particular CYP2C19*2 loss-of-function (LOF) allele is associated with slow metabolism of clopidogrel whereas CYP2C19*1/*1 or CYP2C19*17 are gain-of-function alleles associated with more rapid metabolism. Other polymorphisms such as the membrane transporter ABCB1 have been shown to alter clopidogrel metabolism as well. Unlike clopidogrel, the newer P2Y12 inhibitors, prasugrel and ticagrelor, do not have this limitation. Prasugrel is more potent than clopidogrel, is absorbed faster, and is metabolized into the active metabolite primarily by the P450 enzymes CYP3A4 and 2B6. Ticagrelor is an adenosine triphosphate analog that is not a pro-drug and binds reversibly to the P2Y12 receptor. They both have been shown in large randomized trials to reduce major adverse events to a greater extent than clopidogrel does, although at the expense of more bleeding. Both the American Heart Association and European Society of Cardiology guidelines recommend ticagrelor and prasugrel as preferred agents in patients with acute coronary syndromes (ACSs), whereas clopidogrel is recommended in patients with stable angina and those requiring oral anticoagulation (Class I, Level of Evidence: B). The limitation of prasugrel and ticagrelor is that they are more expensive and both increase bleeding risk, which is an independent risk for adverse outcomes.

Tailored therapy using prasugrel or ticagrelor in patients with HPR on clopidogrel and clopidogrel in those without HPR on clopidogrel and clopidogrel in those without HPR might be a way to maximize effectiveness and safety. Platelet testing has not been common practice because it takes at least 24 h to reach a steady state and this exposes the patient to risk because the greatest risk of thrombotic complications is early after PCI. Genetic testing is an attractive alternative because it could be done prior to initiating therapy. This has not been feasible in the past due to a prolonged assay time and the lack of availability of testing. Recently a rapid bedside genetic test (Spartan RX; Spartan Bioscience Inc., Ottawa, Ontario, Canada) for CYP2C19*1, *2, *3, and *17 has been developed that allows determination of CY2C19 LOF alleles within an hour. This allows genetic testing to be done prior to PCI even in ACS.

The POPular Genetics (Patient Outcome After Primary PCI Genetics Study) was a large trial comparing genotype-guided use versus standard use of prasugrel of ticagrelor in patients with primary PCI (2). The genotyping was done at a central lab or using the Spartan Xl device. It showed that a genotype-guided strategy with use of clopidogrel in patients without...
CYP2C19 LOF alleles and ticagrelor or prasugrel in those with LOF was noninferior for thrombotic events and had a lower incidence of bleeding. Likewise the PHARMCLO (Pharmacogenetics of Clopidogrel in Acute Coronary Syndromes) trial also showed that in patients with ACS, a pharmacogenomic approach compared with a standard approach resulted in lower major adverse cardiac event rates (15% vs. 25%) and lower bleeding (3). This trial was underpowered and should be interpreted cautiously. These and other studies have led to increased enthusiasm to use of a genotype-guided approach.

The use of genotyping to determine the best antiplatelet agent relies on the prasugrel and ticagrelor being highly effective in those with CYP2C19 LOF alleles. This has not been previously studied. In this issue of JACC: Basic to Translational Science, Franchi et al. (4) identified 223 of 781 patients (28.5%) undergoing PCI who had CYP2C19 LOF alleles using rapid bedside genotyping. The patients were randomized to either prasugrel or ticagrelor, and platelet aggregation was measured serially over 24 h and then at 1 to 4 weeks. Both agents showed a rapid inhibition of platelet aggregation by 24 h without loss of effectiveness over time. The study further demonstrated that rapid genotyping was possible even in patients with ACS undergoing urgent catheterization.

Although CYP2C19 genotyping is feasible, is it the optimal way to identify nonresponders to clopidogrel? Studies have suggested that CYP2C19*2 carrier status only explains a small fraction of HPR whereas clinical factors account for most of the variability in platelet function testing. Platelet function testing is the direct way to determine adequate platelet inhibition regardless of the cause. Rapid bedside assay using the VerifyNow device (Accriva, San Diego, California) are readily available in many catheterization labs. The primary limitation is the need to wait for at least 24 h to determine effectiveness, another approach might be to start all patients on ticagrelor or prasugrel and then de-escalate to clopidogrel later. This was tested in the TROPICAL-ACS (Testing Responsiveness to Platelet Inhibition on Chronic Antiplatelet Treatment for Acute Coronary Syndromes) trial where patients were randomized to prasugrel or ticagrelor, and platelet aggregation was measured serially over 24 h and then at 1 to 4 weeks. Both agents showed a rapid inhibition of platelet aggregation by 24 h without loss of effectiveness over time. The study further demonstrated that rapid genotyping was possible even in patients with ACS undergoing urgent catheterization.

The routine use of the more powerful agents in all patients is a simple solution but cost and side effects (particularly with ticagrelor) and increased bleeding is a concern. Cost has become less of an issue recently because prasugrel is now generic in the United States and the cost is only twice that of generic clopidogrel ($20 to $30 vs. $9 to $10 per month) whereas ticagrelor remains 35× more costly.

The relationship between genetic testing and platelet function is loose and neither test alone is adequate for optimizing therapy. For instance, 14% of patients on prasugrel in TROPICAL-ACS had high on-treatment platelet aggregation. Conversely, a significant percentage of patients on prasugrel and ticagrelor have excessive platelet inhibition that has been shown to increase bleeding. One option might be to do an initial screen with genetic testing that would identify those patients with CYP2C19 LOF alleles who would receive prasugrel or ticagrelor and those who do not have LOF alleles who would start clopidogrel. Based on trials, this would preserve efficacy in all patients. This is the approach that the on-going TAILOR-PCI trial (Tailored Antiplatelet Therapy Following PCI; NCT01742117) is studying using ticagrelor. The trial is planned to be presented shortly. An additional option to further reduce bleeding risk would be to de-escalate those patients with LOF on prasugrel or ticagrelor who have a high bleeding risk to clopidogrel after 1 month when the risk of ischemic events falls significantly. Platelet function testing could be done at this point to verify effectiveness. Another approach might be to not de-escalate but to discontinue aspirin at 3 months and continue the potent P2Y12 agent as was done in TWILIGHT (Ticagrelor With Aspirin or Alone in High-Risk Patients After Coronary Intervention). These approaches would maximize antiplatelet therapy while reducing bleeding in those at risk.

The future is to develop more personalized approach to therapy, and integrating rapid genetic testing into the choice of antiplatelet agents is the first step. Further study is clearly needed to determine the optimal integration that can improve efficacy and reduce adverse effects that is practical and cost-effective. Time will tell, but I am optimistic.

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KEY WORDS antiplatelet therapy, clopidogrel resistance high on treatment platelet reactivity, genotyping
Cardiac Fibrosis Is Associated With Decreased Circulating Levels of Full-Length CILP in Heart Failure

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VISUAL ABSTRACT

HIGHLIGHTS

- After in vitro stimulation or in vivo pressure overload injury, activated cardiac fibroblasts express Ltpb2, Comp, and Cilp.
- In ischemic heart disease, LTBP2, COMP, and CILP localize to the fibrotic regions of the injured heart.
- Circulating levels of full-length CILP are decreased in patients with heart failure, suggestive of the potential to use this protein as a biomarker for the presence of cardiac fibrosis.

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Summary

Cardiac fibrosis is a pathological process associated with various forms of cardiac disease that contributes to impaired cardiac function, development of arrhythmias, and ultimately heart failure (1,2). The formation of fibrosis can be initiated by either an acute ischemic event to the heart, such as myocardial infarction, or through a chronic progression driven by increased cardiac load. Cardiac fibroblasts (CFBs) are the main participating cells in the development of myocardial fibrosis (3). Under homeostatic conditions, resident CFBs are responsible for maintaining the structural integrity of the heart by regulating extracellular matrix (ECM) production (4). However, under pathological conditions, CFBs become activated, proliferate, and secrete an excess amount of ECM proteins, contributing to scar tissue (3,4). This scar replaces healthy myocardium, renders the substrate arrhythmogenic, induces stiffening of the heart, and leads to adverse remodeling. Collectively, the sequelae of fibrosis can have deleterious effects on the ability of the heart to pump blood effectively and hinders the recovery of cardiac function. There are currently limited treatment options for the reversal of cardiac fibrosis, and available therapies for heart failure are ineffective at preventing the formation of scar tissue (5). It has been suggested that identifying diagnostic markers for fibrosis may provide prognostic value for clinicians (6). Considering the critical role of CFBs in myocardial fibrosis, we hypothesized that CFBs may release factors that could serve as promising biomarkers for cardiac fibrosis (7). Identification of circulating biomarkers would serve as a noninvasive clinical tool to determine the presence, extent, and progression of fibrosis in cardiac disease patients.

In the present study, we isolated CFBs from C57BL/6J mice that underwent transverse aortic constriction (TAC), a pressure overload injury model, or sham operation and performed ribonucleic acid (RNA) sequencing to identify key up-regulated genes in response to injury (8). From this data, we identified 3 genes encoding secreted proteins that could be potential biomarkers for myocardial fibrosis: latent transforming growth factor (TGF)-β binding protein 2 (Ltbp2), cartilage oligomeric matrix protein (Comp), and cartilage intermediate layer protein 1 (Cilp). Ltbp2 is part of the LTBP family, which consists of key regulators of TGF-β signaling (9). Comp and Cilp are mainly known for their roles in the binding of specific ECM proteins, such as collagens, in cartilage (10,11). Ltbp2, Comp, and Cilp were upregulated in cultured murine CFB and in the fibrotic regions of TAC hearts, suggesting that their expression is specific to the formation of scar. Furthermore, there was an increase in expression of these proteins in stimulated human CFBs and within the scar tissue of transverse aortic constriction (TAC) mice, suggesting that their expression is specific to the formation of scar. Furthermore, there was an increase in expression of these proteins in stimulated human CFBs and within the scar tissue of transverse aortic constriction (TAC) mice, suggesting that their expression is specific to the formation of scar.
METHODS

STUDY APPROVALS. All mouse surgery procedures were carried out with the approval of the University of California, Los Angeles Animal Research Committee or the Institutional Animal Care. The study was approved by an institutional review board (12-001164) and human participants gave written informed consent.

RNA SEQUENCING AND ANALYSIS. CFBs were isolated from murine hearts, as previously described, for RNA sequencing (8). These data are publicly available on Gene Expression Omnibus (GSE51620). Downstream analysis was conducted using the DESeq2, Enhanced Volcanoplot and gplots R packages (12,13). Detailed strategy for identifying potential gene targets is described in the Supplemental Methods.

MICE. Adult C57BL/6J mice (age 8 to 12 weeks) were used for all experiments. For the in vivo experiments, mice were randomly assigned into sham, TAC, and ischemic reperfusion treatment groups. No phenotypic differences were observed between male and female mice. Details of surgery are described in the Supplemental Methods. All procedures were carried out with the approval of the University of California, Los Angeles Animal Research Committee or the Institutional Animal Care.

CFBs CULTURE AND TGFβ TREATMENT (MURINE AND HUMAN). For mouse explant fibroblast cultures, hearts were collected, digested, and plated as described in the Supplemental Methods. Twelve hours after plating, the floating cells were removed, and the media was replaced. Media changes were done every other day until cells reached 80% confluency, at which point, they were passaged and cultured in serum-free media for 24 h prior to TGF-β treatment (50 ng/ml; Cell Signaling Technology, Danvers, Massachusetts). Throughout the TGF-β treatment, the media was changed daily. Human fibroblasts were cultured according to the company’s instructions (Cell Applications, San Diego, California) and similarly passaged for TGF-β treatment (10 ng/ml; R&D Systems, Minneapolis, Minnesota).

RNA EXTRACTION AND REVERSE TRANSCRIPTION-QUANTITATIVE PCR. RNA was extracted from cells using TRizol LS Reagent (Thermo Fisher Scientific, Waltham, Massachusetts) and following the manufacturer’s instructions. RNA was quantified by NanoDrop, and complementary DNA was prepared using the iScript Reverse Transcription Supermix kit (BioRad Laboratories, Hercules, California). Reverse transcription-quantitative polymerase chain reaction (PCR) reactions were prepared using SYBR Green (Bio-Rad) and primers (Integrated Device Technology, San Jose, California) unique for each gene of interest (Supplemental Table 1). The reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad) and relative gene expression data were calculated by double delta computed tomography analysis.

IMMUNOCYTOCHEMICAL AND IMMUNOFLUORESCENCE STAINING. Cells were cultured on 8-well chamber slides (Falcon, Fisher Scientific, Thermo Fisher Scientific) and washed with phosphate-buffered saline prior to fixation with 4% paraformaldehyde. For in vivo staining, murine hearts were isolated and fixed with 4% paraformaldehyde overnight prior to being incubated in 30% sucrose and embedded in Optimal Cutting Temperature compound (Fisher). Hearts were sectioned at a thickness of 8μm in a cryostat, mounted on Colorfrost Plus microscope slides (Fisher), and stored at −20°C until ready to stain. Detailed staining protocol is provided in the Supplemental Methods. Slides were incubated with antibodies outlined in Supplemental Table 2.

ELISA AND WESTERN BLOT. Protocols for conditioned media and serum sample preparation are described in the Supplemental Methods. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from MyBiosource (San Diego, California) and the manufacturer's protocol was followed. For Western blot, protein concentration was measured by a Pierce BCA Protein assay kit (Thermo Fisher Scientific) and 20 μg was loaded into each well of 4% to 20% Mini-PROTEAN TGX Precast Protein gels (Bio-Rad). After transferring the gel onto a polyvinylidene fluoride membrane, detection of CILP was conducted by incubating the membrane with primary antibody followed by secondary antibody conjugated with horseradish peroxidase (Supplemental Table 2). The signal was developed using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

STATISTICAL ANALYSIS. Continuous data are presented using the mean ± SEM and comparisons between groups were performed using Student’s t-test. A p value < 0.05 was considered statistically significant, and data were analyzed using GraphPad Prism 6 (San Diego, California).

RESULTS

MURINE CFBs EXPRESS Ltp2, Comp, AND Cilp AFTER INJURY. To identify secreted proteins expressed by CFBs in fibrotic hearts, we conducted RNA sequencing on isolated CFBs from female C57BL/6J adult mice (8 to 12 weeks old) that had undergone either sham or TAC surgery (n = 3). CFBs

434 Park et al.
CF Associated With Decreased Cilp in HF

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 5, NO. 5, 2020
MAY 2020:432-43
were isolated 7 days after surgery to observe gene expression changes in the early stages of fibrosis (8). After TAC, many genes were differentially expressed in CFBs (Figure 1A, Supplemental Table 3). Specifically, CFBs from mice that had undergone TAC showed higher expression of various genes associated with fibrosis (Figure 1B). From these, we selected genes that encoded for secreted proteins and then further filtered the list to those that were novel in the context of heart failure and had previously reported roles in ECM formation and remodeling. We identified Ltbp2, Comp, and Cilp as potential candidate biomarkers. These results were further validated by reverse transcription-quantitative PCR (Figure 1C).

The TGF-β signaling pathway is a major component of injury response in CFBs (14). Treatment of fibroblasts in vitro with TGF-β activates and induces proliferation of cultured cells, imitating in vivo responses (15). To confirm that the TGFβ signaling pathway stimulates a robust increase in the expression of the identified genes, CFBs from uninjured C57BL/6J mice were cultured in media with or without TGF-β for 72 h (Figure 2A). CFBs were isolated by whole explant culture to encompass the entire...
CFBs population in the heart, rather than a subpopulation \((16)\). TGF-\(\beta\) treatment induced expression of fibroblast activation genes, such as periostin (Postn) and \(\alpha\)-smooth muscle actin (Acta2) \((17)\), as well as Ltbp2, Comp, and Cilp (Figure 2B). Furthermore, immunocytochemistry confirmed that expression of Ltbp2, Comp, and Cilp were increased at the protein level in cultured CFBs after exposure to TGF-\(\beta\) (Figure 2C). We observed similar patterns of staining for these proteins when CFBs were isolated by fluorescence-activated cell sorting (Supplemental Figure 1) \((8)\). These data confirm that CFBs are a cellular source of Ltbp2, Comp, and Cilp under stimulatory conditions.

**Ltbp2, Comp, AND Cilp ARE LOCALIZED TO FIBROTIC REGIONS.** Although TAC surgery induces fibrosis, it also causes other cardiac pathologies, such as hypertrophy \((18)\). To confirm that the increase in Ltbp2, Comp, and Cilp expression after injury was specific to scar formation, we analyzed the anatomic location of Ltbp2, Comp, and Cilp in the hearts of mice that had undergone TAC surgery. After 7 days, there was visible perivascular and interstitial fibrosis in TAC hearts, compared with sham hearts, which exhibited no fibrosis (Figure 3A). Immunofluorescence staining showed minimal expression of the 3 proteins in sham hearts. In TAC hearts, Ltbp2, Comp, and Cilp expression appeared to colocalize with discoidin domain-containing receptor 2 (Ddr2), a marker for fibroblasts \((19)\), and \(\alpha\)-smooth muscle actin \((20)\) within the fibrotic regions of the myocardium in TAC hearts (Figure 3B). Areas of nonfibrotic myocardium in TAC hearts did not stain for any of the target proteins (data not shown), indicating that expression of Ltbp2, Comp, and Cilp are expressed by activated CFBs and localized to regions of fibrosis.

We next sought to determine whether the expression of these biomarkers is also observed in other types of cardiac fibrosis, such as replacement fibrosis after myocardial infarction. Our findings were confirmed in an ischemic reperfusion injury model in which the hearts exhibited discrete areas of fibrosis, although not to the severity of TAC injury. Ltbp2, Comp, and Cilp were found to be specifically colocalized with discoidin domain-containing receptor 2 and \(\alpha\)-smooth muscle actin in hearts that had undergone ischemic reperfusion.
Together, these data suggest that Ltbp2, Comp, and Cilp are expressed by activated CFBs and are localized to regions of fibrosis.

**HUMAN CFBs HAVE INCREASED LTBP2, COMP, AND CILP LEVELS IN RESPONSE TO TGF-β1 TREATMENT.** To confirm the clinical utility of our identified proteins as biomarkers for cardiac fibrosis, we sought to assess their expression levels in human ischemic myocardial tissue. RNA sequencing data of human cardiac tissue from ischemic patients with heart failure in a publicly available database (GSE46224) demonstrated that LTBP2, COMP, and CILP are up-regulated in ischemic hearts (Figure 4A) \(^{(21)}\). We next cultured human CFBs and treated them with TGF-β1 to stimulate their in vitro activation (Figure 4B). TGF-β1 treatment led to morphological changes in human CFBs and induced expression of LTBP2, COMP, and CILP, along with fibroblast activation genes (Figure 4C). Immunocytochemistry staining confirmed the up-regulation of LTBP2, COMP, and CILP in response to TGF-β1 treatment, as seen in mouse CFBs (Figure 4D). Conditioned media from cells that had undergone TGF-β1 treatment did not show significant differences in the levels of LTBP2, an increasing trend of COMP levels, and

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**FIGURE 3** Ltbp2, Comp, and Cilp Localize to the Fibrotic Myocardium

(A) Mice that had undergone TAC surgery exhibited myocardial fibrosis as shown by Masson trichrome staining. (B) Immunofluorescence staining shows colocalization of Ltbp2, Comp, and Cilp (red) with fibroblast marker discoidin domain receptor tyrosine kinase 2 (Ddr2) (green, left) and activated fibroblast marker α-smooth muscle actin (SMA) (green, right). Red channel images are shown separately for clarity. DAPI (blue) stain was used for nuclei. Bars = 50 µm. Abbreviations as in Figures 1 and 2.

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(Supplementary Figure 2). Together, these data suggest that Ltbp2, Comp, and Cilp are expressed by activated CFBs and are localized to regions of fibrosis.
decreased levels of CILP (Supplemental Figure 3). These results may be due to unknown mechanisms of protein secretion that affect the presence of these proteins in the context of our culture protocol. The results from the in vitro culture of human CFBs mirrored our data from mice, further supporting the potential of these proteins to be biomarkers for cardiac fibrosis.

**LTBP2, COMP, and CILP ARE POTENTIAL BIOMARKERS FOR CARDIAC FIBROSIS.** We used immunofluorescence staining to observe the expression of LTBP2, COMP, and CILP within the myocardium of patients with heart failure compared with the myocardium of healthy hearts. Myocardial tissue from patients with heart failure (with a documented diagnosis of ischemic cardiomyopathy) exhibited significant amounts of fibrosis compared with in healthy hearts (Figure 5A). In healthy hearts, we observed no or minimal positive staining for the candidate markers throughout the myocardium (Figure 5B). However, sections from diseased hearts demonstrated a significant increase in expression of all 3 proteins (Figure 5B). Staining for these 3 proteins were localized to disarrayed regions of the myocardium, indicative of the specificity of these proteins for fibrotic areas.
FULL-LENGTH CILP IS SIGNIFICANTLY DECREASED IN SERUM FROM PATIENTS WITH HEART FAILURE. In addition to increased expression within the fibrotic myocardium, we sought to determine the utility of LTBP2, COMP, and CILP as novel circulating biomarkers for cardiac fibrosis. We measured the protein levels in serum from healthy volunteers and patients with heart failure by ELISA (Supplemental Table 4). We observed no significant differences in the circulating levels of LTBP2 or COMP (Figure 6A). However, serum from patients with heart failure exhibited significantly decreased levels of CILP (Figure 6B). Mice that had undergone TAC injury, compared with sham mice, exhibited a similar trend in decreased levels of circulating Cilp (Supplemental Figure 4). The CILP gene encodes a precursor protein that undergoes cleavage into an N-terminal fragment of roughly 75 kDa and a C-terminal fragment of about 55 kDa (23). Both of these fragments were shown to inhibit Smad3 phosphorylation, which is normally promoted by active TGFβ signaling. Whereas commercially available ELISA kits target the C-terminal region of CILP (hence detecting both the C-terminal and the full-length fragment), previous work discovered that CFBs secrete the N-terminal fragment as well as the full-length CILP protein (24). We specifically used an antibody that spans the cleavage site of the CILP precursor and performed Western blotting to confirm levels of circulating full-length CILP. Our results showed that serum from patients with heart failure had significantly decreased levels of full-length CILP in circulation (Figures 6C and 6D). Together, these data suggest while activated fibroblasts in the fibrotic regions of human myocardium express high levels of CILP, the circulating level of CILP is significantly reduced when compared with healthy individuals with no evidence of cardiac fibrosis.

DISCUSSION

With the increasing prevalence of cardiac disease worldwide, there is significant value in identifying a robust biomarker panel to noninvasively measure the presence and progression of cardiac fibrosis. We hypothesized that, as key participants of the fibrotic response, CFBs may be a source of novel biomarkers for myocardial fibrosis. We performed RNA sequencing of CFBs from TAC and sham murine hearts and identified Ltbp2, Comp, and Cilp to be upregulated in hearts after pressure overload injury. The expression of these proteins by CFBs in response

(A) Masson trichrome staining show extensive fibrosis in heart sections of patients with heart failure. Insets are higher magnification images of boxed area. (B) LTBP2, COMP, and CILP (red) expression is significantly increased in hearts undergoing heart failure. DAPI (blue) stain was used for nuclei. Bars ≈ 50 μm. Abbreviations as in Figures 1, 2, and 4.
to injury were validated by in vitro studies in both murine and human CFBs. Additionally, we demonstrated that these proteins localize in fibrotic regions in murine hearts after pressure overload and ischemic reperfusion injury. These findings were further confirmed by high levels of these 3 biomarkers in the fibrotic areas of human ischemic myocardial tissue. Notably, the circulating levels of full-length CILP protein were significantly reduced in the serum of patients with ischemic heart failure compared with the serum of healthy individuals, indicating its potential to be a circulating biomarker.

**LTBP2 AND COMP EXPRESSION IS SPECIFIC TO FIBROTIC REGIONS.** LTBP2 is a member of the latent TGF-β-binding protein family, which consists of key regulators of TGF-β signaling. TGF-β has diverse and pleiotropic effects on various cell types through its binding and activation of TGF-β receptors (14,25). TGFβ is secreted from cells as a multiplex form that is covalently bound to latent TGF-β-binding proteins LTBP1, LTBP3, and LTBP4. These proteins target the latent complex to specific sites for storage within the ECM where it awaits activation. Matrix sequestration of latent TGF-β may serve to regulate its immediate bioavailability while achieving critical threshold concentration at sites of intended function (9,25). However, the functional role of LTBP2 is not well understood. Recent studies suggest that LTBP2 does not bind to latent TGF-β but may interact with other ECM proteins (9). Other studies have additionally reported on the competitive role of LTBP2 with LTBP1 for binding sites on fibrillin-1 within the ECM (26). Our data show strong
support for increased expression of LTBP2 in response to injury and a strong localization of LTBP2 in activated fibroblasts within the fibrotic regions of the myocardium. Whether LTBP2 is merely a surrogate for cardiac fibrosis or is involved in its pathogenesis is not entirely known.

COMP is another ECM protein that is mainly studied in the context of tendons and cartilage (27). The main function of COMP is to directly bind with other ECM components, including collagens and TGF-β1, and to facilitate the stability of the ECM network by the formation of collagen fibrils (28). This role is crucial to maintaining homeostasis of the heart as Comp-knockout mice have been shown to develop dilated cardiomyopathy (29). However, the role of COMP in pathological remodeling is less understood. Studies have shown that COMP is up-regulated in the context of idiopathic pulmonary fibrosis (30) and liver fibrosis (31), although there have been conflicting reports as to whether it can serve as an accurate circulating marker for fibrosis in patients (30,32).

Our results suggest that LTBP2 and COMP both have a strong potential for being markers for cardiac fibrosis as the expression of these proteins are specific to scar formation. However, our data does not support their use as circulating biomarkers after cardiac injury. Although these proteins are known to be secreted, it is possible that they remain within the ECM and participate in the process of fibrosis and scar formation. Further research is warranted to investigate the specific functional contributions of LTBP2 and COMP to the development of cardiac fibrosis. Due to their known roles in other organ systems, it is possible that these proteins may be markers for general fibrosis and not specific to cardiac fibrosis (33).

DECREASED LEVELS OF CILP MAY BE INDICATIVE OF HEART FAILURE. The exact function of CILP within cartilage is still unknown, but it has been implicated in cartilage remodeling and maintenance of the ECM (31,34). The up-regulation of CILP has been found in various disease models including osteoarthritis, idiopathic pulmonary fibrosis, and ischemic heart disease (34-37). However, the contribution of CILP to the development of cardiac fibrosis remains unknown. Whereas most studies suggest that CFBs are the major source of CILP expression in the heart, a recent study has shown evidence of cardiomyocytes being another a major contributor (38,39). Although we did not explore the expression of CILP in cardiomyocytes, our data support the claim that CFBs are a major cellular source of CILP. Several studies have reported that cardiac injury causes an up-regulation of CILP in CFBs but the potential for CILP to be a potential biomarker for fibrosis had not been previously explored (24,39).

The CILP gene encodes for a precursor protein containing a furin cleavage site. The precursor is first synthesized and processed by furin proteases intracellularly prior to being secreted (23). The N-terminal fragment has been shown to directly interact with TGF-β, suppressing TGF-β signaling in CFB, whereas the C-terminal fragment is homologous to a porcine nucleotide pyrophosphohydrolase, which has been reported to have limited enzymatic activity (23,40). In contrast to the 2 fragments, the functional role of full-length CILP protein has not been well studied. The full-length Cilp has been shown to inhibit TGF-β signaling, similarly to the N-terminal fragment, most likely due to the common thrombospondin-1 domain, which has been shown to bind to TGF-β (24). However, further studies to determine any functional differences between the N-terminal fragment and the full-length CILP are required. Our data specifically demonstrate that circulating levels of the full-length CILP are attenuated in patients with heart failure but show an abundance of expression in the fibrotic myocardium. A possible mechanism for this paradox is that full-length CILP is sequestered to the ECM by its binding to TGF-β, therefore reducing circulating levels. Studies have reported that while injury induces increased expression of TGFβ in the myocardium of patients with heart failure (41), circulating TGFβ is reduced (42,43). Due to the inhibitory role of full-length CILP in TGF-β signaling, it is possible that increased levels of CILP may reside in the ECM and promote a negative feedback mechanism to suppress CFB activation (24,44). Further studies on the dynamics of CILP turnover in the ECM are required to elucidate the significance of both circulating and interstitial CILP.

STUDY LIMITATIONS. Our data suggests that circulating levels of full-length CILP are reduced in patients with heart failure. However, our study consists of several limitations. The small sample size limits the statistical power of our analysis and supports a need to conduct additional validation studies on a larger cohort of patients. Furthermore, we did not explore levels of CILP throughout a variety of heart failure etiologies. Conducting larger studies with more patients will provide valuable information to better determine the clinical implications of CILP as a biomarker for the presence of cardiac fibrosis.
CONCLUSIONS

The present study confirms the potential for LTBP2, COMP, and CILP as novel markers of cardiac fibrosis in both mouse and human heart failure models. Most notably, we discovered a significant reduction in serum levels of full-length CILP in patients with heart failure. Our results suggest that LTBP2, COMP, and CILP are worthy of future investigation as participants in cardiac fibrosis and as biomarkers for the development of ischemic heart failure.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Biomarkers for cardiac fibrosis will serve as a noninvasive method to gain diagnostic and prognostic information regarding patients with heart failure. Our study confirms the potential utility of several markers of cardiac fibrosis in mouse and human subjects. Nonetheless, further studies are warranted to validate their clinical utility and investigate the functional role of these proteins.

TRANSLATIONAL OUTLOOK: Our work demonstrates reduced serum levels of CILP in patients with heart failure, raising the exciting possibility that it can be used as a circulating biomarker for myocardial fibrosis. However, further validation studies are warranted to confirm the clinical utility of this marker. In our study, we did not measure the dynamic changes in CILP levels in patient serum during the development of fibrosis nor were we able to examine levels of circulating CILP in large cohorts of patients with different heart failure etiologies. Future studies that include large patient populations with cardiac fibrosis will be valuable to investigate the correlation of CILP levels with their disease progression. These data could provide more support for using serum CILP levels as a diagnostic marker.
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**APPENDIX** For supplemental methods, tables, and figures, please see the online version of this paper.

**KEY WORDS** biomarker, cardiac fibrosis, extracellular matrix protein, heart failure
The clinical and socioeconomic relevance of cardiovascular diseases worldwide eventuated in comprehensive research aiming to unravel molecular mechanisms of developing end-stage heart failure. However, investigations toward improved diagnostic options have been prioritized recently as a first step to enable a fine-tuned personalized treatment. Non-invasive clinical tools are mainly focusing on robust circulating biomarkers, which can be easily detected in patient sera. The major challenge, however, is to identify disease- and organ-specific molecules that are dysregulated in different pathological stages.

One significant hallmark of cardiac diseases is the nascent myocardial fibrosis evolving almost independently of the primary disorder. In general, cardiac fibrosis can be classified according to the anatomical region or based on disease progression—acute or chronic—and, in this context, the functional response of the cardiac fibroblasts (1). Beginning with the latter, the initial and physiological response of fibroblasts to mechanical stress or injury is to stabilize the tissue while preserving the cardiac function. This phase is termed compensated, because the pump function remains stationary, but is accompanied by left ventricular hypertrophy and an activation of the fibroblasts to produce a stabilizing extracellular matrix (ECM). Continuous overload or pumping disability results in the following decompensated phase, making it impossible to achieve a sufficient cardiac output (2,3). A transition from one phase to the next is a complicated process; therefore, the classification of different stages of fibrosis and heart failure is very challenging. Nevertheless, two heterogeneous classifications emerged depending on the phenotype: heart failure with: 1) reduced; or 2) preserved ejection fraction (4). Reduced pump efficiency is mainly caused by ischemic heart events triggering an injury-induced immune response and excessive cardiomyocyte loss. Whereas the second disorder is a chronic progression towards hypertrophy and severe fibrosis, related to diastolic dysfunction. This complexity of fibrotic phenotypes can only be observed by visual diagnostic tools and more specifically by invasive operations to take biopsies for analysis.

To overcome the problematic assessment, the group of Park et al. (5) investigated novel circulating biomarkers in the context of early heart failure stages, allowing specific medication at an early time point of the disease. They performed a ribonucleic acid-sequencing analysis of pressure overload-induced hypertrophic mouse hearts 7 days after transverse aortic constriction and compared the gene expression analysis. The ribonucleic acid-sequencing results were filtered on the basis of novelty in context of heart failure, protein secretion, and reported roles in ECM formation or remodeling. Three different proteins, namely LTBP2 (latent TGF-β-binding protein 2),
COMP (cartilage oligomeric matrix protein), and CILP (cartilage intermediate layer protein 1) were highly up-regulated and further characterized in murine and human in vitro systems as well as in cardiac hypertrophy or myocardial infarction mouse models. Specific up-regulation of all Three genes was confirmed by quantitative polymerase chain reaction and immunocytochemistry stainings, where a functional involvement in the fibrotic response seemed to be most likely. As a proof of clinical relevance, protein levels were additionally analyzed in serum of patients with heart failure. LTBP2 and COMP are both associated with ECM turnover by promoting the formation of dense collagen fibers; however, their levels in human plasma were not significantly altered. Park et al. (5) hypothesized, that these proteins are indeed highly induced and secreted, but instead of being released into the blood vessels, they bind to ECM components and reside within the heart, thus making them unavailable as blood-based biomarkers.

However, CILP seemed to be a promising candidate as a biomarker for cardiac fibrosis because it was significantly up-regulated in cardiac fibroblasts (7,8), which was additionally confirmed by immunocytochemistry stainings of transverse aortic constriction or myocardial infarction hearts. The CILP protein is a precursor for two different variants: specific cleavage at a furin cleavage site leads to a larger N-terminal and a shorter C-terminal fragment, whereas full-length CILP at a furin cleavage site leads to a larger N-terminal and precursor for two different variants: specifically, the N-terminal fragment and full-length CILP are capable of binding TGF-β due to the common thrombospondin-1 domain (7). As TGF-β is the major regulator of the profibrotic response, not only in the heart, these findings are contradictory (10). A possible explanation would be a negative feedback loop, where induction with TGF-β primarily results in the activation of profibrotic programs, but subsequently restricts the signaling (7).

Besides this function, specific yet undiscovered roles in promoting fibrosis are conceivable. In terms of significantly reduced serum levels in patients suffering from heart failure, full-length CILP expression is up-regulated, whereas the secretion might be remarkably diminished, preventing the binding-induced inhibition of TGF-β in the interstitial space. Alternatively, the expression of CILP in diseased hearts might be not sufficient to robustly inhibit the strong effect of TGF-β, reinforced by the findings of Zhang et al. (11) that revealed a cardioprotective effect on Cilp overexpression in mice. Therefore, transformed myofibroblasts maintain a strong TGFβ signaling and further promote cardiac fibrosis.

In summary, the study by Park et al. (5) identified three potential biomarkers as indicators for cardiovascular fibrosis in several disease contexts. The analyzed proteins can serve as early signs for cardiac remodeling, as these were already up-regulated 7 days after transverse aortic constriction surgery in mice. Functions of LTBP2 and COMP have already been reported in different organs; therefore, the markers are only suitable as general fibrosis-associated genes (12). However, specific roles especially for the full-length CILP protein still remain unclear. The underlying mechanism of enhanced gene expression on TGF-β stimulation beginning in the initial phase, combined with reduced serum levels in patients with heart failure warrants further in-depth analysis. A temporal gene expression profile is of major interest, which might allow specific attribution to different pathological stages. Finally, a role in other fibrotic diseases should be considered to evaluate the potential of full-length CILP as a specific biomarker for cardiovascular fibrosis.

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**KEY WORDS** biomarker, cardiac fibrosis, CILP (cartilage intermediate layer protein 1), TGF-β signaling
Role of Blood Oxygen Saturation During Post-Natal Human Cardiomyocyte Cell Cycle Activities

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HIGHLIGHTS

- This study used both human samples and iPSC-CMs to evaluate the effects of hypoxia on CM proliferation.
- The degree of hypoxia and the manner in which O₂ decreases both contribute to the cell cycle activity of post-natal human CMs.
- From the perspective of CM proliferation and protection, it has been unclear why moderate hypoxia (SaO₂:75% to 85%) is targeted by many children’s medical centers when children with CHD are being transported.
- A target was provided that can be used for antioxidant DNA damage to protect the proliferation of post-natal human CMs.
SUMMARY

Blood oxygen saturation (SaO$_2$) is one of the most important environmental factors in clinical heart protection. This study used human heart samples and human induced pluripotent stem cell–cardiomyocytes (iPSC-CMs) to assess how SaO$_2$ affects human CM cell cycle activities. The results showed that there were significantly more cell cycle markers in the moderate hypoxia group (SaO$_2$: 75% to 85%) than in the other 2 groups (SaO$_2$ <75% or >85%). In iPSC-CMs 15% and 10% oxygen (O$_2$) treatment increased cell cycle markers, whereas 5% and rapid change of O$_2$ decreased the markers. Moderate hypoxia is beneficial to the cell cycle activities of post-natal human CMs. (J Am Coll Cardiol Basic Trans Science 2020;5:447–60) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Congenital heart disease (CHD) is the leading cause of birth defect–related mortality (1,2). Although corrective surgery enables young patients to survive most forms of CHD, such patients remain at risk of developing chronic heart failure (3), which is characterized by loss of cardiomyocytes (CMs). In addition, after cardiopulmonary bypass, it is possible that these patients will experience low cardiac output syndrome due to anoxia-induced CM impairment. Low cardiac output syndrome is one of the most serious physiological abnormalities arising from cardiac surgery (4). Recently, our research group (along with other groups) showed that there were significantly more cell cycle active CMs in young patients (5–7). Furthermore, CMs are important contributors to post-natal heart growth (7). Thus, it is essential that young CMs are protected and that their limited proliferative function is maintained during CHD surgery.

Blood oxygen saturation (SaO$_2$) is one of the most important indexes of heart and brain protection during the peri-operative period (8). However, oxygen (O$_2$) is deemed a “double-edged sword” when it comes to cardiac function and repair (9); the effects of hypoxia on CM proliferation are associated with its stage of development (10). Paradis et al. (11) reported that hypoxia/anoxia in newborns inhibits CM proliferation. Conversely, Puente et al. (12) showed that the O$_2$-rich post-natal environment induces CM cell cycle arrest, whereas hypoxia facilitates the proliferation of young CMs. One possible explanation for the conflicting results between these 2 studies is that they used different methods. Paradis et al. (11) exposed neonatal mice to an environment that contained approximately 0.2% O$_2$ for 10 min, whereas Puente et al. (12) exposed mice to an environment that contained approximately 15% O$_2$ for 7 days. It is likely that too much or too little O$_2$ inhibits the cell cycle activities of CMs, and that a moderate supply of O$_2$ represents a more optimal scenario. This is also why many children’s centers aim to supply moderate levels of SaO$_2$ during transportation (13).

When O$_2$ levels increase, mitochondrial oxidative phosphorylation increases accordingly. This phenomenon facilitates increased free radical production, causing increased DNA damage (14,15). Conversely, severe hypoxia results in the down-regulation of antioxidant defenses, making cells vulnerable to oxidative damage and promoting...
increased DNA damage (16,17). Current evidence suggests that DNA damage is a critical factor in the suppression of CM proliferation (12,18). We speculated that dramatic increases or decreases in post-natal O2 contribute to an increase in mitochondrial content, thereby activating the DNA damage response and causing permanent cell cycle arrest in CMs.

Yes-associated protein 1 (YAP1) is closely related to the regulation of CM proliferation (19–21). For example, the study by van Gise et al (20) demonstrated that activated YAP1 can promote the proliferation of mouse CMs after birth, and our previous studies showed that YAP1 plays an important role in the post-natal proliferation of human CMs (6). Studies on neuronal cells have shown that YAP1 degradation plays an important role in DNA oxidative damage by inhibiting neuronal cell proliferation (22,23). For example, Lehtinen et al. (22) and Xiao et al. (23) demonstrated that O2 free radical-mediated DNA damage could activate the Hippo/mammalian STE20-like protein kinase (MST) signaling pathway, which, in turn, phosphorylates and leads to the degradation of YAP1, and ultimately, to the inhibition of neuronal cell proliferation (22,23). Thus, we also set out to investigate the role of YAP1 in hypoxia-induced CM proliferation.

METHODS

Primers, reagents, and antibodies are detailed in Supplemental Tables S1 and S2.

STUDY POPULATION AND TISSUE SAMPLING. We collected 30 right ventricular outflow myocardial tissue specimens from resections that were required to relieve obstruction in patients with tetralogy of Fallot (TOF) at the Shanghai Children’s Medical Center (Shanghai, China) between January 2018 and July 2018. Each specimen was preserved in liquid nitrogen and later divided into 3 portions, which were used for DNA extraction, quantitative polymerase chain reaction (qPCR), and immunofluorescence (IF). All procedures conformed to the principles outlined in the Declaration of Helsinki and were approved by The Animal Welfare and Human Studies Committee at the Shanghai Children’s Medical Center. Parental written informed consent was obtained before study initiation.

CM DIFFERENTIATION, MAINTENANCE, AND O2 TREATMENT OF HUMAN-INDUCED PLURIPOTENT STEM CELLS. We purchased the human-induced pluripotent stem cell (iPSC) line del-AR1034ZIMA 001 from Allele Biotechnology (San Diego, California). The cells were differentiated under normal O2 conditions and maintained with the STEMdiff Cardiomyocyte Differentiation Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada) according to the manufacturer’s instructions. After 15-day induction, approximately 90% of the cells were beating and positive for both cardiac troponin T and sarcomeric α-actinin. We re-seeded the cells and cultured them in different O2 concentrations (21%, 15%, 10%, and 5%) in incubators for 7 days. To produce rapid changes in O2, we incubated cells at 21% O2 for 2 days, and then changed the O2 level to 15% for 2 days, 10% for 2 days, and finally, 5% for 2 days. After 7 days of culture, we subjected the cells to DNA extraction, qPCR, and IF.

YAP1 OVEREXPRESS BY ADENOVIRUS HARBORING YAP1–COMPLEMENTARY DNA. We purchased YAP1 complementary DNA and negative control lentiviral vector from GeneChem (Shanghai, China). YAP1-complementary DNA or negative control was cloned into pDC315 plasmid (GeneChem) harboring the cytomegalovirus promoter. The pDC315-YAP1 plasmid or control plasmid was co-transfected with pBHGloX3E1-Cre (GeneChem) into HEK293 cells using Lipofectamine 2000 (Invitrogen, Waltham, Massachusetts). After 2 rounds of virus amplification, the supernatant was filtered at 0.45 μm, and purified using the Adeno-XTM Virus Purification kit (Takara, Clontech, Dalian, China). YAP1 adenovirus transfections were performed over 8 h and confirmed by Western blotting and IF. At 72 h after transfection, we cultured the cells in incubators at an O2 concentration of 5% for 7 days. After washing cells with phosphate-buffered saline, they were harvested and subjected to qPCR, Western blotting, and IF.

YAP1 KNOCKDOWN BY LENTIVIRUS HARBORING YAP SHORT-HAIRPIN RNA. Short-hairpin (sh) RNA (shYAP1:5'-GACTCAGGATGGAGAAATTTA-3') targeting a specific region of human YAP1 mRNA (NM_006106), and a scrambled negative control (sh-con, 5'-TTC TCC GAA CGT GTCA CG T-3'), were cloned into the GV248 vector (GeneChem). Lentivirus (LV) gene transfer vectors encoding gene fluorescent protein (GFP)-shYAP1 (LV-GFP-shYAP1) and a scrambled shRNA used as the negative control (LV-GFP-sh-con) were synthesized by GeneChem. Transfections were performed over 8 h and confirmed by Western blotting and IF. At 72 h after transfection, cells were cultured in incubators at an O2 concentration of 10% for 7 days. After washing cells with phosphate-buffered saline, they were harvested and subjected to qPCR, Western blotting, and IF.

IF. Slides or cells were washed 3 times with phosphate-buffered saline, fixed with 4%
paraformaldehyde for 10 min, permeated with 0.5% Triton X-100 for 15 min, blocked in 10% donkey serum for 30 min, and stained with primary antibodies overnight at 4°C. After an additional 3 washes, we incubated the sections or cells with secondary antibodies and 4’,6-diamidino-2-phenylindole for 30 min. Three researchers who were blinded to the sample identity quantified cellular Ki67, phospho-histone H3 (pHH3), and aurora B by either manual counting or digital thresholding (image segmentation and creation of a binary image from a grayscale). We analyzed the converted binary images using ImageJ software (National Institutes of Health, Bethesda, Maryland).

**qPCR ANALYSIS.** For mRNA quantification, mRNA was extracted and purified using the PureLink RNA Micro Scale Kit (Life Technologies, Carlsbad, California). Reverse transcription was performed using the PrimeScriptTM reagent kit. The qPCR reactions were carried out using SYBR Green Power Premix Kits (Applied Biosystems, Foster City, California) according to the manufacturer’s instructions. The reactions were performed with the 7900 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 1 cycle at 95°C for 10 s, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. The primers were obtained from Generay Biotech Co., Ltd (Shanghai, China).

For mitochondrial DNA quantification (24), DNA was extracted and purified from tissue samples following proteinase K digestion and phenol and/or chloroform extraction. Mitochondrial DNA was quantified by qPCR, and quantification was performed using the SYBR Green PCR Master Mix and 7900 Sequence Detection System (Applied Biosystems). The relative mitochondrial DNA copy number was calculated from the ratio of mitochondrial DNA copies to nuclear DNA copies per gram of tissue. Then the relative fold change was then calculated using the ΔΔCT method.

**WESTERN BLOT ANALYSIS.** Proteins were extracted with Radio Immunoprecipitation Assay (RIPA) (Beyotime, Shanghai, China) Lysis Buffer according to the manufacturer’s instruction, separated on 10% sodium dodecyl sulfate(SDS) (Beyotime, Shanghai, China) polyacrylamide gels, and transferred onto polyvinylidene fluoride membranes (Merck, Millipore, Billerica, Massachusetts). Then, the membranes were blocked in 5% nonfat milk in Tris-buffered saline with Tween 20 for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. After 3 washes with Tween 20, the membranes were incubated with second antibodies for 1 h at room temperature, and proteins were detected using the Bio-Rad ChemiDoc Imaging Systems (Bio-Rad, Hercules, California).

**STATISTICAL METHODS.** Continuous data were expressed as mean ± SD. Differences were tested with 1-way analysis of variance test and Student’s Newman-Keuls for post hoc test. Categorical variables were compared by the Wilcoxon test. The values of p < 0.05 were considered statistically significant. Statistical analyses were performed using SAS (software version 9.4, SAS Institute Inc., Cary, North Carolina).

**RESULTS**

**BASELINE PATIENT CHARACTERISTICS.** Thirty infants with TOF were included in the study. Because age and pressure load could affect CM proliferation (6,8,15), patients were selected to ensure that there were no significant differences in age, sex, or pulmonary arterial pressure (increasing right ventricular pressure load) among the 3 groups (Supplemental Table S3). The only factor that significantly differed among the groups was SaO2 (p < 0.001; n = 10). In clinical practice, patients were divided into 3 groups according to SaO2 levels: >85% was defined as mild hypoxia (group A); 75% to 85% was defined as moderate hypoxia (group B); and <75% was defined as severe hypoxia (group C) (13). Ten patients from each group were analyzed to test our hypothesis that SaO2 influenced CM cell cycle activities. Patient characteristics were deemed well balanced and suitable for studying the effects of SaO2 on CM cell cycle activities.

**CELL CYCLE ACTIVITY OF CMs IN THE DIFFERENT SaO2 GROUPS.** Because Ki67 is present during all active phases of the cell cycle (G1, S, G2, and mitosis), we measured Ki67-positive cells in all 3 groups. As shown in Figures 1A and 1B, the percentage of Ki67-positive CMs in groups A, B, and C was 0.99 ± 0.25%, 6.08 ± 2.51%, and 2.47 ± 2.64%, respectively (p < 0.001), which suggested that cell cycle activities in those with moderate hypoxia CMs were upregulated. Next, we detected the mitotic marker pH3 and found that the percentages of pH3-positive CMs in groups A, B, and C was 0.10 ± 0.04%, 0.53 ± 0.13%, and 0.23 ± 0.10%, respectively (p < 0.001) (Figures 1C and 1D). This indicated that CMs in the mitotic stage in group B also increased, although the percentage of pH3-positive CMs was only 10% of Ki67-positive CMs. A combination of aurora B-positive, midbody position, and daughter nuclei distance that indicated CM proliferation was recently demonstrated (25), so we also counted aurora B-positive CMs. None was found in most of the sections from groups A and C (mild and severe hypoxia, respectively), but the
FIGURE 1  Higher Cell Cycle Activity of Human CMs in Moderate SaO₂ Conditions

(A) Representative Ki67-positive cardiomyocytes (CMs) in group B; cardiac troponin-T (cTnT) (red), Ki67 (green), and 4',6-diamidino-2-phenylindole (DAPI) (blue) stainings are shown. The arrow indicates proliferating CMs. (B) Quantification of Ki67-positive CMs: 1-way analysis of variance (ANOVA), Student Newman Keuls (SNK), n = 10; **p < 0.01. (C) Representative phospho-histone H3 (pHH3) – positive CMs in group B; cTnT (red), pHH3 (green), and DAPI (blue) stainings are shown; the arrow indicates proliferating CMs and hatch sign indicates proliferating non-CMs. (D) Quantification of pHH3-positive CMs; 1-way ANOVA, SNK, n = 10; **p < 0.01. (E) Representative aurora B-positive CMs in group B; cTnT (red), aurora B (green), and DAPI (blue) stainings are shown; the arrow indicates proliferating CMs. (F) Quantification of aurora B-positive CMs; 2-way ANOVA, SNK, n = 10; **p < 0.01. We used quantitative polymerase chain reaction (qPCR) to analyze the expression of mRNA levels of (G) Ki67, (H) cyclin D2, and (I) AURKB in CMs treated with different levels of oxygen saturation (SaO₂). Our results indicated that Ki67, cyclin D2, and AURKB mRNA were significantly increased in the moderate SaO₂ group compared with the other 2 groups. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control; 1-way ANOVA, SNK, n = 10; **p < 0.01.
percentage of aurora B–positive CMs in group B was 0.04 ± 0.03% (Figures 1E and 1F). These results indicated that moderate SaO₂ levels promoted CM proliferation.

To confirm these results, we also performed qPCR to detect the mRNA levels of Ki67, cyclin D2, and aurora B. As shown in Figures 1G to 1L, the relative expression of Ki67, cyclin D2, and aurora B (AURKB) in group B was significantly increased. This result confirmed that moderate SaO₂ increased the cell cycle activities of CMs.

**CELL CYCLE ACTIVITY OF HUMAN iPSC-CMS CULTURED AT DIFFERENT O₂ CONCENTRATIONS**

The previously mentioned results suggested that moderate SaO₂ was beneficial to CM cell cycle activity; however, it remains unclear if this is true in vitro. Therefore, we set up 5 different O₂ concentrations and treatment methods to observe the effect of SaO₂ on CM proliferation. As shown in Figure 2, 15% O₂ and 10% O₂ treatment for 7 days significantly increased the percentage of Ki67+, pHH3+, and aurora B–positive CMs, whereas 5% O₂ and rapidly changing O₂ (21% to 5%) treatment decreased this percentage compared with normal O₂ (21%) treatment. This suggested that both O₂ concentrations and treatment methods contributed to the cell cycle activities of human iPSC-CMs in vitro. The percentage of Ki67–positive human iPSC-CMs cultured in vitro in normal O₂ was as high as 20%, which suggested that the cell cycle activity of iPSC-CMs in vitro was much higher than that of human CMs in vivo.

**REDUCED OXIDATIVE DNA DAMAGE IN MODERATE SaO₂-TREATED CMs.** Puente et al. (12) previously reported that reduced mitochondrial DNA content might be an indicator of reduced oxidative DNA damage. The latter form of damage is a critical inhibitor of cell proliferation. In this study, we analyzed mitochondrial DNA content in heart tissue from patients with TOF. As expected, mitochondrial DNA levels were significantly reduced in the moderate SaO₂ group compared with the other 2 groups (Figure 3A), which suggested that moderate SaO₂ levels might protect CMs by inhibiting DNA damage. To validate this result, we monitored levels of 8-oxoguanine (one of the most common DNA lesions that results from reactive oxygen species) in all 3 groups (26). As shown in Figures 3B and 3C, 8-oxoguanine levels were significantly reduced in the moderate SaO₂ group. We also detected a DNA damage response marker, namely, phosphorylated ataxia telangiectasia mutated (pATM). Similarly, pATM levels were significantly reduced in the moderate SaO₂ group (Figures 3D and 3E). Because paired like homeodomain 2 (Pitx2) is a critical antioxidant factor and promotes CMs proliferation (27), we also detected its expression in the 3 different SaO₂ groups. The results showed that Pitx2 was significantly upregulated in the moderate SaO₂ group (Figures 3F and 3I). These results suggested that moderate SaO₂ levels facilitated the protection of CMs by reducing DNA damage.

In accordance with the cell cycle activity of human iPSC-CMs cultured at different levels of O₂, expression of 8-oxoguanine, mitochondrial DNA, and pATM was reduced in the 15% and 10% O₂-treated groups but was increased in the 5% and 21% to 5% O₂-treated groups compared with the normal O₂-treated groups (Figures 4A to 4E). The expression of Pitx2 was upregulated in the 15% and 10% O₂-treated groups but was downregulated in the 5% and 21% to 5% O₂-treated groups compared with the normal O₂-treated groups (Figures 4F to 4I).

**HIGHER EXPRESSION AND OVEREXPRESSION OF YAP1.** Higher expression of YAP1 occurred in the moderate hypoxia group, overexpression of YAP1 rescued the cell cycle activities of human iPSC-CMs in the 5% O₂-treated group, and knockdown reduced activities in the 10% O₂-treated group.

YAP1 is a critical co-transcription factor in the regulation of CM proliferation. To investigate its role in inhibiting oxidative DNA damage to CMs, we first investigated the expression of YAP1 in human heart samples. We found a significant increase of YAP1 expression in the moderate SaO₂ group compared with the other 2 groups (Figures 5A and 5B). Consistent with our observations in heart samples, there was also more abundant expression of YAP1 in the 10% O₂-cultured human iPSC-CMs, especially in the nuclei (Figures 5C and 5E). When YAP1 was overexpressed in 5% O₂-treated iPSC-CMs (Figures 5F to 5H), the percentage of Ki67+, pHH3+, and aurora B–positive CMs significantly increased (Figures 5I and 5J) (p < 0.01; n = 10). In contrast, when YAP1 was knocked down in 10% O₂-treated iPSC-CMs (Figures 5K to 5M), the percentage of Ki67−, pHH3−, and aurora B–positive CMs significantly decreased (Figures 5O and 5P) (p < 0.01; n = 10).

**REDUCED OXIDATIVE DNA DAMAGE AFTER OVER-EXPRESSION OF YAP1 IN HUMAN iPSC-CMS CULTURED IN 5% O₂.** To cross-check the effects of YAP1 overexpression, we detected mitochondrial DNA and 8-oxoguanine expression in human iPSC-CMs and found that both markers were downregulated in the YAP1 overexpression group (Figures 6A to 6C).
Higher Cell Cycle Activity of Human iPSC-CMs in 10% and 15% O₂-Treated Conditions

(A) Ki67-positive CMs, sarcomeric α-actinin (SAA) (red), Ki67 (green), and DAPI (blue) stainings are shown; the arrow indicates proliferating CMs, and the hatch sign indicates proliferating non-CMs. (B) Quantification of Ki67-positive CMs for each group: 1-way ANOVA, SNK; **p < 0.01, n = 10 fields for each group from 3 independent experiments. (C) pH3-positive CM: SAA (red), pH3 (green), and DAPI (blue) are shown; the arrow indicates proliferating CMs, and the hatch sign indicates proliferating non-CMs. (D) Quantification of pH3-positive CMs for each group: 1-way ANOVA, SNK; **p < 0.01, n = 10 fields for each group from 3 independent experiments. (E) Aurora B-positive CM: SAA (red), aurora B (green), and DAPI (blue) stainings are shown; the arrow indicates proliferating CMs, and the hatch sign indicates proliferating non-CMs. (F) Quantification of aurora B-positive CMs for each group: 1-way ANOVA, SNK; **p < 0.01, n = 10 fields for each group from 3 independent experiments. We used qPCR to analyze mRNA levels of (G) Ki67, (H) cyclin D2, and (I) AURKB in CMs treated with different levels of oxygen (O₂). Our results indicated that Ki67, cyclin D2, and AURKB mRNA were significantly increased in 15% and 10% O₂-treated human induced pluripotent stem cell–cardiomyocytes (iPSC-CMs). GAPDH served as a control; 1-way ANOVA, SNK, n = 3; *p < 0.05; **p < 0.01. Abbreviations as in Figure 1.
FIGURE 3  Oxidative DNA Damage Was Significantly Reduced in Moderate Hypoxia Human CMs

(A) We used qPCR to analyze mitochondrial DNA (mtDNA) levels in the hearts of patients with tetralogy of Fallot (TOF). Our results indicated that mtDNA was significantly decreased in the moderate SaO2 group compared with the other 2 groups: 1-way ANOVA, SNK, n = 10 samples; **p < 0.01. (B) 8-oxoGuanine (8-oxoG) in mild, moderate, and severe hypoxia heart samples; cTnT (white), 8-oxoG (red), and DAPI (blue) stainings are shown. (C) Quantification of 8-oxoG immunofluorescence (IF) intensity in mild, moderate, and severe hypoxia heart samples: 1-way ANOVA, SNK, n = 10 samples, **p < 0.01, compared with group B (moderate hypoxia). (D) Phosphorylated ataxia telangiectasia mutated (pATM) in mild, moderate, and severe hypoxia heart samples; cTnT (green), pATM (red), and DAPI (blue) staining are shown. (E) Quantification of pATM IF intensity in mild, moderate, and severe hypoxia heart samples. One-way ANOVA, SNK, n = 10 samples; **p < 0.01, compared with group B (moderate hypoxia). (F) IF graph of Pitx2 in mild, moderate, and severe hypoxia heart samples; cTnT (red), Pitx2 (green), and DAPI (blue) staining are shown. (G) Quantification of Pitx2-positive CMs in mild, moderate, and severe hypoxia heart samples. One-way ANOVA, SNK, n = 10 samples; **p < 0.01, compared with group B (moderate hypoxia). (H) Western blot graph of Pitx2 in mild, moderate, and severe hypoxia heart samples. (I) Quantification of Pitx2 densitometry in mild, moderate, and severe hypoxia heart samples. One-way ANOVA, SNK, n = 10 samples; **p < 0.01, compared with group B (moderate hypoxia). Abbreviations as in Figures 1 and 2.
FIGURE 4 Oxidative DNA Damage Was Significantly Reduced in 10% and 15% O₂-Treated Human iPSC-CMs

(A) 8-oxoG in normal and 15% O₂-Treated Human iPSC-CMs; cTnT (red), 8-oxoG (green), and DAPI (blue) stainings are shown. (B) Quantification of 8-oxoG IF intensity in differently O₂-treated human iPSC-CMs; 1-way ANOVA, SNK; *p < 0.05; **p < 0.01, n = 10 fields from 3 independent experiments, compared with normal. (C) Quantification of mtDNA content in differently O₂-treated human iPSC-CMs; 1-way ANOVA, SNK; **p < 0.01, n = 3 replicates compared with normal. (D) pATM in normal, 10%, and 5% O₂-treated human iPSC-CMs; cTnT (green), pATM (red), and DAPI (blue) stainings are shown. (E) Quantification of pATM IF intensity in differently O₂-treated human iPSC-CMs; 1-way ANOVA, SNK; *p < 0.05; **p < 0.01, n = 10 fields from 3 independent experiments compared with normal. (F) IF graph of Pitx2 in normal, 10% hypoxia, and 5% hypoxia iPSC-CMs; cTnT (green), Pitx2 (red), and DAPI (blue) staining are shown. (G) Quantification of Pitx2 positive CMs in normal, 10% hypoxia, and 5% hypoxia iPSC-CMs. One-way ANOVA, SNK, n = 10 fields from 3 independent experiments; **p < 0.01, compared with group B (moderate hypoxia). (H) Western blot graph of Pitx2 in normal, 10% hypoxia, and 5% hypoxia iPSC-CMs. (I) Quantification of Pitx2 densitometry in normal, 10% hypoxia, and 5% hypoxia iPSC-CMs. One-way ANOVA, SNK, n = 3 samples; **p < 0.01, compared with group B (moderate hypoxia). Abbreviations as Figures 1 to 3.
FIGURE 5 Higher Expression of YAP1 in Moderate SaO2 Human CMs and 10% O2-Treated Human iPSC-CMs; Overexpression of YAP1 Protected the Proliferative Potential of Human iPSC-CMs From Oxidative DNA Damage
CM proliferation contributes to post-natal heart growth in young humans, and their protective attributes are important in cardiac surgery and heart failure therapy (28). Currently, pharmacological treatment regimens are still the mainstay for heart failure in children. However, many of the drugs that
have been developed for heart failure (including beta-blockers and angiotensin-converting enzyme inhibitors) in adult patients are ineffective in pediatric patients (28,29). Furthermore, newer, more promising drugs have not been subjected to randomized clinical trials in pediatric patients (30,31). Because prevention is better than therapy, there is increasing awareness of the importance of protecting the young human heart. Therefore, new therapeutic paradigms for pediatric heart failure are now under investigation (32). This study is the first to focus on the relationship between CM proliferation and heart protection in children. We are hopeful that this research will provide a platform from which the potential pediatric heart failure therapies may be further investigated.

O2 balance is extremely important in CHD therapy; too much or too little O2 can be harmful to neonates with CHD. For example, excessive O2 administration to newborns with single ventricle physiology can lead to circulatory collapse by increasing pulmonary blood flow at the expense of systemic perfusion, whereas insufficient O2 may cause acidosis and subsequent brain damage (33). However, until now, researchers did not realize that balanced O2 is also required for myocardial protection. During cardiopulmonary bypass, the myocardium is protected by 2 methods, hypothermia and potassium-induced electromechanical cardiac arrest, both of which reduce O2 consumption in the heart (34,35). This study suggested that during the peri-operative period, moderate SaO2 may be beneficial to CM cell cycle activities. Clinical investigations from other institutions also demonstrated that children who had severe SaO2 deprivation needed more inotrope intervention (31). The underlying mechanism might be associated with oxidative DNA damage. We found that both too much and too little O2 increased oxidative DNA damage (Figures 3A to 3J). As suggested by Schoots et al. (36), an imbalance between reactive O2 species and antioxidants, because of hypoxia, caused damage to DNA.

In addition to observing how O2 affects human CM proliferation, we investigated the effects of different concentrations of O2 on the proliferation of iPSC-CMs in vitro. The results showed that 15% and 10% O2 cultures significantly promoted the proliferation of CMs, but the effects of these 2 levels were similar. Conversely, 5% O2 culture and a progressively hypoxic culture inhibited CM proliferation. These results suggested that a proper level of hypoxia was necessary during clinical monitoring, but excessive hypoxia or a rapid change in O2 levels was not conducive to the recovery of the child’s heart.

This study also helped reconcile the current international controversy of whether hypoxia is beneficial to CM proliferation. Our research showed that the answer is not simply yes or no but depends on the specific degree of hypoxia and whether it is acute or chronic progressive hypoxia. Studies at the Sadek Laboratory (University of Texas Southwestern Medical Center, Dallas, Texas) showed that progressive hypoxia in vivo is beneficial for CM proliferation. Our study contradicted those observations. There are 2 possible reasons for this. First, the differences between in vivo and in vitro experiments, and second, the Sadek Laboratory researchers induced progressive hypoxia by reducing the O2 concentration by 1%/h, whereas we reduced it by 5% every other day. Thus, changes to the O2 concentration in our study were more dramatic; such intense changes are not conducive to CM growth. Consistent with the previously mentioned observations, the manner of hypoxia exposure caused oxidative DNA damage (Figures 3F to 3J). Cai et al. (37) found that chronic intermittent hypoxia increased oxidative damage in the neonatal rat liver.

**STUDY LIMITATIONS.** Our results indicated that YAP1 served as an anti-oxidative DNA damage transcription factor in the regulation of CM proliferation. However, by what signaling pathway YAP1 plays such a role is still unknown, although some studies indicated that it might participate in the DNA base excision repair pathway (38). Another limitation of this study is that we still do not know how SaO2 is converted into O2 concentration in vitro. This limitation may impede the translation of our results into clinical application.

When considering the effects of hypoxia, the role of hypoxia-inducible factor 1 alpha must be considered. As expected, higher expression of hypoxia-inducible factor 1 alpha was found in the severe hypoxia group (Supplemental Figure S5); however, it has not been positively correlated with CM proliferation rate. The role of hypoxia-inducible factor-1 alpha in the regulation of CMs proliferation is complex. Paradis et al. (11) showed that when neonatal rat hearts were exposed to hypoxia in vivo, there was a significant increase in hypoxia-inducible factor-1 alpha protein but CMs proliferation was inhibited. Their results showed that in this process, endothelin-1 might be the major responsive factor. In contrast, Kimura et al. (39) showed that stabilization of hypoxia-inducible factor-1 alpha was
Critical for stem or progenitor cells, mapping the proliferating CMs. How hypoxia-inducible factor-1 alpha, YAP1, and Pitx2 work together in the heart to regulate CMs proliferation needs more investigation.

CONCLUSIONS

Moderate hypoxia (SaO2: 75% to 85%) is beneficial for the cell cycle activities of postnatal human CMs and a reduction in DNA damage and upregulation of YAP1 appear to be the underlying mechanisms.

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KEY WORDS blood oxygen saturation, cardiomyocyte, congenital heart disease, pediatric patients, proliferation

APPENDIX For supplemental figures and tables, please see the online version of this paper.
Hypoxia Induces Cardiomyocyte Proliferation in Humans*

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The mammalian heart has a substantial regenerative capacity in the first few days of life mediated by cardiomyocyte proliferation; however, this capacity is lost shortly after birth (1). Transition from the embryonic to postnatal circulation results in a rapid and substantial increase in oxygen tension coinciding with separation of the right and left sided circulations (2). This is accompanied by a shift from anaerobic glycolysis to mitochondrial oxidative phosphorylation, particularly toward fatty acid (FA) utilization by mitochondria. Previous studies have demonstrated that changes in oxygenation and metabolism are important regulators of cardiomyocyte maturation and cell cycle (3). For example, the postnatal window of cardiomyocyte proliferation in mice can be modulated by changes in the fraction of inspired oxygen immediately after birth, where hypoxia prolongs the window and hyperoxia shortens it (3). Similarly, exposure to gradual severe hypoxia results in reversion to anaerobic metabolism, decreased DNA damage, and reactivation of cardiomyocyte proliferation in adult mice (4). However, these studies and others were performed in rodents or in cultured cells, and it is unknown whether a similar phenomenon occurs in the human heart.

In this issue of JACC: Basic to Translational Science, Ye et al. (5) showed in an elegant report that hypoxia induces cardiomyocyte proliferation in the human heart in a population of patients with cyanotic heart diseases. The authors collected 30 ventricular outflow myocardial tissue specimens (obtained from the surgical procedure required to relieve obstruction) from Tetralogy of Fallot patients and categorized them into 3 main groups based on SaO2 levels: mild hypoxia >85%, moderate hypoxia 75% to 85%, and severe hypoxia <75%. The authors are to be commended for the careful patient selection, which included parameters like age and sex matching as well as matching pulmonary pressure. They found that moderate hypoxia reduced mitochondrial oxidative DNA damage, reduced DNA damage response, and promoted cardiomyocyte proliferation. The authors also demonstrate a similar role of Hippo pathway in regulation of cardiomyocyte proliferation, not unlike previous landmark studies by Jim Martin’s group (6) that implicated the antioxidant role of Pitx2 in regulation of cardiomyocyte proliferation. Overall, these results are well in line with previous observations in rodents. However, there is a significant unanswered question here: why are lower levels of oxygen associated with increased oxidative DNA damage? It is well accepted that acute hypoxia can result in bursts of mitochondria-derived reactive oxygen species, which might have been the case here. The current paper does not provide data with regard to fluctuations in oxygen levels in Group C patients, for example, which would at least partially explain these findings. Nevertheless, the mere demonstration of the correlation among hypoxia, decreased DNA damage, and human myocyte proliferation in vivo is a significant finding that moves the field forward.

The authors also performed studies on human induced pluripotent stem cell (iPSC)-derived cardiomyocytes. They exposed the cells to 1 of 4 levels of oxygen: 21%, 15%, 10%, or 5%. Oxygen levels were...
dropped every 2 days. They also showed that in the moderate range of hypoxia there is an increase in cardiomyocyte proliferation. Again, and similar to their in vivo results, the peculiar finding here was that lower levels of oxygen increase DNA damage. Although these results are also in line with hypoxia-induced regeneration studies in rodents, in our view these types of in vitro studies are difficult to interpret for several reasons. First, myocytes in culture are not exposed to mechanical stress, and it is well established that under regular culture conditions, these cells lack critical metabolic characteristics of adult cardiomyocytes. For example, a recent report by Porrello and Hudson’s groups (7) showed convincingly that under regular culture conditions, levels of DNA damage in iPSC-derived myocytes are low, and that forcing fatty acid oxidation in the absence of changes in oxygenation is critical for induction of an oxidative metabolic phenotype and enhanced differentiation of iPSC-derived myocytes. Second, how does one translate an in vivo systemic oxygen level to that at a cellular level? For example, although 7% FiO2 in mice for 2 weeks can induce myocyte proliferation in vivo, and in the current study by Ye et al. (5), 75% to 80% systemic arterial saturation was associated with myocyte proliferation, what is the equivalent oxygen level in cultured cells? The short answer is that we simply do not know. Third, previous studies have shown that it is not the oxygen level per se, but rather the metabolic programming that occurs in response to these oxygen levels that affects myocyte proliferation (3). Therefore, rapid changes in oxygenation over 2 days might not be sufficient to affect metabolic reprogramming. Finally, was there a differential effect on ROS production, whether mitochondrial or otherwise, at different oxygen levels? Thus, the level of hypoxia, the timeline of induction of hypoxia, the duration under hypoxia, and the mechanism of changes in DNA damage are all necessary factors to consider before reaching conclusions about a certain protocol of hypoxia in vitro. The authors thus suggest in their discussion that the protocol they used in vitro was perhaps too rapid to induce sufficient metabolic reprogramming, as an attempt to explain the discrepancy between their in vitro data and the in vivo data in mice. This might be true; however there simply is no way to correlate workload and oxygenation in a working heart in vivo to unloaded single-layer cardiomyocytes in vitro.

In conclusion, the findings presented in this report by Ye et al. (5) represent an important extension of the role in hypoxia in cardiomyocyte cell cycle regulation to the human heart, and they solidify the role of hypoxia in blunting DNA damage as a mechanism of myocyte proliferation. The studies also highlight the notion that careful consideration of the degree of hypoxia and the model used is warranted. Not all hypoxias are created equally.

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**KEY WORDS** blood oxygen saturation, cardiomyocyte, cardiomyocyte proliferation, congenital heart disease, DNA damage, hypoxia.
Changes in Myocardial Microstructure and Mechanics With Progressive Left Ventricular Pressure Overload

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HIGHLIGHTS

- A large animal model of progressive PO was created that evolved into phenotypic features of HFpEF.
- The progression from PO to the HFpEF pathophysiology was accompanied by specific shifts in the collagen matrix microstructure over and above collagen content (i.e., fibrosis).
- Using early changes in regional myocardial stiffness measurements by speckle tracking methodology predicted the extent and magnitude of the HFpEF phenotype at later timepoints.
SUMMARY

This study assessed the regional changes in myocardial geometry, microstructure, mechanical behavior, and properties that occur in response to progressive left ventricular pressure overload (LVPO) in a large animal model. Using an index of local biomechanical function at early onset of LVPO allowed for prediction of the magnitude of left ventricular chamber stiffness (Kc) and left atrial area at LVPO late timepoints. Our study found that LV myocardial collagen content alone was insufficient to identify mechanisms for LV myocardial stiffness with progression to heart failure with preserved ejection fraction (HFpEF). Serial assessment of regional biomechanical function might hold value in monitoring the natural history and progression of HFpEF, which would allow evaluation of novel therapeutic approaches. (J Am Coll Cardiol Basic Trans Science 2020;5:463–80) © 2020 Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

The rate and incidence of heart failure (HF) continues to escalate, and recent statistics have identified this disease as the leading cause of morbidity and mortality—eclipsing all other chronic diseases (1). Although significant progress has been achieved in therapeutic retardation or elimination of other major illnesses (e.g., such as cancer), these advancements have not been as forthcoming for HF. One contributory factor for this is that, unlike cancer, which is classified by morphology, cell type, and molecular and/or genetic profiles, HF has been historically considered to be a single entity fundamentally defined by symptomatology. However, it must be recognized that HF arises from distinctly different etiologies, which, in turn, have demonstrated distinctly different therapeutic responses and clinical outcomes (2,3). Although the precise categorization of HF is continuously evolving, a generalized dichotomy can be made between patients with HF who present with or without significant left ventricular (LV) pump dysfunction. Specifically, if LV ejection fraction (EF) is impaired, this is defined as HF with reduced EF (HFrEF), whereas if HF symptoms are present and EF is within normal limits, then the definition of HF with a preserved EF (HFpEF) is used. It has been estimated that patients with these HF phenotypes are equally distributed (2). Randomized clinical trials using combinatorial pharmacology or device-driven therapies have demonstrated significant improvement in clinical outcomes in patients with HFrEF but not in patients with HFpEF (4–11). In addition, although the functional progression of HFrEF can be monitored by serial assessment of changes in EF, the natural history of HFpEF is not as easily assessed or well-understood (12,13).

One-rate limiting step in the research progress for HFpEF is a paucity of translationally relevant animal models that recapitulate key phenotypical features of the clinical syndrome of HFpEF (14,15). Specifically, HFpEF can arise from progressive and prolonged LV pressure overload (LVPO) rather than from an acute pathological stimulus (e.g., myocardial ischemia) that progresses to HFrEF (16–19). In addition, a key component in the progression of HFpEF is the development of LV diastolic dysfunction, for which detection requires sensitive measures of LV diastolic performance that can be difficult to assess in a serial manner (20,21).

We used a previously established porcine model of progressive LVPO that resulted in significant LV...
hypertrophy with stable EF but with evidence for impaired diastolic function that was consistent with the clinical phenotype of HFrEF (22). The central hypothesis of this study was that LV myocardial remodeling in response to progressive LVPO is characterized by an increase in LV myocardial collagen content and a change in collagen fiber microstructure, which together will affect both regional LV myocardial mechanical properties and LV chamber stiffness. To test this hypothesis, we developed and deployed novel and innovative approaches based on noninvasive imaging to assess LV regional diastolic function together with advanced optical analysis of LV myocardial collagen microstructure. We used obtained data to build a mechanics-based mathematical model that predicts the rate and magnitude of LV chamber stiffness elevations with progressive LVPO. Our findings supported the continued consideration of progressive LVPO as a preclinical model for the clinical syndrome of HFrEF and demonstrated the potential clinical value of assessing regional LV myocardial biomechanical function to monitor HFrEF progression.

**METHODS**

Progressive LVPO was induced in pigs for up to a 5-week period, during which regional LV myocardial strain and stiffness were quantified using speckle tracking echocardiography (STE). At terminal time-points, LV myocardial collagen content was quantified using quantitative histomorphometry, LV myocardial microstructure was assessed using second harmonic generation (SHG) imaging, and select biochemical markers were quantified using immunohistochemistry and targeted gene analysis (23-29). LV myocardial functional response variables were assessed in a serial fashion focusing on identifying factors that would contribute to and potentially predict changes in LV chamber stiffness, a hallmark of HFrEF progression (22,30-37).

**LVPO INDUCTION.** Yorkshire pigs (n = 14; 15.8 ± 0.6 kg; Hambone Farms, Orangeburg, South Carolina) were anesthetized with isoflurane (3%/1.5 l/min), and through a left thoracotomy, a 12-mm inflatable silastic vascular cuff (Access Technologies, Skokie, Illinois) was secured around the supracoronary ascending aorta without inducing aortic constriction. A length of silastic tubing was connected to a subcutaneous access port (Access Technologies) for subsequent hydraulic expansion of the aortic cuff. After a 1-week recovery period, the cuff was inflated through the access port (via 0.45 ml glycerol) to achieve a specific target gradient of 75 mm Hg across the cuff and thus induce LVPO. At weekly intervals thereafter, the cuff was further inflated (0.25-ml increments) to cause a stepwise increase in the pressure gradient (25 mm Hg increase and/or inflation). At either 4 weeks (n = 7) or 5 weeks (n = 7) post-LVPO, the animals were anesthetized (5% isoflurane), and the LV was harvested. Age-matched referent control pigs (n = 12) were used for comparative analyses. All animals were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Eighth Edition; Washington, DC: 2011), and all experimental protocols were approved by Institutional Animal Care and Use Committees at the University of South Carolina.

**SERIAL LV ECHOCARDIOGRAPHY.** LV echocardiography was performed weekly throughout the study (GE Vivid E9 with XDclear Ultrasound System: M5S [1.5 to 4.6 MHz] transducer probe; GE, Boston, Massachusetts). LV and left atrial (LA) dimensions and function were assessed by 2-dimensional and M-mode echocardiographic studies, as described previously (28,39). From the obtained LV measurements, LV end-diastolic volume (EDV), end-systolic volume (ESV), and EF were calculated using the biplane method of disks. LA area was determined from the anteroposterior dimensions acquired from the parasternal long-axis view. LV wall thickness was determined and LV mass computed using conventional formulas (40). Finally, LV isovolumic relaxation time (IVRT) was measured by tissue Doppler imaging M-mode through the mitral leaflet (41).

**DEFINITION OF NORMAL RANGE FOR LV MASS.** LV mass measurements and body weights were examined from an archived data set of control pigs (100 pigs; body weight: 13 to 33 kg). These data allowed for the construction of a normogram for LV mass as a function of body weight (linear regression and a 95% confidence interval) and thus provided a basis to assess LVPO-mediated changes in LV mass with respect to normal growth.

**STE.** Three consecutive digital loops of the 2-dimensional echocardiography for the LV long and short-axes were stored on a hard disk and transferred to a workstation (EchoPac, Vingmed, General Electric) for post-processing. For each echocardiographic digital loop, a region of interest was defined at the onset of the R-wave and then discretized with a spatial mesh of acoustic clusters on a frame-to-frame basis throughout the cardiac cycle (R-R interval) (26). Following grouping of the acoustic clusters into 6
predetermined anatomical regions, tracking of the region of interest allowed for the definition of segmental lengths at end-diastole ($L_0$) and continuously throughout the cardiac cycle ($L$). Regional LV myocardial segmental strains ($ε$) and strain rates ($γ$) were then computed as:

$$e = \frac{L - L_0}{L_0} = \frac{ΔL}{L_0} \quad \text{(Eq. 1)}$$

$$γ = \frac{ΔL/Δt}{L_0} \quad \text{(Eq. 2)}$$

where $Δt$ is the relative time in the cardiac cycle. LV myocardial peak strains and diastolic strain rates were quantified in 2 normal directions, circumferential (short axis) and longitudinal (long axis), at the endocardial, mid-wall, and epicardial surfaces. Global LV myocardial strain values were calculated based on the total length change of all 6 segments. Obtained LV myocardial segmental strain and strain rate data were assessed for intraobserver variance by calculating the intraclass correlation coefficient for a single operator under the assumption that systematic differences were relevant. The intraclass correlation coefficient for all measures of LV myocardial strain and strain rate ranged from 0.94 to 0.99.

**REGIONAL LV MYOCARDIAL WALL STRESS.** We modified a thick-walled ellipsoidal model proposed by Janz (42) to compute regional LV myocardial wall stress in the circumferential and longitudinal directions. The regional circumferential LV myocardial wall stress ($σ_C$) was computed as:

$$σ_C = \frac{Pr^2}{2f(τ + \frac{1}{2})} \quad \text{(Eq. 3)}$$

where $r$ is the inner radius, $τ$ is the regional wall thickness, and $P$ is LV pressure. For the purposes of this study, LV pressure was assumed to equal zero at the onset of diastole. An estimated value at end-diastole (described in the following) regional longitudinal LV myocardial wall stress ($σ_L$) was computed as:

$$σ_L = \frac{Pr^2}{2f(\sin Φ)(τ + \frac{τ\sin Φ}{2})} \quad \text{(Eq. 4)}$$

where $Φ$ is the angle between the normal vector from the endocardium at the region of interest and the axis of revolution in the truncated ellipsoid model.

**REGIONAL LV DIASTOLIC MYOCARDIAL STIFFNESS.** Obtained values for regional LV myocardial wall stress and segmental myocardial strain at the onset of diastole and at end-diastole enabled calculation of regional LV diastolic myocardial stiffness in both the circumferential and longitudinal directions. The slope of the line between these 2 points in the stress-strain plane, developed in the defined anatomical regions, was used to compute the regional LV diastolic myocardial stiffness ($K_{MR}$) as:

$$K_{MR} = \frac{σ_{ED}}{ε_{DO}} \quad \text{(Eq. 5)}$$

where $σ_{ED}$ and $ε_{DO}$ are the regional end-diastolic LV myocardial wall stress and LV segmental myocardial strain at the onset of diastole, respectively (39).

**NONINVASIVE ESTIMATION OF LV END-DIASTOLIC PRESSURE AND LV CHAMBER STIFFNESS.** To facilitate serial biomechanical analyses of the LV, we developed a noninvasive estimation of LV end-diastolic pressure ($P_{ED}$) via relation of the LA area to invasively measured pulmonary capillary wedge pressure. Briefly, a cohort of referent control (n = 5) and LVPO (n = 8) pigs were anesthetized (fentanyl 50 and propofol 100 mg), and a multilumen thermocatheter (7.5F, Baxter Healthcare Corp., Irvine, California) was then advanced through the right external jugular vein and positioned in the pulmonary artery. Pulmonary capillary wedge pressure, a surrogate for $P_{ED}$, was measured using conventional methods (43). Data were used to identify an expression for noninvasive estimation of LV end-diastolic pressure, specifically $P_{ED} = 1.88e^{0.16(\text{LA area})} (r^2 = 0.91)$, which was subsequently applied across the study groups. This pressure estimation was used to compute a noninvasive measure of LV chamber stiffness ($K_C^*$) as follows (39):

$$K_C^* = \frac{P_{ED}}{EDV} \quad \text{(Eq. 6)}$$

**INVASIVE MEASURES OF LV PRESSURE AND LV CHAMBER STIFFNESS.** To validate noninvasive biomechanical measures, invasive LV pressure and dimensions were obtained in a subset of referent control (n = 6) 4 weeks post-LVPO (n = 4), and 5 weeks post-LVPO (n = 4) pigs. A pre-calibrated microtip transducer (7.5F, Millar Instruments Inc., Houston, Texas) was placed in the LV through a small apical stab wound. LV pressure data were recorded at a sampling frequency of 100 Hz and digitized (Ponemah, Harvard Bioscience Inc., Holliston, Massachusetts). Following the placement of instrumentation, an ultrasound transducer (GE Vivid E9 with XDclear Ultrasound System: M5S [1.5 to 4.6 MHz] transducer probe) was positioned for
transthoracic imaging of the LV short axis and measurement of continuous LV volume and wall thickness (44). Steady-state LV function was determined with simultaneous acquisition of LV pressure and echocardiographic data, with the ventilator temporarily suspended to prevent respiratory artifact. After steady-state measurements, LV pre-load was altered with sequential occlusion and release of the inferior vena cava with vascular ligature. Again, simultaneous LV pressure and echocardiographic data were collected during occlusion and release.

After data acquisition was complete, the digitized LV pressure and dimension data were aligned using the R-wave of the simultaneously recorded electrocardiogram from each modality. The aligned data for the steady-state and caval occlusion cardiac cycles were then used to compute the regional LV myocardial stiffness constants ($K_C$) and LV chamber stiffness ($K_{Ch}$), respectively (45,46). In addition, data were processed to compute classic indexes of LV systolic function and LV diastolic function using established methods (47-49).

**SHG IMAGING.** LV myocardial collagen microstructure was analyzed in subset of the referent control (n = 5), 4 weeks post-LVPO (n = 6), and 5 weeks post-LVPO (n = 7) pigs using SHG imaging. Briefly, the pigs were euthanized (5% isoflurane), the LV removed, and a 1-inch thick circumferential section was fixed in 10% formalin—preserving the native orientation and configuration of the LV myocardial collagen fibers—for 24 h at 4°C. The LV lateral free-wall myocardium was then sectioned into 200 μm sections and placed on the motorized stage of a multiphoton microscope (Leica TCS SP8 MP, Leica Microsystems). Short-pulsed infrared lasers were used to create nonlinear polarization effects in the form of SHG signals at a magnification of 25×. SHG images were acquired for a fixed sample area of 0.2 mm² at depth intervals of 1.5 μm.

**AUTOMATED TRACING OF INDIVIDUAL COLLAGEN FIBERS.** Two-dimensional images acquired from the multiphoton microscope were converted to 8-bit grayscale and virtually stacked in 3 dimensions using the Amira software package (50). For automated tracing of individual collagen fibers, we modified the previously described techniques developed for electron tomography (51,52), which were packaged into the XTracing filament detection extension available for Amira (50). In brief, this tracing algorithm consists of 2 main steps: template matching and a line search approach. Template matching uses a graphics processing unit (NVIDIA Quadro M2000, Santa Clara, California) to compute the voxel cross-correlation field in a 3-dimensional image stack, with a cylindrical template that mimics a collagen fiber segment with a mask radius of 5 μm and a length of 20 μm. The line search approach identifies the voxels that form the centerline of each collagen fiber. From an initial seed point, a search cone spanning 20 μm and 45 degrees identifies candidate collagen fiber points that are scored for continuity, curvature, and deviations from the orientation field. The line search stops when the search cone fails to identify candidate points with scores above the minimum threshold.

**CHARACTERIZATION OF THE COLLAGEN FIBER ORIENTATION AND MICROSTRUCTURE.** Each collagen fiber was evaluated with respect to orientation and undulation. Two angles were used to define the fiber orientation. The azimuthal angle ($\phi$) refers to the angle from the positive radial axis of the fiber’s projection on the longitudinal-radial plane, where $\phi = 0$ degrees is then the radial direction. The elevation angle ($\theta$) refers to the angle from the positive circumferential axis of the fiber’s projection on the circumferential-longitudinal plane, where $\theta = 0$ degrees and $\theta = 90°$ correspond to the circumferential and longitudinal directions, respectively. Fiber undulation ($u$) is defined as the ratio between the path length ($l_p$) and the end-to-end length ($l_e$) of a given fiber.

$$u = \frac{l_p}{l_e} \quad \text{(Eq. 7)}$$

**LV MYOCARDIAL COLLAGEN CONTENT BY LIGHT MICROSCOPY.** LV myocardial collagen content in lateral free-wall sections was analyzed in subset of the referent control (n = 9), 4 weeks post-LVPO (n = 6), and 5 weeks post-LVPO (n = 7) pigs. Collagen was visualized via picrosirius red staining under polarized light at a magnification of 40×. Viewing fields were chosen at random from the LV epicardium, mid-myocardium, and endocardium. Fields with large blood vessels were excluded from the analysis. Collagen content was calculated as the percentage of positively stained area in the field of view (ImageJ software) (53).

**PLASMA N-TERMIAL PRO-BRAIN NATRIURETIC PEPTIDE.** Plasma samples were obtained in a subset of the 4 weeks post-LVPO (n = 8) and 5 weeks post-LVPO (n = 8) pigs, as well as a referent control group that included pigs from a previous study (n = 19) (54). Plasma was thawed on ice and diluted 1:10 with phosphate-buffered saline. Pig-specific N-terminal pro-brain natriuretic peptide (NT-
proBNP) was measured using an enzyme-linked immunoassay format (Kamiya Biomedical Company, Seattle, Washington).

**TARGETED GENE EXPRESSION.** Targeted mRNA levels for the calcium regulatory proteins sarcoplasmic reticulum calcium adenosine triphosphatase and phospholamban were measured (55,56), as well as the cytoskeletal protein titin (57,58). Briefly, RNA was extracted from LV mid-myocardium (RNeasy Fibrous Tissue Mini Kit, Qiagen, Valencia, California), and the quality and concentration confirmed (Experion Automated Electrophoresis System, Bio-Rad, Hercules, California). RNA samples were then converted to cDNA (RT2 First Strand Kit, Qiagen, Cat. #330401, Valencia, California) and quantitative real-time polymerase chain reaction (qPCR) was performed (RT2 qPCR Primer Assay, Qiagen Cat. #33001) with pig-specific mRNA expression primers (Qiagen). The qPCR reactions (Bio-Rad CFX96) were carried out using cycling parameters designated by the manufacturer. Cycle time (Ct) values of the mean for Gapdh were used as reference gene values for normalization.
Fold-change expression was calculated using the $\Delta \Delta Ct$ method, in which $2^{\Delta \Delta Ct}$ is the fold-change value.

**Statistical Analysis.** Data are reported as the mean ± SEM. Comparative analyses were performed using a 1-way analysis of variance followed by post hoc pairwise comparisons using the least significant difference method. For the assessment of the relative change from baseline, a Student’s $t$-test was performed in which significance of the transformed data were determined using a null hypothesis of a zero mean value. For SHG-based collagen fiber characterization, at each timepoint, all values of $\varphi$, $\theta$, and $\gamma$ were pooled and binned into 100 equally sized bins. The resulting frequency distributions were subjected to a Kruskal-Wallis 1-way analysis of variance for nonparametric comparisons across different timepoints. A multiple linear regression model with inputs derived at 1 week post-LVPO was used to predict the relative change in $K_C$ at 2, 3, 4, and 5 weeks post-LVPO, and the $p$ value and $t$-statistic were calculated for each of the input variables. Spearman’s rank correlation analysis was used to interrelate experimental response variables at 4 and 5 weeks post-LVPO. All statistical analyses were performed with SPSS software version 24.0 (IBM, Armonk, New York).
TABLE 1  LV Function and Biochemistry With Progressive LVPO

|                          | Referent Control | 4 Weeks Post-LVPO | 5 Weeks Post-LVPO |
|--------------------------|------------------|------------------|------------------|
| LV pressures             |                  |                  |                  |
| Peak-systolic pressure   | 117 ± 5          | 149 ± 14*        | 153 ± 7*         |
| End-diastolic pressure   | 7.4 ± 0.7        | 12.5 ± 0.6*      | 12.5 ± 0.9*      |
| LV systolic function     |                  |                  |                  |
| Stroke work (kg/cm²)     | 4.6 ± 0.5        | 7.0 ± 0.6*       | 7.9 ± 0.6*       |
| PRSW (g/cm²)             | 37.3 ± 8.7       | 88.9 ± 11.4*     | 88.5 ± 12.7*     |
| Max – dP/dt (mm Hg/s)    | 2,165 ± 431      | 2,096 ± 867      | 1,194 ± 131      |
| Normalized max – dP/dt   | 18.5 ± 3.9       | 13.1 ± 4.2       | 7.8 ± 0.8        |
| End-systolic elastance   | 1.3 ± 0.3        | 3.9 ± 1.0*       | 3.3 ± 1.1*       |
| Normalized end-systolic  | 0.016 ± 0.005    | 0.028 ± 0.008    | 0.024 ± 0.01     |
| end-systolic elastance   |                  |                  |                  |
| LV diastolic function    |                  |                  |                  |
| Max – dP/dt (mm Hg/s)    | 2,168 ± 385      | 1,356 ± 177      | 1,371 ± 108      |
| Normalized max – dP/dt   | 18.3 ± 2.9       | 9.0 ± 0.5*       | 8.9 ± 0.3*       |
| Tau (ms)                 | 25.3 ± 2.5       | 32.6 ± 2.0       | 34.7 ± 2.6*      |
| Biochemistry             |                  |                  |                  |
| Plasma NT-proBNP (pg/ml)| 604 ± 63         | 676 ± 68         | 1347 ± 497*      |
| SERCA (mRNA)§            | –                | 1.1 ± 0.2        | 1.1 ± 0.1        |
| Phospholamban† (mRNA)§   | –                | 1.2 ± 0.2        | 1.5 ± 0.4        |
| Titin (mRNA)§            | –                | 1.2 ± 0.1        | 1.8 ± 0.3*       |

Values are mean ± SD. Sample sizes are n = 4 or greater/group; see Methods for details. *p < 0.05 vs. referent control. †Normalized by peak left ventricular (LV) systolic pressure. §Normalized by LV mass. ‡Results expressed as 2–ΔΔCT fold change. §p < 0.05 vs. 4 weeks post-left ventricular pressure overload (LVPO).

LV BIOMECHANICAL FUNCTION. LV peak myocardial strain in the circumferential direction was generally retained post-LVPO, whereas slight initial (1 week post-LVPO) increases remained unchanged by 5 weeks post-LVPO (Figure 3A). In the longitudinal direction, LV peak myocardial strain was preserved or moderately elevated throughout the LV myocardium with significant elevations occurring in only the endocardial layer (Figure 3A). Thus, similar to indexes of LV systolic function (Table 1), LV strain patterns were not diminished by LVPO.

LV diastolic myocardial strain rate was largely preserved in the circumferential direction, but monotonically declined in the longitudinal direction, with significant reductions in the LV epicardium and mid-myocardium by 3 and 4 weeks post-LVPO, respectively (Figure 3B). Mean LV diastolic myocardial stiffness (κMR), taken as the average value among all regions, significantly increased by 4 weeks post-LVPO in the circumferential direction and by 2 weeks post-LVPO in the longitudinal direction. Mean κMR in the circumferential direction exhibited a significant late elevation with an approximate 30% increase occurring between 4 and 5 weeks post-LVPO (Figure 3C). Regional measurements of κMR determined via STE exhibited significant correlation with analogous measurements obtained from invasive catheterization (p = 0.70, p = 0.024), supporting the use of STE for serial evaluation of LV myocardial mechanical properties.

LV CHAMBER STIFFNESS. LV pressure-volume relations were notably altered post-LVPO, including increased at 4 and 5 weeks post-LVPO (Table 1). Load-independent indexes of LV function such as pre-load recruitable stroke work (PRSW) and end-systolic elastance followed a similar pattern (Table 1). LV end-diastolic pressure was increased from referent control values at 4 and 5 weeks post-LVPO (Table 1) and paralleled the relative magnitude in LA area at these timepoints (Figure 2D). Indexes of active LV relaxation were impaired with progressive LVPO, which included prolongation of IVRT (Figure 2E). Other indexes of LV relaxation derived from invasive LV pressure measurements, such as peak negative pressure development and tau, were both changed from referent control values by 5 weeks post-LVPO (Table 1). Thus, in this model of progressive LVPO, LV systolic function was not compromised. However, indexes of LV diastolic function were significantly affected, which is consistent with the functional phenotype of HFpEF (27,49,59).

RESULTS

LV GEOMETRY AND FUNCTION. LVPO induced a monotonic increase in LV mass exceeding normal developmental growth by 2 weeks post-LVPO with a relative doubling of LV mass at 5 weeks post-LVPO (Figure 1). In contrast, the LV EDV/body weight ratio was preserved, and LVEF was modestly increased post-LVPO (Figures 2A and 2B). LV mass/volume ratio increased by 2 weeks post-LVPO (Figure 2C), whereas LA area more than doubled by 5 weeks post-LVPO (Figure 2D). In comparison, LA area of age-matched referent control pigs (5.7 ± 0.6 cm²) was unchanged with respect to the baseline value (5.4 ± 0.4 cm²). Thus, with LVPO, significant LV hypertrophy and LA dilation occurred over and above that of normal growth.

As expected, LV peak systolic pressure was increased at 4 and 5 weeks post-LVPO (Table 1). Additional indexes of LV pump function (LVEF shown in Figure 2B), such as stroke work and LV pressure development (+dP/dt), either increased or remained unchanged from the referent control values (Table 1).
changes in the diastolic phase (Figure 4A). At 5 weeks post-LVPO, significant increases in LV chamber stiffness relative to referent control values were indicated by changes in both KC and KC/C3 (Figure 4B). A steady rise in KC resulted in a doubling of the baseline value by 5 weeks post-LVPO, with a significant late rise occurring between 4 and 5 weeks post-LVPO (Figure 4C). There was significant correlation between KC and KC/C3 (r = 0.75, p = 0.013) that supported the use of KC/C3 for serial evaluation of LV chamber stiffness. Thus, the progressive rise in LV chamber stiffness, which is considered a key determinant of HFrEF progression (32,59), occurred post-LVPO and could be noninvasively monitored.

**LV MYOCARDIAL COLLAGEN CONTENT AND ORGANIZATION.** LV myocardial collagen content increased in the epicardium, mid-myocardium, and endocardium by 4 weeks post-LVPO with no further elevations observed at 5 weeks post-LVPO (Figure 5). Layer-specific differences in myocardial collagen content emerged at 5 weeks post-LVPO, with significantly greater content in the endocardium compared with the epicardium. The increased collagen content post-LVPO was coupled with changes in collagen fiber microstructure (Figure 6A). In all 3 LV myocardial layers, collagen fiber undulation distributions indicated a significantly higher coincidence of less undulated collagen fibers at 5 weeks post-LVPO, with significant changes occurring between 4 and 5 weeks post-LVPO.
post-LVPO in the endocardial and epicardial layers (Figure 6B). In terms of collagen fiber organization, collagen fiber angle distributions on the circumferential-longitudinal plane in the endocardium and epicardium were significantly shifted toward the circumferential axis by 4 and 5 weeks post-LVPO, respectively (Figure 6C). Collagen fiber angle distributions on the radial-longitudinal plane were generally preserved post-LVPO (Figure 6D).

Thus, although total collagen content increased at both 4 and 5 weeks post-LVPO, shifts in fibrillar collagen architecture occurred at 5 weeks post-LVPO and were coincident with the significant rise in LV chamber stiffness.

**BIOCHEMICAL MARKERS.** Plasma NT-ProBNP levels were maintained at referent control values at 4 weeks post-LVPO but increased significantly at 5 weeks post-LVPO. LV myocardial mRNA levels for
sarcoplasmic reticulum calcium adenosine triphosphatase and phospholamban were unchanged at 4 or 5 weeks post-LVPO (Table 1). The cytoskeletal protein titin, which was previously shown to be increased with LVPO and HFpEF (58) was increased by 5 weeks post-LVPO (Table 1).

PREDICTING CHANGES IN LV CHAMBER STIFFNESS. To examine the relationship between LVPO-induced regional changes in $\kappa_{MR}$ and LV wall thickness ($H$), we computed the slope of a linear regression between these response variables ($\partial K_{MR}/\partial H$), which was developed with measurements from the 6 defined LV myocardial regions at 1 week post-LVPO. When considered as a composite, changes in regional $\kappa_{MR}$ and $H$ were significantly correlated, whereas $\partial K_{MR}/\partial H$ was negative (Figure 7A). Individual values for $\partial K_{MR}/\partial H$, along with the LV chamber stiffness determined at 1 week post-LVPO ($K_{C,1-week}$), were incorporated into a multiple linear regression model of the form

$$K_{C, pred} = a \frac{\partial K_{MR}}{\partial H} + b K_{C,1-week} + cT + d$$  \hspace{1cm} (Eq. 7)

where $K_{C, pred}$ is the predicted percent increase in chamber stiffness at time $T$ (post-LVPO time, in days), and $a$, $b$, $c$, and $d$ are fitting parameters. The regression model, built with data from 2 weeks post-LVPO through 5 weeks post-LVPO, yielded a strong linear relation between $K_{C, pred}$ and $K_{C}$ (Figure 7B), with all model fitting parameters ($a$, $b$, $c$, and $d$) exhibiting statistical significance ($p < 0.05$). A repeated measures (rm) correlation analysis was performed to account for the within-individual association for paired measures, with a positive correlation coefficient between $K_{C, pred}$ and $K_{C}$ ($r_{rm} = 0.42$, $p = 0.011$) (60). Furthermore, a linear mixed model was generated, and a Bonferroni adjustment revealed pairwise
significant statistical differences between the predicted values at all 4 timepoints.

**CORRELATION BETWEEN LV MYOCARDIAL COMPOSITION AND BIOMECHANICS.** A correlation matrix was developed to relate the relative change in LV biomechanical response variables and geometrical, compositional, and microstructural features of the LV myocardium (Figure 8). LV wall thickness and myocardial collagen content were not correlated to biomechanical measurements, whereas some changes in matrix microstructure were associated with these measurements. Specifically, a reduction in collagen fiber undulation ($u \rightarrow 1$) was correlated with increased LV myocardial stiffness in all 3 layers of the LV. Furthermore, an inverse correlation was observed between the collagen fiber elevation angle and LV myocardial stiffness in the epicardial layer, linking a preferential fiber orientation toward the circumferential axis ($\theta \rightarrow 0^\circ$) to higher circumferential LV myocardial stiffness.
FIGURE 7 Predicting LVPO-Induced Increase in LV Chamber Stiffness Based on Early Noninvasive Measurements

(A) Region-matched changes in wall thickness (H) and circumferential diastolic myocardial stiffness (kMR) obtained at 1 week post-LVPO exhibited an inverse correlation among all post-LVPO animals (n = 14). (B) Predicted values for LVPO-induced changes in LV chamber stiffness relative to baseline (Kpre/c3) were generated through multiple linear regression modeling. Kpre/c3 significantly correlated with experimental values, suggesting that noninvasive measurements obtained at 1 week post-LVPO can predict the severity of subsequent LV chamber stiffening. RV = right ventricular; other abbreviations as in Figure 1.

FIGURE 8 Biomechanical, Geometrical, Compositional, and Microstructural Correlations

A Spearman’s rank correlation analysis was used to interrelate region-matched biomechanical response variables (peak strain, diastolic strain rate, and diastolic myocardial stiffness (kMR)) with geometrical (wall thickness), compositional (collagen content), and microstructural (collagen undulation (u), undulation (v) and orientation (w)) response variables. All correlations refer to data means at the terminal study timepoints; (4 weeks post-LVPO: n = 6; 5 weeks post-LVPO: n = 7). *p < 0.05 for Spearman’s rank correlation coefficient (ρ). Abbreviations as in Figures 1 and 6.
DISCUSSION

HFpEF is a specific HF phenotype that afflicts millions of patients annually, although specific and effective therapeutic strategies have not been forthcoming (1,2,61). This issue is also compounded by the fact that the indexes of LV function that contribute to the development and progression of HFpEF require specific measures of LV myocardial diastolic performance (20,21). The present study addressed these issues by using a large animal model of progressive LVPO, which over time, recapitulated many of the key phenotypical features of HFpEF. The significant findings from this study are 3-fold. First, progressive LVPO induced a gradual and significant rise in LV diastolic myocardial stiffness, which was noninvasively determined and thus holds potential for clinical assessment of HFpEF. Second, these LVPO-induced elevations in LV diastolic myocardial stiffness appeared not only to be modulated by myocardial collagen content, but also by changes in collagen microarchitecture (shifts in alignment and undulation). Third, our data suggested that with LVPO, a relationship between early changes in regional LV diastolic myocardial stiffness and LV wall thickness could be predictive of the subsequent rise in LV chamber stiffness. Taken together, these novel findings elucidated the effects of LVPO on LV myocardial biomechanics and hold promise as a translational approach to monitor and predict HFpEF progression as well as evaluating new therapeutic targets.

LV MYOCARDIAL BIOMECHANICS. LV longitudinal myocardial strain has been used as a clinical index for HF diagnosis and phenotyping, wherein changes in global values are indicative of LV systolic function, and changes in regional strain patterns can provide information for HF sub-classification (62–65). Although past clinical studies suggested that HFpEF is accompanied by a reduction in LV global longitudinal myocardial strain, the present study (along with others) observed modest, if any, early changes in regional or global myocardial strain and diastolic strain rate (27,29,62,66–68). In addition to LV regional strain measurements, the present study examined several indexes of LV systolic function. Overall, these measurements, such as EF and stroke work, were either preserved or modestly changed with progressive LVPO. Load-independent measures of LV function, such as PRSW and end-systolic elastance, followed a similar trend. Thus, using multiple approaches, this model of LVPO was shown to not to be associated with a compromise in LV pump function or systolic performance. The relative increase in load-independent indexes of LV function, such as PRSW, were likely due to the increased LV mass, and as such, increased mass of contractile units. Furthermore, at the timepoints measured in this study, LVPO was not associated with transcriptional shifts in key determinants of sarcoplasmic reticular Ca++ function. Thus, this progressive LVPO model appeared to initially present overall with intact LV myocardial contractile function, with clear declines in both active and passive components of LV diastolic function, which is a key functional feature of HFpEF.

With progressive LVPO and LV hypertrophy, other key characteristics of HFpEF development occurred, including LA dilation, increased LV diastolic pressure, and elevated NT-proBNP. From a biomechanical perspective, hypertrophy is the primary adaptive response of the LV myocardium to LVPO, mitigating the elevated values LV myocardial wall stress (67). Partially as a result of LV hypertrophy, we observed progressive and significant elevations in $K_C$ with LVPO, similar to previous reports (22,30–32,68,69). Our serial analyses enabled novel comparison of late study timepoints (4 weeks post-LVPO vs. 5 weeks post-LVPO), which suggested that late $K_C$ elevations were not solely governed by LV myocardial collagen accumulation (i.e., “fibrosis”) but also the local changes in LV myocardial mechanical properties as reflected by $K_M$. However, it must be recognized that changes in LV chamber stiffness are governed by both active relaxation as well as passive myocardial compliance. In the present study, a prolongation of both IVRT and tau occurred at the LVPO timepoints, which were indicative of changes in active relaxation. Thus, it is likely that the rise in LV chamber stiffness with LVPO was due to both changes in LV myocardial collagen content and structure, as well as active relaxation.

Although the co-dependence of aberrant LV structural mechanics on both LV geometry and myocardial mechanical properties is expected in all modes of HF, these results provided novel directional and regional detail on LVPO-induced elevations in $K_M$, and importantly, informed temporal relation to elevations in $K_C$ (22,68,70–72). Specifically, late LVPO-induced elevations in $K_M$ were coupled with an abrupt rise in $K_C$, which suggested that image-based determination of LV myocardial mechanical properties holds
potential usefulness to identify a key transition point and progression of HFpEF.

**MICROSTRUCTURAL CHANGES IN THE LV MYOCARDIUM WITH LVPO.** One of the hallmarks of the LV myocardial remodeling with LVPO is increased fibrillar collagen content, which is due in part to shifts in collagen synthesis and turnover. Moreover, these changes in LV myocardial collagen content with LVPO contributed to diastolic dysfunction and were not readily reversible (73–77). Specifically, increased LV myocardial collagen content was associated with increased LV chamber stiffness and LV myocardial stiffness (78–81). In the present study, a correlation between LV myocardial stiffness and collagen content with progressive LVPO was observed, but our studies moved beyond this observation. Specifically, our analysis identified that changes in collagen fiber undulation and orientation, which are the key microstructural features that govern collagen engagement in load-bearing, were also associated with changes in LV myocardial stiffness at late LVPO timepoints (82). Moreover, the late emergence of coinciding, layer-specific differences in LV myocardial collagen content and fiber undulation distributions further suggested that both factors contributed to increased passive LV myocardial stiffness with LVPO.

The present study also identified that LV myocardial collagen fiber realignment occurred as a function of time with LVPO, which further supported the concept that the microarchitecture of the assembled collagen fibers with LVPO was abnormal. However, it must be recognized that past studies with LVPO identified significant shifts in myocyte cytoarchitecture, such as titin, which were associated with changes in LV myocardial stiffness properties (83). Although examined only at the transcriptional level, the present study identified that titin mRNA was increased at the late (5 weeks) post-LVPO timepoint. Thus, shifts in titin content and potentially phosphorylation states might have occurred in this LVPO model and warrant future study (83). Although due to a different LV remodeling stimulus, recent findings identified a similar shift in collagen microarchitecture within the hypertrophied remote region in the post-infarcted LV myocardium (84). When coupled with the significant late increase in $\kappa_{MR}$ in the circumferential direction, our findings suggested that LV myocardial collagen fiber realignment was a key factor underlying increased LV myocardial stiffness with LVPO and was an independent and/or additive mechanism that contributed to the progression of HFpEF.

**PREDICTING LATE INCREASES IN LV CHAMBER STIFFNESS DUE TO LVPO.** In general, muscle growth and remodeling are adaptive, cell-mediated processes that restore local mechanical homeostasis (i.e., baseline values of stresses and strains) under sustained deviations in mechanical loading (85–89). Thus, elevations in local stress result in a compensatory increase in mass while stress reductions result in the opposite. Using the present study as a platform, we postulated that if growth and remodeling processes also act to retain normal structure function relations at the LV chamber level (i.e., LV chamber stiffness), then LV mass changes must be coupled with opposing changes in LV myocardial stiffness. At 1 week post-LVPO, increased regional LV wall thickness was inversely correlated to regional changes in $\kappa_{MR}$. Using this observation as a framework, we then examined the relation between early changes in regional LV wall thickness and $\kappa_{MR}$ with LVPO and identified a predictive model of $K_2$ elevations over later post-LVPO timepoints. This image-based biophysical analysis has the potential to yield a clinically relevant approach to predict the rate and extent of increased LV chamber stiffness with prolonged LVPO, and hence, the relative risk for HFpEF. Defining early determinants for the risk of developing HFpEF have been identified as an important area of development for disease management (33–37).

**STUDY LIMITATIONS.** Although the present study identified unique ultrastructural and regional biophysical relationships in a large animal model of LVPO, several limitations should be recognized. Although indexes of LV diastolic function (i.e., LA area, IVRT, tau, LV chamber stiffness) suggest that the LVPO pigs were on a trajectory to develop symptomatic HFpEF (e.g., pulmonary edema), these longitudinal studies were not carried out to this endpoint. However, by 5 weeks post-LVPO, a biomarker feature for the progression to HF was identified by increased NT-proBNP (90). As such, this LVPO model appeared to meet the criteria for emulating key features of the natural history of the progression to HFpEF. Because of the inherent sensitivity of indexes of LV diastolic function to measurements of LV filling pressure, it must be recognized that our analysis was limited by the estimations of LV pressures that were used at both the onset of diastole and end-diastole. Future work will
focus on developing new methods of characterizing LV filling pressure in a serial manner and incorporating those measurements into this analytical framework.

CONCLUSIONS

The present study, using STE, provided a serial method for assessing regional LV myocardial stiffness, but future work will be required to examine whether and to what degree these measurements are sensitive to and predictive of pharmacotherapies. Past studies have identified that in isolated myocyte preparations, passive stiffness properties are affected with LVPO (91). In light of the fact that steady-state mRNA levels for the cytoskeletal protein titin increased at the late LVPO timepoints, these myocyte based studies would be an appropriate future direction. These limitations notwithstanding, the unique findings from this study were that assessment of global changes in LV function with LVPO might be insensitive to identifying key transition points in HFP EF development and that quantifying the relative fibrosis through LV myocardial collagen content alone was insufficient to identify the mechanistic underpinnings that govern increases in LV myocardial stiffness and LV chamber stiffness, which are biophysical milestones of this disease.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The underlying mechanisms that contribute to the development and progression of HFP EF remain incompletely understood. This is due in part to a paucity of animal models that recapitulate key phenotype features of HFP EF, which can be measured in a serial fashion. The present study developed a pig model of progressive PO, which developed into HFP EF phenotype. Key biophysical parameters such as collagen microstructure and regional stiffness were associated with the transition to the HFP EF phenotype. Early changes in regional myocardial stiffness predicted the extent of the HFP EF phenotype.

TRANSLATIONAL OUTLOOK: This study provides the foundation by which new directions in diagnostics and therapeutics can be developed.
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KEY WORDS Heart failure, echocardiography, pressure overload
Interstitial Fibrosis and Diastolic Dysfunction in Aortic Stenosis*

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Continuous and progressive left ventricular (LV) pressure overload from hemodynamically significant aortic stenosis or severe sustained hypertension results in a well-characterized progression from compensated to decompensated pathophysiology (1). The classical response to pressure overload is characterized by variable degrees of LV hypertrophy, which normalizes wall stress to maintain systolic function and ejection fraction in the face of markedly elevated LV afterload. Increases in myocardial oxygen consumption arise from the increased LV mass and sustained elevations in systolic pressure. These changes, along with the inability of the coronary microcirculation to proliferate in the hypertrophied ventricle, reduce coronary flow reserve leading to repetitive subendocardial ischemia and angina in the absence of obstructive coronary artery disease (2). Episodes of transient ischemia and increased diastolic myocyte strain from elevations in LV end-diastolic filling pressure lead to myocyte apoptosis as well as myocardial fibrosis, which becomes particularly prominent in the subendocardium. Whereas this compensation can be maintained for some time, sustained pressure overload eventually leads to decompensation characterized by a reduction in systolic function resulting in heart failure with a reduced ejection fraction.

Whereas myocardial fibrosis was originally envisioned to be a relatively late manifestation of pressure overload, recent clinical studies using cardiac magnetic resonance (CMR) imaging have suggested that fibrosis may occur when systolic function is preserved and may provide prognostic significance affecting the timing of aortic valve replacement (1). Both fibrosis and LV hypertrophy alter diastolic properties and can contribute to symptoms of dyspnea in aortic stenosis. In this issue of JACC: Basic to Translational Science, Torres et al. (3) provide insight into the temporal evolution of LV diastolic properties in a large animal model of supravalvular aortic stenosis. In contrast to the numerous studies evaluating how aortic banding and hypertensive pressure overload leads to heart failure with a reduced ejection fraction, the experiments were conducted over a time frame before ejection fraction falls with systolic function presumably preserved. Temporal changes in diastolic properties were assessed noninvasively with speckle tracking echocardiography and complemented with invasive diastolic LV pressure-volume relations assessed at 4 or 5 weeks after producing moderately severe aortic stenosis. The in vivo physiological studies were paired with histologic measurements of myocardial collagen content and ex vivo 3-dimensional studies of transmural collagen fiber orientation. The results demonstrate a late shift in indices of collagen microstructure stiffness that reduced global LV chamber stiffness and could be predicted by the analysis of circumferential diastolic stiffness present 1 week after chronic pressure overload. Most of these changes occurred in the...
subendocardium. Torres et al. (3) conclude that noninvasive assessment of regional biomechanical function may provide a sensitive approach to monitor the progression of diastolic function when systolic function is preserved.

These are technically demanding studies and the investigators should be congratulated on combining sophisticated and translationally relevant measurements of in vivo LV diastolic function with postmortem characteristics of myocardial fibrosis, collagen quality, and fiber orientation in a chronic swine model. Interestingly, despite significant changes in diastolic properties, the magnitude of the increase in interstitial fibrosis was quite small by picrosirius staining (~6% vs. 4% in normal subjects). Whereas additional analysis supports the investigators’ conclusions regarding the importance of collagen quality and fiber orientation, other potential determinants of diastolic properties were not assessed and could contribute to altered diastolic properties. For example, increased titin messenger ribonucleic acid was demonstrated in animals studied with 5 versus 4 weeks of pressure overload, but titin protein content, isoform expression, and phosphorylation status were not assessed. These are determinants of early diastolic properties and may be contributory, particularly because the LV end-diastolic pressure range studied with pressure-volume relations during preload manipulation was low (<12.5 mm Hg, probably reflecting the isoflurane anesthetized state). In addition, the changes in diastolic properties between 4 and 5 weeks after instrumentation are most prominent in the subendocardium and accompanied by transmural variations in collagen with subendocardial collagen content exceeding subepicardial collagen content at 5 weeks. This raises the question as to whether subendocardial ischemia in the hypertrophied heart could be contributing to the observed changes. Finally, alterations in collagen cross-linking may also be playing a role and were not determined but may also contribute to changes in diastolic distensibility.

From a clinical standpoint, the results of this study further inform our understanding of the importance of LV diastolic dysfunction in aortic stenosis. Recent clinical research has focused on the role of diastolic properties in aortic stenosis by evaluating interstitial fibrosis (1). This can be quantified by CMR and may be the result of the aortic stenosis or concomitant comorbidity from heart failure with preserved ejection fraction (HFpEF) or amyloid deposition, which are particularly common in the elderly patient population. Quantifying fibrosis in aortic stenosis can also predict the likelihood of reversible systolic dysfunction and improvement in heart failure prior to valve replacement. The increases in collagen content demonstrated by pathology in the present study would be difficult to quantify with precision using current CMR imaging techniques. Thus, the proposed biomechanical assessment developed by Torres et al. (3) may identify the progression of diastolic myocardial disease before fibrosis develops. Comparative prospective serial studies correlating echocardiographic indices of LV diastolic distensibility, CMR fibrosis, and prognosis or persistent symptoms after aortic valve replacement will be required to address this.

Clinical studies demonstrate that patients with HFpEF infrequently progress to systolic dysfunction and usually this is related to underlying coronary artery disease (4). In contrast, prior animal models of sustained pressure overload from aortic banding as well as models of severe hypertension usually demonstrate a progression from preserved to depressed systolic function. This transition is dependent on a number of variables including the severity and duration of pressure overload. Whereas ejection fraction did not decline within the 5-week time frame of the present study, the 5-week time point may be at the threshold of the transition to systolic dysfunction. This is suggested by a reduction in circumferential systolic strain, a marked reduction in LV contractility assessed by LV +dP/dt and an increased N-terminal pro-B-type natriuretic peptide in animals studied at the 5- versus 4-week time point.

Whether this would remain stable is unclear, and it seems plausible that a longer duration of pressure overload may have resulted in the predictable transition from a phenotype-dominated diastolic dysfunction to systolic dysfunction. Understanding the longer-term progression of this model along with studies to assess subendocardial perfusion would help address whether the alterations in biomechanical properties are related to diastolic properties, subendocardial ischemia, or reflect an early predictor of systolic dysfunction.

Most animal models of HFpEF developed to date have significant limitations in that they employ variations of severe systolic pressure overload with or without a variety of proinflammatory risk factors (e.g., diabetes, obesity, and hyperlipidemia) associated with endothelial dysfunction. Whereas Torres et al. (3) conclude that aortic stenosis with sustained LV pressure overload and preserved ejection fraction recapitulates multiple phenotypical features of HFpEF, there are differences with the clinical disease state that are also germane to other hypertensive models. First, whereas most patients with HFpEF...
have a history of hypertension, it is usually well-controlled and severe sustained pressure overload is quite uncommon. Second, severe LV hypertrophy is uncommon and, depending on enrollment criteria, as many as one-half of patients in clinical trials of HFpEF have a structurally normal echocardiogram without hypertrophy. Finally, increases in interstitial fibrosis in biopsies of patients with HFpEF are typically greater (~10%) than the small increase demonstrated in the present aortic stenosis model (~2%).

How is hypertension related to the development of HFpEF in the absence of sustained systolic pressure elevations? Whereas hypertension is prevalent, antihypertensive therapy and blood pressure control has intensified. Targets for control have fallen from 160 to 120 mm Hg over the last 50 years. Yet, as severe uncontrolled hypertension has declined, the incidence of HFpEF has actually increased to where it now accounts for well over one-half of the patients with congestive heart failure. This disconnect supports the role of proinflammatory states such as diabetes and obesity, which have become more common over this time period. Nevertheless, in preclinical studies, nonhypertensive animal models of obesity and diabetes exhibit minimal impairment of myocardial diastolic properties indicating a key role for a history of hypertension in altering LV distensibility. One possible explanation is that repetitive transient systolic hypertension arising from reduced aortic compliance (particularly common in elderly patients) may be the stimulus that produces HFpEF. In support of this mechanism, brief repetitive pressure overload in swine can lead to a 2-fold increase in interstitial fibrosis and reduced LV compliance (5). This is associated with concentric LV remodeling that develops in the absence of anatomic LV hypertrophy and recapitulates the structural phenotype of many patients with HFpEF.

Whereas all animal models of human disease have strengths and limitations, large animal models continue to play an important role in terms of their translational relevance as well as utility in studying therapeutic interventions prior to clinical application in humans. The work of Torres et al. (3) provides further insight into the complexity of transmural myocardial diastolic properties in compensated aortic stenosis that may ultimately be confirmed to relate to mechanisms of diastolic dysfunction in patients with HFpEF. Understanding whether these are modulated by proinflammatory stimuli may allow the importance of hypertension versus other risk factors to be defined in preclinical studies of HFpEF.

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KEY WORDS aortic stenosis, diastolic dysfunction, heart failure with a preserved ejection fraction, myocardial fibrosis
Activation of Oxytocin Neurons Improves Cardiac Function in a Pressure-Overload Model of Heart Failure

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HIGHLIGHTS

- Hypothalamic OXT neurons were chronically activated using a chemogenetic approach in an animal model of HF.
- Synaptic release of OXT onto parasympathetic autonomic targets was reduced in animals with HF but restored with daily treatment consisting of activation of OXT neurons.
- Long-term daily OXT neuron activation increased parasympathetic activity to the heart and reduced mortality, cardiac inflammation, and fibrosis and improved critical longitudinal in vivo indices of cardiac function.
- The benefits in cardiac function and autonomic balance in HF closely tracked the study-designed differences in initiation of OXT neuron activation in different groups.
This work shows long-term restoration of the hypothalamic oxytocin (OXT) network preserves OXT release, reduces mortality, cardiac inflammation, fibrosis, and improves autonomic tone and cardiac function in a model of heart failure. Intranasal administration of OXT in patients mimics the short-term changes seen in animals by increasing parasympathetic—decreasing sympathetic—cardiac activity. This work provides the essential translational foundation to determine if approaches that mimic paraventricular nucleus (PVN) OXT neuron activation, such as safe, noninvasive, and well-tolerated intranasal administration of OXT, can be beneficial in patients with heart failure. (J Am Coll Cardiol Basic Trans Science 2020;5:484–97) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

SUMMARY

The 9-amino acid neuropeptide, oxytocin (OXT), is only synthesized in a limited number of discrete brain regions: the paraventricular (PVN), supraoptic, and accessory nuclei of the hypothalamus (1). In addition to the classic effects of OXT—such as uterine contraction, milk ejection during lactation, sexual arousal, and penile erection—recent work indicates that OXT has an important role in both behavior and cardiovascular homeostasis, particularly during anxiety and stress (2). In human volunteers in unstressed conditions, intranasal administration of OXT increased parasympathetic—decreased sympathetic—cardiac control (3). Administration of OXT to subjects with obstructive sleep apnea shortened the duration of obstructive events and increased sleep satisfaction and parasympathetic activity to the heart (4).

The potential benefit of long-term OXT network activation in protracted cardiovascular diseases has not been tested. Heart failure (HF) affects nearly 23 million people worldwide, and prevalence is projected to increase 46% in the next 15 years (5). Approximately 50% of patients diagnosed with HF die within 5 years, necessitating the development of new treatments (5). A hallmark of HF is elevated cardiac sympathetic activity and parasympathetic withdrawal (6,7), an imbalance that contributes to ventricular dysfunc- tion, structural remodeling, and electrical instability (8). In the initial stages of HF, parasympathetic tone decreases as early as 3 days after the development of cardiac dysfunction, typically preceding increases in sympathetic activity (9,10).

Here, using OXT-sensitive sniffer cells, we test the hypothesis that endogenous release of OXT from hypothalamic PVN neurons onto brainstem autonomic targets is blunted in an animal model of HF. Furthermore, we examine if chronic chemogenetic activation of PVN neurons restores OXT release and if novel treatment paradigms of restoring OXT neuron activity, initiated at different intervals during the disease-progression timeline, reduces mortality, cardiac inflammation and fibrosis, and longitudinal indices of cardiac dysfunction compared with untreated animals with HF.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors’ institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the JACC: Basic to Translational Science author instructions page.
METHODS

ETHICAL APPROVAL. All animal procedures were completed in agreement with the George Washington University institutional guidelines and in compliance with the panel of Euthanasia of the American Veterinary Medical Association and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

SURGICAL PROCEDURE FOR TRANSASCENDING AORTIC CONSTRICTION. Pressure overload induced left-ventricular (LV) hypertrophy was initiated in male Sprague-Dawley rats using a minimally invasive transascending aortic constriction (TAC) procedure, similar to our previous study (11). Rats at 1 week of age were anesthetized by hypothermia and underwent TAC surgery. A 0.5-cm incision was made at the level of the chest, the chest was opened, and the thymus was retracted to reveal the aorta. A 4-0 silk suture was passed around the ascending aorta, and with a 25-gauge needle temporarily placed adjacent to the aorta—the suture was tied around both the aorta and needle (TAC and TAC + OXT groups). The needle was then removed, leaving the constricting suture around the aorta. Buprenorphine was applied as an analgesic. Successful constriction was confirmed with high aortic-velocity post constriction, using high-resolution echocardiography as well as upon examination of the aorta after each animal was killed.

SELECTIVE EXPRESSION AND ACTIVATION OF ChR2 AND DREADDs IN PVN OXT NEURONS. Selective expression of both channelrhodopsin (ChR2) and an excitatory chemogenetic receptor for chronically activating PVN OXT neurons, designer receptors exclusively activated by designer drugs (DREADDs), was accomplished using 3 viral vectors in combination with the Cre-Lox system (NIH, Bethesda, MD). Expression of the enzyme Cre recombinase was exclusively driven by the OXT promoter (rAAV1-OXT-Cre), stereotactically coinjected into the PVN of 1-week old pups with both floxed excitatory ChR2 (AAV1-EF1a-DIO-hChR2) and floxed excitatory DREADDs (AAV2-hSyn-DIO-hM3D(Gq), (30 to 50 nl of each virus selectively microinjected over a 20-min period), as previously described (12).

ACTIVATION OF PVN OXT NEURONS IN VIVO. PVN OXT neurons expressing DREADDs were exclusively activated by clozapine-N-oxide (CNO), 1 mg/kg intraperitoneally (IP). Our previous work has demonstrated that injections of CNO increases the firing of PVN OXT neurons for at least 1 h (12).

IN VIVO ASSESSMENTS OF CHANGES IN AUTONOMIC TONE AND CARDIAC FUNCTION. Sprague-Dawley rats at 2 weeks post-TAC/Sham surgery were anesthetized (isoflurane) and implanted with a telemetry device (DSI wireless transmitters, ETA-F10 [Data Sciences International, St. Paul, Minnesota]) with electrocardiographic (ECG) leads inserted subcutaneously to measure heart rate (HR). The following protocol was used to measure peak effort capacity, defined as the time that animals are able to run on the treadmill and the speed at time of exhaustion. Animals began with an initial warm-up period of 5 min at 6 cm/s with 1 min of recovery. The treadmill speed then quickly ramped up to 12 cm/s and increased by 6 cm/s every 3 min until exhaustion. Heart rate recovery (HRR) was assessed by subtracting the instantaneous HR following 20 s of recovery from that at peak exercise (Naughton protocol).

Echocardiographic measurements were obtained using a VisualSonics high-resolution small-animal system (Vevo 3100 Imaging System, FUJIFILM VisualSonics Inc, Toronto, Ontario, Canada) that continuously acquired the ECG, respiratory waveform, and body temperature. Imaging was performed at frame rates up to 1KHz, using 25MHz linear array transducer to obtain 2-dimensional (2D) pulse and continuous-wave Doppler and Motion-mode (M-mode) imaging. A 2D parasternal long-axis view of the LV was used to obtain LV outflow tract diameter and cardiac functional measures including percent LV ejection fraction (EF %). M-mode images were obtained from the parasternal short-axis view of the LV, at the level of papillary muscles to measure LV structural variables including LV diastolic posterior-wall diameter (LVPWd), interventricular septal diameter (IVSd), LV diameter in diastole (LV DD) and systole (LV Dds), and LV systolic posterior-wall (LVPWs) thickness. These parameters were used to calculate percent of fractional shortening (FS). Pulse-wave Doppler imaging in an apical 4-chamber view was used to obtain inflow velocities through mitral valves for assessing diastolic function, including early diastolic mitral inflow (E-wave), late diastolic mitral inflow (A-wave), and early-to-late diastolic mitral inflow ratio (E/A ratio). Continuous-wave Doppler modality was used to measure functional variables, such as velocity and pressure of blood flow in the ascending and descending aorta, and HR and cardiac output.

EX VIVO ASSESSMENTS OF CARDIAC FUNCTION. At 16 weeks post-Sham/TAC, rats were anesthetized with an IP injection of pentobarbital (50 mg/kg) and isoflurane inhalation anesthesia. Following cessation of pain reflexes, hearts were rapidly excised and Langendorff perfused via the aorta at constant pressure (65 mm Hg) and temperature (37°C [98.6°F]) with a
Krebs-Henseleit solution containing (in mM) 118 NaCl, 4.7 KCl, 1.25 CaCl₂, 0.57 MgSO₄, 1.17 K₂HPO₄, 25 NaHCO₃, and 6.0 glucose. Perfusate was oxygenated with 95% O₂-5% CO₂. After a stabilization period of 10 min, a size-5 balloon (Harvard Apparatus, Holliston, MA) was inserted into the LV to measure isovolumic LV-developed pressure (LVDP), as we have previously described (13,14). Diastolic pressure was set to 7 mm Hg, and LVDP was computed as the difference between systolic and diastolic pressures. HR and LVDP were measured for at least 15 min during sinus rhythm. After each study, contractility and relaxation were calculated as the maximum and minimum values of the first derivative of the LVDP waveform, respectively, to assess inotropy and lusitropy.

ANATOMIC MEASUREMENTS AND HISTOLOGY. Hearts from ex vivo studies were sliced longitudinally, halfway between the anterior and posterior sides. One-half of the heart was preserved in 10% formalin and stained with Masson’s trichrome to visualize collagen deposition.

WESTERN BLOTTING. Hearts from ex vivo studies were sliced longitudinally, halfway between the anterior and posterior sides. LV tissue from one-half
FIGURE 2 Long-Term Chemogenetic Activation of PVN Oxytocin Neurons Attenuates Cardiac Dysfunction Seen in Animals With Heart Failure

A. Sham (6) - TAC saline (6) - TAC CNO (5)

B. Early Treatment (6) - Late Treatment (7)

C. SV (microL)

D. % EF

E. CO (mL/min)

F. % FS

Continued on the next page
of the heart were flash frozen in liquid nitrogen and stored at -80°C (-112°F). Samples were thawed and homogenized using the Qproteome Mammalian Protein Prep Kit (QIAGEN, Hilden, Germany) in tubes containing metallic beads. Samples were centrifuged at 14,000 g for 10 min, and protein concentrations were determined by the Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, Massachusetts). Laemmli buffer (Bio-Rad, Hercules, California) with 10% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri) was added to the samples, which were then heated to 98°C (208.4°F). Equal protein concentration was loaded into wells containing 4% to 15% PROTEAN Criterion TGX Gels (Bio-Rad). The samples were then run between 50V to 100V for up to 2 h to separate proteins by electrophoresis.

After electrophoresis, the samples were then transferred to polyvinylidene fluoride (PVDF) membranes, using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes to be probed for collagen III (Abcam, Cambridge, United Kingdom) were blocked with 5% milk for 18 h and then incubated for 1 h at room temperature with collagen III, at a concentration of 1:1,000. The membranes to be probed for interleukin(IL)-1β were blocked with 5% bovine serum albumin (BSA) for 2 h. They were then incubated with IL-1β (Cell Signaling Technology, Danvers, Massachusetts) at a concentration of 1:1,000 overnight at 4°C (39.2°F). All membranes were washed and incubated for 1 h with the horseradish peroxidase (HRP)-conjugated secondary antibodies anti-rabbit HRP-conjugated secondary antibody (Sigma-Aldrich) 1:6,000 for 1 h. The membranes were washed and incubated for 1 h with the anti-mouse HRP-conjugated secondary antibody. Band intensities for collagen III and IL-1β were measured using NIH ImageJ or Li-Cor Image Studio (LI-COR Biosciences, Lincoln, Nebraska) and normalized to the corresponding band intensity for GAPDH; collagen III bands were normalized to Sham values as well as GAPDH.

Because of the large number of animals in each group, not all samples could be loaded on the same gel. We therefore ran 2 to 3 gels and included samples from each of the 3 groups (Sham, TAC, and TAC + OXT) on each gel and repeated selected samples on each gel. We then normalized signal intensity between membranes based on the repeated sample expression. This method provides consistent normalization of all samples both within each set and across the minimum number of required gels. Refer to Supplemental Figure 1 for more details.

**Statistical Methods.** Data were presented as mean ± standard error mean and analyzed using GraphPad Prism (GraphPad, San Diego, California) statistical software (version 8). Longitudinal data were compared among all groups using mixed-effects model (restricted maximum likelihood method) with Tukey’s postmultiple comparison test. For survival analysis, Kaplan-Meier curves were plotted and compared, using the log-rank test. One-way analysis of variance (ANOVA) was used to compare in vitro heart data statistically from Langendorff and Western-blots studies. A p value of ≤0.05 was considered statistically significant.  

**Figure 2.** Continued  

(A) The top panel depicts high-resolution 2D echocardiograms in parasternal longitudinal-axis B mode taken from 16-week post-Sham, TAC, early activation of DREADDs-expressing PVN oxytocin neurons (initiated at 4 weeks post-TAC), and late treatment (starting at 6 weeks post-TAC) animals showing left ventricle (LV) in mid-systole with directional vectors in green. Vertical scale bar represents 3 mm. The middle panel shows 2D echocardiograms in parasternal short-axis M-mode at 16 weeks post-Sham, TAC, early- and late-treatment animals, used to measure the LV posterior-wall thickness and interventricular septal-wall thickness, LV internal diameter, and percent fractional shortening. Vertical and horizontal scale bar represents 3 mm and 0.1 sec, respectively. The bottom panel displays pulsed Doppler measurements of descending aortic flow velocities taken immediately after TAC point in Sham, TAC, and treatment animals. (B) Quantitative longitudinal Doppler descending aortic flow velocities in Sham, TAC saline (expressing DREADDs), TAC CNO (not expressing DREADDs), early- and late-treatment animals taken bivweekly from 2 to 16 weeks post-Sham/TAC surgery. The velocities were 3-fold greater in TAC and treatment animals compared with Sham animals. Graphs displaying longitudinal echocardiographic measurements of stroke volume (C), percent ejection fraction (D), cardiac output (CO) (E) from parasternal longitudinal axis B-mode and percent fractional shortening from parasternal short-axis M-mode measured bivweekly from 2 to 16 weeks post-Sham, TAC saline (expressing DREADDs), TAC CNO (not expressing DREADDs), and early- and late- treatment animals. Data were analyzed using mixed-effects model with Tukey’s multiple comparison post-test. Sham (n = 6); TAC (n = 6); early treatment (n = 6); late treatment (n = 7); TAC CNO (n = 5). *Sham vs. TAC; †TAC vs. early treatment; ‡TAC vs. late treatment; ‡‡Sham vs. early treatment; ‡rSham vs. late treatment; *p < 0.05; **p < 0.01; ***p < 0.001. CNO, dizosipine-N-oxide, DREADDs, designer receptors exclusively activated by designer drugs; PVN, paraventricular nucleus of the hypothalamus; TAC, transcending aortic constriction.
FIGURE 3 Long-Term Activation of PVN Oxytocin Neurons Improves Heart Rate Recovery and Survival Rates in Animals With Heart Failure

- Sham (7)
- Early Treatment (9)
- TAC saline (9)
- Late Treatment (7)
- TAC CNO (6)

A

B

C

**p<0.01**

Continued on the next page
RESULTS

To test the hypothesis that synaptic release of OXT from hypothalamic OXT neurons at downstream autonomic targets is reduced in an animal model of HF, we quantified OXT release using engineered sniffer cells sensitive to OXT, in combination with photoactivation of ChR2 selectively expressed in PVN OXT neurons. Chinese hamster ovary (CHO) cells that expressed both OXT receptors and the red fluorescent genetically encoded Ca2+ indicator (R-GECO) were used as “sniffer cells,” which are highly sensitive and selective biological targets for the evoked photo-stimulated synaptic release of OXT (15). Selective expression of both ChR2 and an excitatory chemogenetic receptor for chronically activating PVN OXT neurons, DREADDs, was accomplished using 3 viral vectors in combination with the Cre-Lox system. Expression of the enzyme, Cre recombinase, was exclusively driven by the OXT promoter (rAAV1-OXT-Cre), stereotactically injected into the PVN of 1-week old pups with both floxed excitatory ChR2 (AAV1-EF1a-DIO-hChR2) and floxed excitatory DREADDs (AAV2-hSyn-DIO-hM3D(Gq)), as previously described (12).

PVN synaptic terminals in the dorsal motor nucleus of the vagus (DMNX), a site of origin for preganglionic parasympathetic cardiac vagal neurons (CVNs), were identified, then surrounded by sniffer OXT-sensitive CHO cells, and photostimulated (Figure 1A). Photostimulation of ChR2 PVN OXT synaptic endings in the DMNX activated sniffer cells in Sham-operated animals with no significant changes in CHO cell responses at 2, 4, 6, 8, and 10 weeks postsurgery (Figure 1B and C). Pressure overload-induced HF was initiated in another group of animals using a TAC procedure to initiate progression to HF (11). In TAC animals, the photo-stimulated release of OXT, as detected by OXT-sensitive CHO cells, was significantly diminished at 6 and 10 weeks post-TAC (Figures 1B and 1C).

We next examined if chronic selective activation of PVN OXT neurons could restore OXT release in the DMNX following TAC. In this third group of animals, PVN OXT neurons were exclusively activated with chemogenetics via stimulation of excitatory DREADDs activated by daily intraperitoneal injections of CNO, 1 mg/kg/day, beginning at 4 weeks post-TAC, until animals were killed. In TAC + PVN OXT neuron-activated treatment animals, the release of OXT upon photoactivation of PVN synaptic terminals in the DMNX was similar to that of Sham animals at all time points, effectively attenuating TAC-induced diminished OXT release (Figures 1B and 1C).

In our previous work using this HF model, we have shown that there are no off-target effects of the DREADDs agonist CNO, or any biologically active metabolites, in nondisease (Sham) animals (16). In this study, we further tested for off-target effects of CNO or any biologically active metabolites by comparing 2 groups of TAC animals: a TAC group expressing DREADDs but receiving saline (TAC-saline) and a second TAC group, receiving CNO but not expressing DREADDs (TAC-CNO) (Figure 2). Treatment animals were divided into 2 groups: 1 with “early” PVN OXT neuron stimulation initiated at the onset of disease (4 weeks post-TAC) (Figure 2A) and the other with “late” treatment initiated at advanced progression of disease (6 weeks post-TAC). This was an important therapeutic time point because release...
FIGURE 4  Hearts From TAC Animals With PVN OXT Neuron Activation Had Improved LV Function and Less Collagen III and IL-1β Expression Compared with Untreated TAC Animals

A

B

C

D

E

F

Continued on the next page
of OXT in the DMNX was lowest at 6 weeks post-TAC and cardiac dysfunction and mortality had been established (Figures 2 and 3). We examined longitudinal indices of cardiac function beginning at 2 weeks post-TAC, using high-resolution echocardiography (echo) in Sham, TAC (saline), TAC (CNO), and TAC + PVN OXT-activated animals. As expected, aortic velocity was 3-fold higher in each of the animal groups with TAC (TAC [saline], TAC [CNO], early and late treatments) compared with Sham animals (Figure 2B). Importantly, the degree of aortic narrowing, quantified by the increase in aortic velocity immediately downstream of the constriction, was not different in either of the TAC groups and treatment groups (Figure 2B). Indices of cardiac function—including stroke volume, EF, cardiac output, and FS—indicate preserved function with PVN OXT neuron treatment (early or late) compared with TAC (Figures 2C to 2F).

The benefit in cardiac function closely followed the timed initiation of PVN OXT neuron activation. In animals in which PVN oxytocin neurons were activated at 4 weeks, at the initial stage of disease initiation the benefit in cardiac function was observed beginning at the onset of treatment at 4 weeks post-TAC (Figure 2). Likewise, those animals that had late treatment (PVN OXT neuron-activated at 6 weeks post-TAC) when cardiac dysfunction was established, also showed benefits to cardiac function only beginning when treatment was started. From 8 to 16 weeks post-TAC, there were no significant differences in the improved cardiac function when comparing the 2 treatment groups, and both PVN OXT neuron-activated groups showed significant improvements in cardiac function compared with the untreated TAC group.

HRR after peak effort capacity is a common clinical assessment of autonomic balance and risk of adverse cardiovascular events, with a lower HRR associated with depressed parasympathetic activity and increased mortality (17). To assess HRR in each of the 5 groups of animals, ECG wireless transmitters were implanted 2 weeks post-TAC to measure the ECG and HR immediately following—and 20 s after—peak exercise treadmill tests (Naughton protocol). HRR values were lowest in TAC animals and significantly greater in animals in which PVN OXT neurons were activated both early (at 4 weeks post-TAC) and late (6 weeks post-TAC) compared with untreated TAC animals (Figure 3A). We then tested whether the change in HR responses to either daily CNO delivery or long-term activation of PVN OXT neurons were preserved throughout the treatment regimen. As shown in Figure 3B, the increase in parasympathetic activity to the heart, assessed as decreases in HR upon activation of PVN OXT neurons, shown previously to be blocked by muscarinic receptor blockade (16), persisted each week during the entire 16-week duration of this study. We next examined if PVN OXT neuron treatment reduced mortality following TAC-induced HF. There were no fatalities in the Sham group of animals during the course of the 16-week study (Figure 3C). The survival rate in TAC animals 16 weeks post-TAC was 50% and was significantly increased to 66% and 70%, respectively, in the early- and late-treatment animals (Figure 3C).

To elucidate potential intracardiac mechanisms responsible for the improvement in cardiac function following PVN OXT neuron activation, the expression of collagen III and the inflammatory marker, IL-1β, was measured by Western-blot analysis at the end of the study (16 weeks post-TAC). In addition, the isovolumic contractile function of hearts excised from animals was assessed during normal sinus rhythm. As cardiac function in both early- and late-treatment groups were not significantly different from each other at the end of the study, both treatment groups were combined for further analysis.

As expected, IL-1β expression was significantly higher in TAC animals compared with Sham-operated animals. PVN OXT treatment significantly attenuated...
Collagen III was likewise significantly higher in TAC animals than in Sham animals, and the expression of this fibrotic protein was significantly blunted in PVN OXT-treated animals (Figure 4E, Supplemental Figure 1). Trichrome histological sections suggest increased fibrosis in all disease groups compared with Sham; however right-ventricular wall thinning was more apparent in TAC animals compared with TAC treatment groups (Figure 4F). Cardiac function in excised hearts from these animals was examined using a Langendorff preparation in which the heart was perfused via the aorta at constant pressure (65 mm Hg). A 5.5-mm balloon (Harvard Apparatus) was inserted into the LV to measure isovolumic LVDP. Contractility and relaxation were measured as the maximum and minimum values of the first derivative of the LVDP waveform, respectively, to assess inotropy and lusitropy, as we have previously described (16). TAC significantly impaired LVDP, and PVN OXT treatment attenuated this dysfunction (Figure 4A). Similarly, cardiac contractility and relaxation (Figures 4B and 4C) were significantly compromised in TAC compared with Sham animals; however, Sham- and PVN-treated animals did not vary significantly.

**DISCUSSION**

Parasympathetic activity to the heart originates in the brainstem and acts to reduce heart rate (18) and increase coronary flow (19,20). Our previous work has shown CVNs receive powerful excitation from a population of PVN OXT neurons that corelease OXT and glutamate (Glut) to excite parasympathetic cardiac vagal neurons (CVNs) in the brainstem. The synaptic release of OXT was assessed using sniffer CHO cells that express both OXT receptors and the red fluorescent Ca²⁺ indicator (R-GECO) that were placed in close proximity to both PVN OXT synapses and their targeted CVNs. Increases in cardiac parasympathetic activity by activation of CVNs excites downstream parasympathetic cardiac ganglia neurons that release acetylcholine and activate muscarinic (M2) receptors in the heart.
activation of PVN OXT neurons with DREADDs in which OXT release is assessed by sniffer CHO cells surrounding CVNs—and the OXT network that increases parasympathetic activity to the heart—is shown in Figure 5. We have recently demonstrated that, in rats with LV hypertrophy that progresses to HF, CVNs have diminished excitation owing to both an increase in spontaneous inhibitory gamma amino-nobutyric acid (GABA)ergic neurotransmission frequency and a decrease in amplitude and frequency of excitatory glutamatergic neurotransmission to CVNs (11). Taken together, these findings suggested increasing excitatory input to CVNs—such as via the oxytocinergic PVN OXT/glutamate pathway—could be a promising approach to maintain cardiac parasympathetic activity, autonomic balance, and cardiac function during HF.

Using sniffer CHO cells as a novel approach to detect OXT, we have shown that photoactivated synaptic release of OXT from ChR2-expressing PVN fibers at brainstem targets (DMNX) where CVNs are localized is blunted in TAC animals but that this release can be restored with DREADDs-mediated selective activation of PVN OXT neurons. In Figure 1, we show that CHO cell responses were significantly blunted at 6 and 10 weeks but not 8 weeks post-TAC. Although we do not know the reason that the decrease at 8 weeks was not significantly different, it is likely due to the experimental design necessity of nonlongitudinal use of different groups of animals at each time point post-TAC. As PVN OXT release in the DMNX was lowest at 6 weeks post-TAC, we examined whether treatment by chronic PVN OXT neuron activation would benefit cardiac function, assessed both in vivo and ex vivo, as well as improve autonomic balance and reduce mortality. We began treatment in 1 group of animals early, at 4 weeks post-TAC, and another group late, at 6 weeks post-TAC, to reflect treatment further in progression of disease at a time when cardiac dysfunction has been established and mortality to the disease has begun.

Our results show that both early and late PVN OXT neuron activation improved mortality, as the survival rate in TAC animals—50%—was significantly improved to 66% and 70%, respectively, in the early- and late-treatment animals. PVN OXT neuron activation significantly mitigated the progression of cardiac dysfunction following TAC. Cardiac function indices, including EF, stroke volume, cardiac output, and FS all showed very similar improvements in animals with PVN OXT neuron activation, and those improvements followed a similar time course. Fibrosis, assessed by expression levels of collagen III, was significantly higher in TAC animals than in Sham animals, and this index of fibrosis was significantly blunted in PVN OXT-treated animals. Increased myocardial fibrosis is indicative of diffusive loss of working myocardium, which would increase wall stiffness while also having a negative impact on contractile function. Reduced ventricular compliance and increased perivascular collagen would also have a negative effect on vasodilatory reserve (23,24). Reduced coronary flow, plus the increased metabolic demands of pressure overload, would motivate ischemia, causing further myocardial damage. This would further increase fibrosis as myocytes were lost from ischemic injury, then replaced by collagen to maintain structural integrity (23), further reducing working myocardial mass. Indeed, in our ex vivo assessments of cardiac function, LVDP was significantly impaired in TAC animals and higher in treated animals. The LV hypertrophy observed in the untreated TAC animals was not reduced by PVN OXT neuron activation in the treated animals, but fibrosis and IL-1β levels were reduced, likely contributing to compensation mechanisms in the treatment groups that maintained a healthier myocardium.

HRR, an index of parasympathetic activity to the heart and a common clinical assessment of the risk of adverse cardiovascular events (with a lower HRR associated with increased mortality [17]), was lowest in TAC animals and significantly greater in early- and late-treatment animals. Consistent with these findings, HR responses to either daily CNO delivery or long-term activation of PVN OXT neurons were preserved throughout the treatment period as the decreases in HR upon activation of PVN OXT neurons was consistent upon testing each week throughout the entire 16-week duration of this study.

Beneficial outcomes observed with OXT neuron activation could be due to a number of downstream parasympathetic cardioprotective benefits. In addition to evidence that increasing parasympathetic activity to the heart decreases HR and increases coronary flow, PVN OXT neuron activation may have initiated a parasympathetic muscarinic receptor-mediated reduction in sympathetic activity. M2 muscarinic-receptor activation attenuates the production of cyclic adenosine monophosphate (AMP) to reduce the inotropic effects of β-adrenergic receptor activation in the ventricles (25,26). During long-term sympathetic stimulation, M2 activation reduces myocardial stress by lowering cyclic AMP to reduce the cellular hypercontractile state and increase relaxation during diastole, thereby improving myocyte viability (27) and slowing the progression of hypertrophy (28,29).

Although our work used an approach of selectively activating PVN OXT neurons, studies that have
activated downstream targets of PVN OXT neurons report similar increases in parasympathetic activity to the heart. Electrical stimulation of the parvocellular PVN evokes a bradycardia that is suppressed by application of an OXT receptor antagonist into the DMNX (30). In addition, a bradycardia is elicited upon injection of OXT into the DMNX, a response that is blocked by atropine (31). Basal HR varies inversely with dorsal brainstem oxytocin content, with the normal resting HR of normotensive rats associated with higher OXT content than in hypertensive animals (32), suggesting an important role of endogenous OXT release in the autonomic control of HR and the maintenance of a normal HR, autonomic balance, and blood pressure.

Overall, the improvements in cardiac function in HF we observed with PVN OXT neuron activation is consistent with the effects reported for other more invasive and poorly tolerated approaches to increase parasympathetic activity to the heart. Recent device-based approaches—such as implantable vagal stimulators that stimulate a multitude of visceral sensory and motor fibers in the vagus nerve (33) are being evaluated as new therapeutic approaches for HF (34,35). In animal studies, long-term vagal stimulation improves LV function, reduces infarct size, and decreases mortality (6). Despite initial promising clinical trials, a recent large-scale clinical study, NECTAR-HF (Neural Cardiac Therapy for Heart Failure), provided a negative result for chronic vagal nerve stimulation in patients with HF (36). There are many potential reasons for the lack of effect seen in this clinical study, which includes ineffective and/or nonspecific stimulation parameters (electrical stimulation amplitude and frequency) as well as the inherent disadvantage of activating noncardiac parasympathetic efferent fibers along with the sensory afferent fibers in the vagus nerve with vagal nerve stimulators (37). Our work in targeted activation of OXT neurons in the PVN provides an important step forward to determine if approaches that mimic PVN OXT neuron activation, such as noninvasive and well-tolerated intranasal administration of OXT, can increase parasympathetic activity to the heart in patients with HF, as it does in patients with obstructive sleep apnea (4), with, it is hoped, similar benefits of PVN OXT neuron activation shown in this animal model of HF.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Long-term restoration of the hypothalamic OXT network preserves OXT release, reduces mortality, cardiac inflammation, fibrosis, and improves autonomic tone and cardiac function in a model of heart failure.

TRANSLATIONAL OUTLOOK: This work provides the essential translational outlook to determine if approaches that mimic PVN OXT neuron activation, such as safe, noninvasive, and well-tolerated intranasal administration of OXT, can be beneficial in patients with HF.

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KEY WORDS heart failure, oxytocin, parasympathetic

APPENDIX For a supplemental figure, please see the online version of this paper.
Heart failure is a major public health problem, affecting more than 23 million people worldwide. The prevalence of heart failure is increasing throughout the world, driven by a variety of factors including aging populations and increased incidence of hypertension, diabetes, and obesity. Paradoxically, better treatment of coronary artery disease and myocardial infarction has also contributed, as survivors may eventually develop heart failure. Almost one-half of the patients diagnosed with heart failure die within 5 years, highlighting the need for new interventions.

A hallmark of human heart failure is autonomic imbalance, characterized by elevated sympathetic activity and parasympathetic withdrawal. This imbalance contributes to disease progression and is the focus of many therapeutic interventions. Blunting sympathetic transmission with β adrenergic receptor antagonists is a cornerstone of therapy, prolonging life in patients who can tolerate β receptor blockade. Likewise, angiotensin converting enzyme inhibitors, angiotensin receptor 1 antagonists, and several other types of therapeutics decrease sympathetic nerve activity in humans with heart failure. Finally, exercise training is effective at both increasing cardiac vagal tone and decreasing sympathetic tone in patients who are able to undertake exercise. In addition to these proven treatments, several forms of neural modulation are under investigation to restore autonomic equilibrium including vagal nerve stimulation, electrical activation of the carotid baroreceptor reflex, destruction of afferent renal nerves, and device-based inhibition of carotid chemoreceptors (1).

A new study from Dyavanapalli et al. (2) in this issue of JACC: Basic to Translational Science identifies an intriguing new target for enhancing parasympathetic transmission during heart failure: oxytocin. Oxytocin (OXT) is a 9 amino acid peptide that is made in the hypothalamus and released into the circulation from the posterior pituitary. Its classic effects relate to social bonding, reproduction, and childbirth, but newer studies have identified roles for OXT neurotransmission within the central nervous system in regulating cardiovascular homeostasis, metabolic homeostasis, and bone density. OXT-producing neurons in the paraventricular nucleus (PVN) of the hypothalamus mediate these effects via projections throughout the brain stem, including the dorsal motor nucleus of the vagus, the nearby nucleus of the solitary tract, and multiple sympathetic targets including the intermediolateral cell column. With regard to cardiovascular homeostasis, distinct populations of OXT-releasing neurons stimulate parasympathetic transmission via the dorsal motor nucleus of the vagus (with possible involvement of the nucleus of the solitary tract), or they stimulate sympathetic outflow via the intermediolateral cell column and other targets (3). The study by Dyavanapalli et al. (2) builds on work showing that glutamatergic neurons in the PVN that coexpress the peptide OXT activate cardiac parasympathetic neurons in the dorsal motor nucleus of the vagus. Cardiac
vagal neurons are less active in heart failure, due in part to decreased excitatory glutamatergic inputs. The current study provides evidence that the release of OXT from PVN neurons onto cardiac vagal motor neurons decreases during heart failure and that chemogenetic activation of glutamatergic OXT neurons increases parasympathetic transmission and blunts the development of cardiac pathology.

The study began by asking the simple but technically daunting question “Does OXT release from hypothalamic PVN neurons onto cardiac vagal neurons decrease in heart failure?” There are no good methods to quantify synaptic peptide levels, so Dyavanapalli et al. (2) combined multiple methods to address this question. First, they selectively expressed channelrhodopsin-2 in PVN oxytocin neurons so that OXT release could be stimulated by light activation of channelrhodopsin-2. Then they added a “sniffer cell” bioassay to the dorsal motor nucleus region of their brain stem slice preparation, using cells engineered to link OXT receptor activation to the red fluorescent genetically encoded Ca$^{2+}$ indicator R-GECO. This sensitive bioassay revealed that OXT release declined as heart failure developed in animals with transaortic constriction-induced pressure overload. A drawback of this method is the need to use a different set of sniffer cells for each time point, generating variability as each batch exhibits different levels of reporter expression and sensitivity. However, the general trend was clear: release of OXT was impaired in animals that developed heart failure compared with in sham animals, and the physiological changes were consistent across time.

Injecting OXT directly into the dorsal motor nucleus of the vagus triggers parasympathetic activation and bradycardia. The loss of OXT release onto those neurons during heart failure may therefore contribute to the loss of parasympathetic transmission. The investigators showed previously that expressing an excitatory chemogenetic receptor (designer receptor exclusively activated by designer drugs) in OXT neurons within the PVN could stimulate cardiac parasympathetic activity (4). Here they chronically activated excitatory DREADDs (designer receptor exclusively activated by designer drugs) in PVN OXT neurons with the ligand clozapine N-oxide beginning 4 or 6 weeks after pressure overload. They assessed cardiac and autonomic parameters in conscious animals throughout the 16-week study. Although the study did not measure vagal nerve activity, ongoing treatment with clozapine N-oxide lowered heart rate and improved heart rate return after exercise throughout the study duration, suggesting that cardiac parasympathetic tone was increased. In addition to enhancing parasympathetic transmission, the DREADD activation of hypothalamic OXT release decreased mortality, improved indices of cardiac function, and decreased cardiac fibrosis. Expression of inflammatory cytokines was also lowered, consistent with cholinergic suppression of cardiac inflammation in other contexts.

The translational potential of oxytocin use in humans makes this study especially intriguing. Although OXT can modulate both parasympathetic and sympathetic outflow from the central nervous system, systemic administration of OXT decreases heart rate, suggesting that the parasympathetic effects predominate. Indeed, intranasal administration of oxytocin in healthy humans increases parasympathetic and decreases sympathetic transmission to the heart (5). Oxytocin also increases cardiac parasympathetic drive in patients with obstructive sleep apnea. These human data and the new study from Dyavanapalli et al. (2) raise the possibility that intranasal OXT administration might be useful for increasing parasympathetic tone in heart failure.

The observation of deficient OXT release in the brain stem during heart failure is important, because replacing OXT back to a normal level may be less likely to cause unwanted side effects than adding excess OXT. In animal studies, the lack of OXT or its receptor leads to profound bone defects and late-onset obesity, and replacing OXT exogenously restores normal function. Similarly, OXT reverses ovariectomy-induced osteopenia and body fat gain in mice. In humans, the concentration of circulating OXT is positively associated with bone mineral density and lean mass, and heart failure patients are more likely than age-matched control subjects to have decreased bone mineral density and osteoporosis. Heart failure patients are also more likely to experience fractures even though many therapeutics used to treat heart failure decrease fracture risk (6). The observation of decreased OXT in this rat model of heart failure suggests a potential contributor to bone loss in humans with heart failure. Thus, boosting OXT levels in heart failure patients could potentially contribute to multiple beneficial outcomes, including enhanced parasympathetic transmission in the heart.

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COVID-19 Clinical Trials
A Primer for the Cardiovascular and Cardio-Oncology Communities

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HIGHLIGHTS

- SARS-CoV-2, the virus that causes COVID-19, is a novel CoV that infects humans by binding to ACE2, which degrades angiotensin II, and hence plays a critical role in modulating the renin angiotensin system (RAS).
- The emerging epidemiology of COVID-19 suggests that patients with cardiovascular risk factors, including older age, cardiovascular disease, or cancer may be more susceptible to infection and suffer from worse clinical outcomes.
- Because of the limited understanding with respect to the interaction of RAS inhibitors and SARS-CoV-2 infectivity, we endorse current society recommendations to continue RAS antagonists for clinical indications for which these agents are known to be beneficial.
- Treatments for COVID-19 that are undergoing clinical trials range from therapies that block the entry of SARS-CoV-2 into host cells, to repurposed antiviral therapies such as protease inhibitors and nucleoside analogs that block viral replication by inhibiting viral RNA-dependent RNA polymerase.

SUMMARY

The coronavirus disease-2019 (COVID-19) pandemic has resulted in a proliferation of clinical trials designed to slow the spread of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2). Many therapeutic agents that are being used to treat patients with COVID-19 are repurposed treatments for influenza, Ebola, or for malaria that were developed decades ago and are unlikely to be familiar to the cardiovascular and cardio-oncology communities. Here, the authors provide a foundation for cardiovascular and cardio-oncology physicians on the front line providing care to patients with COVID-19, so that they may better understand the emerging cardiovascular epidemiology and the biological rationale for the clinical trials that are ongoing for the treatment of patients with COVID-19. (J Am Coll Cardiol Basic Trans Science 2020;5:501–17) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
The coronavirus disease-2019 (COVID-19) pandemic has resulted in a proliferation of clinical trials that are designed to slow the spread of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), the virus that causes COVID-19. These therapies range from vaccines, to repurposed treatments for influenza, to drugs that were not effective in patients with Ebola, to treatments for malaria that were developed decades ago. Recognizing that patients with underlying cardiovascular risk factors, cardiovascular disease, or cancer have an increased risk for adverse outcomes with COVID-19, and recognizing that these vulnerable populations may be enrolled in COVID-19 clinical trials, here we present a critical review of the rationale for the different therapeutics that are currently being employed. As background, we first review the epidemiology of COVID-19, followed by the biology of CoV. We then briefly define the complex interplay between the CoV and the renin-angiotensin system (RAS), which is directly relevant to the care of the majority of patients with cardiovascular disease or cancer who are receiving drugs that modulate this system. Finally, we review the mechanisms of action of the multiple therapies that are currently being studied in clinical trials. Given the breadth of information that is emerging, we will not discuss the role of vaccines.

### Epidemiology of COVID-19

The current impact of the novel CoV, SARS-CoV-2 is unquantifiable. The number of confirmed cases and deaths from the global COVID-19 pandemic increase daily (1,2). Although there is a great deal that still remains to be understood, initial reports from 552 hospitals in China describing 1,099 of the 7,736 patients infected with COVID-19 provide some insight into the disease (3). In this multicenter retrospective analysis, the majority were Wuhan residents or had contact with Wuhan residents, although 25.9% were neither. The median age of patients was 47 years (interquartile range [IQR]: 35 to 58 years), and 41.9% were female. Patients with more severe disease, compared with those with nonsevere disease, tended to be older and tended to suffer from at least 1 comorbidity. In this retrospective analysis, patients commonly received intravenous antibiotics (58.0%). Oseltamivir was administered in 35.8%, systemic steroids in 18.6%, and oxygen in 41.3% of patients. The median duration of hospitalization was 12.0 days (IQR: 10.0 to 14.0 days); however, the majority of the patients (93.6%) remained hospitalized at the time of data analysis and as such, the clinical course still largely remains to be defined.

### COVID-19 and Cardiovascular Complications.

Epidemiologic data thus far suggest that patients with cardiovascular risk factors, including older age, cardiovascular disease, or cancer are more susceptible to infection and suffer from worse clinical outcomes (4). COVID-19 can also directly result in a number of cardiovascular complications, including fulminant myocarditis, myocardial injury, heart failure, and arrhythmia (3,5,6). There have been a number of published case reports of clinically suspected myocarditis as suggested by: markedly elevated troponin levels, ST-segment elevation on electrocardiogram without obstructive coronary disease, severely decreased left ventricular systolic function, and shock (7), with cardiac magnetic resonance imaging evidence of diffuse myocardial edema and gadolinium enhancement (8). However, in another isolated autopsy report from a patient who suffered from SARS-CoV-2-related pneumonia and cardiac arrest, no obvious histological changes in the myocardium were observed with the exception of few interstitial mononuclear inflammatory infilrates (9).

Elevated troponin levels have also been observed in those with worse clinical outcomes. In a retrospective, single-center analysis of 416 hospitalized patients with confirmed COVID-19, 19.7% displayed evidence of cardiac injury, as defined by elevated high-sensitivity troponin I levels greater than the 99th percentile upper limit. Those with confirmed cardiac injury tended to be older (median age of 74 vs. 60 years) and suffer from hypertension (59.8% vs. 23.4%), diabetes (24.4% vs. 12.0%), coronary heart disease (29.3% vs. 6.0%), heart failure (14.6% vs. 1.5%), or cancer (8.5% vs. 0.6%) (10).

### COVID-19 in Patients with Cardiovascular Risk Factors or Disease.

Patients with cardiovascular risk factors or disease are at increased risk of suffering from worse clinical outcomes with COVID-19. In an analysis of 2 cohorts from Jinyintan Hospital and Wuhan Hospital of 191 patients, patients with hypertension, diabetes, or coronary heart disease were at increased risk of in-hospital mortality (11). The prevalence of hypertension among nonsurvivors was 48% as compared to 30% in survivors; 31% versus 19% for diabetes, and 13% versus 8% for cardiovascular disease. These comorbidities were also more likely to be present in patients who required intensive care unit admission (4). Other studies, including a
recently published meta-analysis of 46,248 infected patients, have corroborated the observation that patients with cardiovascular risk factors or cardiovascular disease have worse clinical outcomes (12) and also suggest that hypertension (17 ± 7%; 95% confidence interval [CI]: 14% to 22%), diabetes (8 ± 6%; 95% CI: 6% to 11%), and cardiovascular disease (5 ± 4%; 95% CI: 4% to 7%) were prevalent comorbidities among infected patients. Recent studies have also demonstrated that age and hypertension were predictors of an increased likelihood of cardiovascular complications, and cardiovascular complications were associated with a 4.26-fold increased risk of death (95% CI: 1.9 to –9.49) (10).

**COVID-19 IN PATIENTS WITH HEART TRANSPLANTATION.** There have been case series published on COVID-19 infection in heart transplant recipients. Two confirmed cases suggest similar presentations to nontransplant recipients and both patients demonstrated clinical improvement. A questionnaire of 87 heart transplant recipients in China, of which importantly 96.6% undertook quarantine procedures, did not suggest a markedly elevated rate of SARS-CoV-2 infection in this population (13,14).

**COVID-19 IN PATIENTS WITH CANCER.** In a retrospective medical review of 1,524 patients with cancer who were admitted to the Department of Radiation and Medical Oncology in Zhongnan Hospital of Wuhan University from December 30, 2019, to February 17, 2020, the infection rate of SARS-CoV-2 in patients with cancer was 0.79% (95% CI: 0.3% to 1.2%) (15). In contrast, the estimated cumulative incidence of all COVID-19 cases in Wuhan was 0.37%. As a result, the odds of infection in patients with cancer were estimated to be 2.31 (95% CI: 1.89 to 3.02) greater. Patients with cancer who were infected had a median age of 66 years and were more likely to have non-small cell lung cancer (58.3%). Five of these patients were being treated with chemotherapy, immunotherapy, or radiation therapy. Three deaths were recorded.

In a multicenter, prospective cohort study of 2,007 cases from 575 hospitals, 1% of the 1,590 COVID-19 cases had a history of cancer (15). This in contrast to an incidence of cancer in the Chinese population of 0.29% per 100,000 people. Again, among those infected, lung cancer was most common, and patients tended to be older. Patients with cancer also suffered from an increased risk of adverse events that tended to occur earlier, including admission to the intensive care unit, need for invasive ventilation, or death, which occurred in 7 of 18 patients (39%), compared with 124 of 1,572 patients without cancer (8%). Patients with cancer who were recently treated with chemotherapy or surgery were also more likely to suffer from clinically severe adverse events. However, there is a critical need for additional studies to validate these early observations.

**THE CoV FAMILY**

CoVs represent a large family of hundreds of enveloped, single-stranded, positive-sense ribonucleic acid (RNA) viruses that establish an infection primarily by targeting the mucosal surfaces of respiratory and intestinal tracts of a wide range of mammals and birds. There are 4 main subgroupings of CoVs: alpha, beta, gamma, and delta (16). The 7 CoVs that are capable of infecting humans include 229E (alpha CoV), NL63 (alpha CoV), OC43 (beta CoV), HKU1 (beta CoV), Middle East respiratory syndrome (MERS)-CoV (beta CoV), SARS-CoV (beta CoV), and SARS-CoV-2 (beta CoV). The prototype human CoV isolates 229E and OC43 have been causally linked to the common cold. SARS-CoV is the cause of the SARS, whereas MERS-CoV was established as the cause of MERS. Identification and sequencing of the virus responsible for COVID-19 established that it was a novel CoV that shared 88% sequence identity with 2 bat-derived SARS-like CoVs (16). Subsequently, the 2019 novel CoV was shown to share a 79.5% sequence homology with SARS-CoV and was subsequently renamed SARS-CoV-2 (16). The genome of the CoVs encodes 4 major structural proteins: the spike (S) protein, nucleocapsid protein, membrane protein, and the envelope protein (Central Illustration). The S protein is responsible for facilitating entry of the CoV into the target cell (16,17) and is composed of a short intracellular tail, a transmembrane anchor, and a large ectodomain that consists of a receptor binding S1 subunit and a membrane-fusing S2 subunit (16).

**CoV VIROLOGY**

Given that far more is known with respect to the virology of SARS-CoV than of SARS-CoV-2, and given that these 2 CoVs appear to have some overlapping biology and clinical presentations, we will discuss these 2 viruses together, with an emphasis on the most recent studies that have revealed unique biological aspects of SARS-CoV-2. We will review viral attachment, entry, and replication of SARS-CoV and SARS-CoV-2 in host cells. This discussion will be integrated with a review of the ongoing clinical trials that target these different aspects of the biology of SARS-CoV-2 (see Tables 1 to 5).

Angiotensin-converting enzyme 2 (ACE2) is the entry receptor for SARS-CoV and SARS-CoV-2. Viruses
enter cells by binding to host cell-encoded proteins that facilitate the entry of the virus into the cell, as well as allow the virus to survive and replicate within the cell. Some viruses, including certain strains of CoVs are capable of down-modulating the entry receptor once they gain access to the cell. Receptor down-modulation is a strategy broadly used by many viruses to escape the immune system, as well as establish the best environment for viral replication and spread (18). Receptor down-modulation may also disrupt many of the natural physiologic functions of the host cell, resulting in cell death leading to organ level dysfunction.

The entry receptor utilized by both SARS-CoV and SARS-CoV-2 is ACE2 (Central Illustration), which is a type I transmembrane carboxypeptidase with 40% homology to ACE. ACE plays a critical role in activation of the RAS, by processing angiotensin I (angiotensin 1-10) to angiotensin II (angiotensin 1-8), the major effector peptide of RAS, which mediates its effects through selective interactions with G-protein-coupled angiotensin II type 1 (AT1) and type 2 (AT2) receptors (19). ACE, however, has not been implicated in the entry of human CoVs into cells. ACE2 is highly expressed in the mouth, tongue, and types I and II alveolar epithelial cells in the lungs. ACE2 is also abundantly expressed by cardiovascular endothelium, cardiac myocytes, cardiac fibroblasts, as well as epithelial cells of the kidney and testis. The major substrate of ACE2 is angiotensin II, which is cleaved to angiotensin 1-7 (Figure 1) and functions through association with the G-protein-coupled receptor Mas receptor. The ACE2-angiotensin (1-7)-Mas receptor axis is regarded as the counter-regulatory

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus genome encodes 4 major structural proteins: the spike (S) protein; the nucleocapsid (N) protein; the membrane (M) protein; and the envelope (E) protein. The S protein is responsible for facilitating entry of the CoV into the target cell. The routes employed by SARS-CoV include endocytosis and membrane fusion. The route employed by SARS-CoV-2 is via endocytosis; whether SARS-CoV-2 enters cells by membrane fusion is not known. Binding of the S protein of SARS-CoV to angiotensin-converting enzyme 2 (ACE2) leads to the uptake of the virions into endosomes, where the viral S protein is activated by the pH-dependent cysteine protease cathepsin L. Activation of the S protein by cathepsin L can be blocked by bafilomycin A1 and ammonium chloride, which indirectly inhibit the activity of cathepsin L by interfering with endosomal acidification. Chloroquine and hydroxychloroquine are weak bases that diffuse into acidic cytoplasmic vesicles such as endosomes, lysosomes, or Golgi vesicles and thereby increases their pH. MDL28170 inhibits calpain and cathepsin L. SARS-CoV can also directly fuse with host cell membranes, after processing of the virus spike protein by transmembrane protease serine 2 (TMPRSS2), a type II cell membrane serine protease. Camostat mesylate is an orally active serine protease inhibitor. Modified from Simmons et al. (25).

Ky, B. et al. J Am Coll Cardiol Basic Trans Science. 2020;5(5):501-17.
arm of the RAS by opposing the effects of the ACE-angiotensin II axis-AT1. Although the precise role of ACE2 is still being evaluated, studies have shown that ACE2 exerts protective effects in the pulmonary and the cardiovascular systems, where it serves to oppose the deleterious effects of RAS activation (20–22).

Infection with SARS-CoV and SARS-CoV-2 is triggered by binding of the S protein on the surface of the CoV to ACE2 that is expressed on the cell surface. The receptor binding domain of the S protein of SARS-CoV-2 is located on the S1 subunit, which undergoes a conformational change when it binds to ACE2, which facilitates viral attachment to the surface of target cells (17). Binding of SARS-CoV-2 to ACE2 can result in uptake of virions into endosomes (Central Illustration). Viral entry into the cell requires priming of the S protein by the serine protease transmembrane protease serine 2 (TMPRSS2), which cleaves the viral S protein at the S1/S2 and the S2’ site and allows fusion of viral and cellular membranes (23). The S proteins of SARS-CoV-2 can also use pH-sensitive endosomal proteases (cathepsin B and L) for priming and entry into cells. Interestingly, the binding affinity of the SARS-CoV-2 S ectodomain to ACE2 is 10- to 20-fold higher than the binding of the SARS-CoV ectodomain to ACE2 (17). The increase in stickiness of the SARS-CoV-2 capsid S protein makes disease transmission more likely and might explain the increased person-to-person transmission with SARS-CoV-2 compared with that of SARS-CoV. Insofar as the viral S proteins are the part of the virus that interacts with the immune system, they may serve as a promising target for vaccines. Relevant to this discussion, convalescent sera from patients with SARS have been shown to block the entry of SARS-CoV-2 entry into cultured cells, albeit with less efficiency that SARS-CoV (23). However, monoclonal antibodies raised against the receptor binding domain of the S1 protein of SARS-CoV do not bind to the receptor binding domain of the S1 protein of SARS-CoV-2, suggesting that SARS-directed antibodies are not cross reactive and that SARS-CoV-2 proteins are necessary to develop effective antibodies. Although ACE inhibitors do not inhibit ACE2, Hoffman et al. (23) demonstrated that anti-ACE2 antibody prevented entry of viral vectors into cell lines expressing the SARS-CoV-2 S protein.

**INTERACTION OF CoV WITH THE RAS.** An additional layer of complexity to understanding the pathophysiology of the SARS-CoV-2 in humans stems from the complexity of the interactions of CoVs with the RAS (Figure 1), as well as the widespread use of drugs that interfere with the RAS, including ACE inhibitors, angiotensin-receptor antagonists, or angiotensin receptor-neprilysin inhibitors. Each of these drugs has different effects on the expression of the various components of the RAS in different tissue beds. Here we will briefly discuss these important interactions, as well as their implications for the treatment of patients with COVID-19.

Previous studies have shown that SARS-CoV S proteins induce the expression of a cell surface metalloenzyme termed a disintegrin and metalloproteinase-17, which was originally described as the enzyme that cleaves membrane-bound tumor necrosis factor-α from the cell surface and allows it circulate in the soluble form of tumor necrosis factor-α (24). As shown in Figure 1, activation of a disintegrin and metalloproteinase-17 results in the proteolytic cleavage of ACE2 (referred to as shedding) from the cell surface, with the release of the catalytically active
| Drug Name          | Mechanism of Action | NCT Number  | Title                                                                 | Study Population                                                                 | Targeted Enrollment | Study Design                                      | Primary Outcome Measure                  |
|--------------------|---------------------|-------------|----------------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------|-------------------------------------------------|------------------------------------------|
| Camostat Mesylate  | Viral entry         | NCT04321096 | The Impact of Camostat Mesylate on COVID-19 Infection (CamoCo-19)     | Age 18–110 yrs, COVID-19–confirmed hospitalized patients (~48 h) if hospital-acquired COVID-19 is suspected, ~48 h since onset of symptoms | 180                 | Randomized, double-blind placebo controlled, phase IIa trial | Time to clinical improvement at 30 days |
| Hydroxychloroquine| Viral entry         | NCT04315896 | Hydroxychloroquine Treatment for Severe COVID-19 Pulmonary Infection (HYDRA Trial) | Age 18–80 yrs, COVID-19 confirmed by RT-PCR in any respiratory sample; severe disease defined by pulse O₂< 91%, 3% decline from baseline pulse O₂, or need for increased supplemental O₂, mechanical ventilation, or sepsis | 500                 | Randomized, double-blind, placebo controlled | All-cause hospital mortality at 120 days |
| Hydroxychloroquine| Viral entry         | NCT04316377 | Norwegian Coronavirus Disease 2019 Study (NO COVID-19)                 | Age ≥18 yrs, hospitalized, moderately severe disease (NEWS score ≥6); SARS-CoV-2-positive test | 202                 | Randomized, open, single arm                    | Rate of decline in SARS-CoV viral load at 96 h |
| Chloroquine phosphate | Viral entry       | NCT04303507 | Chloroquine/ Hydroxychloroquine Prevention of Coronavirus Disease (COVID-19) in the Healthcare Setting (COPCOV) | Age ≥16 yrs; health care worker or front-line participant with patient contact working in a health care facility; inpatient or relative of a patient and likely exposed to COVID-19; agree to not self-medicate with potential antivirals | 40,000              | Randomized, double-blind, placebo controlled | Number of symptomatic COVID-19 infections Severity of symptoms |
| Hydroxychloroquine| Viral entry         | NCT04308668 | Post-exposure Prophylaxis/Pre-emptive Therapy for SARS-Coronavirus-2 (COVID-19 PEP) | Age ≥18 yrs; exposure to a COVID-19 case within 4 days as either a health care worker or household contact; symptomatic COVID-19 case with confirmed diagnosis within 4 days of symptom onset; or symptomatic health care worker with known COVID-19 contact and within 4 days of symptom onset | 3,000               | Randomized, double-blind, placebo controlled | Incidence of COVID-19 disease at 14 days Ordinal Scale of COVID-19 disease severity at 14 days |
| Hydroxychloroquine| Viral entry         | NCT04318444 | Hydroxychloroquine Post Exposure Prophylaxis for Coronavirus Disease (COVID-19) | Age ≥18 yrs; household contact of index case: currently residing in the same household as an individual evaluated at NYP via outpatient, ED, or inpatient services who: 1) tests positive for COVID-19, or 2) is defined as suspected case, or PUI, by the treating physician | 1,600               | Randomized, double-blind, placebo controlled | Symptomatic, lab-confirmed COVID-19 |
| Hydroxychloroquine| Viral entry         | NCT04318015 | Hydroxychloroquine Chemoprophylaxis in Healthcare Personnel in Contact With COVID-19 Patients (PHYDRA Trial) | Age ≥18 yrs; health care personnel exposed to patients with COVID-19 respiratory disease (physicians, nurses, chemists, pharmacists, janitors, stretcher-bearer, administrative, and respiratory therapists) | 400                 | Randomized, double-blind, placebo controlled | Symptomatic COVID-19 infection rate at 60 days |

For an up-to-date listing of trials, search for "COVID-19" at the ClinicalTrials.gov website.

ED = emergency department; NEWS = National Early Warning Score; NYP = New York Presbyterian; PUI = person under investigation; RT-PCR = reverse transcriptase-polymerase chain reaction; other abbreviations as in Table 1.
soluble angiotensin-converting enzyme 2 (sACE2) ectodomains into the circulation (22,25). A decrease in ACE2 levels on the cell surface would be expected to result in a decrease in the levels of angiotensin 1-7 (cytoprotective) and a corresponding increase in tissue levels of angiotensin II (proinflammatory and profibrotic). The importance of SARS-CoV2-induced down-regulation of cell surface ACE2 was demonstrated in experimental studies, wherein administration of recombinant human ACE2 protein, genetic deletion of the AT1 receptor, or administration of an AT1 receptor antagonist were shown to be protective in acute lung injury models (21,22). These and other observations have suggested that the use of AT1 receptor antagonists may be beneficial in patients with COVID-19 (26), and consistent with this, losartan is currently being tested in randomized, double-blind placebo controlled studies as a potential therapy in hospitalized infected patients (Table 1). Relevant to this discussion, the ACE inhibitors in clinical use do not directly affect ACE2 activity (27). The biological significance of circulating sACE2 is not known. Of note, sACE2 retains its ability to bind the S protein of SARS-CoV and was shown to prevent entry of SARS-CoV into cells in vitro (28). Thus, sACE2 may act as a decoy receptor that prevents SARS-CoV-2 from binding to ACE2 on the cell surface. APN01 is a human recombinant sACE2 that has been shown to block the early stages of SARS-CoV-2 infections in cell culture and human tissue organoid cultures (29). APN01 has already undergone safety and tolerability testing in a phase II trial of healthy volunteers (NCT00886353), but at the time of this writing is not being tested clinically in patients with COVID-19.

The recognition that many patients with COVID-19 have underlying medical conditions that are treated with ACE inhibitors and AT1 receptor antagonists (30), coupled with the knowledge that higher urinary ACE2 levels have been observed in patients treated with AT1 receptor antagonists (25), has given rise to the concern that pharmacologic up-regulation of ACE2 by RAS inhibitors may influence the infectivity of SARS-CoV-2 in a patient population that is already at high risk for severe COVID-19 infection (31). However, as noted in a recent review (32) on this topic, the experimental and clinical data often yield conflicting results with respect to the role of ACE inhibitors and AT1 receptor antagonists on ACE2 levels in different pathophysiological contexts. These conflicting results suggest that the effects on RAS inhibitors on ACE2 are complex and nuanced and should not be assumed to be the same for all RAS inhibitors, nor should it be assumed that changes in ACE2 levels in the heart or other tissues necessarily reflect changes in ACE2 levels in the lung, which is the portal of entry for SARS-CoV-2. Given that we have limited understanding with respect to the interaction of RAS inhibitors, ACE2 levels, and SARS-CoV-2 infectivity in humans, we do not believe that it is possible to make definitive statements that go beyond the joint statement issued on March 17, 2020, by the Heart Failure Society of America, American College of Cardiology, and American Heart Association, that recommended “continuing renin-angiotensin-aldosterone system antagonists for those patients who are currently prescribed such agents for indications for which these agents are known to be beneficial” (33).

**ENTRY OF SARS-CoV AND SARS-CoV-2 INTO CELLS.** The entry of enveloped viruses into host cells occurs through 2 primary mechanisms: the first is direct fusion of the viral membrane with the plasma membrane of the host cells, which allows the virus to directly deliver its genomic material into the cytosol; and the second is that the virus hijacks the cell’s endocytic machinery, by binding to a cell surface receptor, which then triggers endocytosis of the virus-receptor complex (Central Illustration). In the endocytic pathway, the endocytosed virions are subjected to an activation step within the endosome, which is typically mediated by the acidic environment of the endosome, resulting in fusion of the viral and endosomal membranes, which allows for the release of the viral genome into the cytosol. Several viruses, including human immunodeficiency virus and SARS-CoV use direct membrane fusions at the cell surface or endocytosis to enter cells. As noted, recent studies suggest that SARS-CoV-2 binds to ACE2, which leads to endocytosis of the receptor-virus complex (23). What is not known at this time is whether SARS-CoV-2 is also capable of directly fusing with the lipid membrane of cells. However, based on the similarities of how SARS-CoV and SARS-CoV-2 behave, it is likely that their modes of entry into cells will be similar. Understanding these differences in cell entry has implications for developing novel therapeutics.

**THERAPEUTICS TARGETING ENDOCYTOSIS.** The entry of SARS-CoV into cells was shown to occur by direct fusion of the viral membranes with the plasma membrane of the host cell (Central Illustration), through a process that requires processing of the viral S protein by TMPRSS2 at or near the cell surface. Processing of the S protein exposes the fusion peptide of the S protein that inserts into the cell membrane, which brings the envelope of the viral membrane into closer approximation with the membrane of the host cell, thereby facilitating fusion (34). At the time of this writing, the uptake of SARS-CoV-2 into cells has
been shown to occur through endocytosis of the SARS-CoV-2-ACE2 complex, which also requires priming of the S protein by TMPRSS2. It is not known whether SARS-CoV-2 also enters through direct fusion.

Based on the evidence linking TMPRSS2-mediated SARS-CoV-2 activation to SARS-CoV-2 infectivity (23,35), the small molecule serine protease inhibitor camostat mesylate may also be an attractive target for clinical trials with SARS-CoV-2 (Table 2). Camostat mesylate has already been shown to inhibit replication of influenza and parainfluenza viruses and to prevent the development of pneumonia and viral myocarditis in infected mice (36). Given that the SARS-CoV-2 S protein is activated by the pH-dependent cysteine protease cathepsin L, this processing step may be sensitive to inhibition with drugs that indirectly inhibit cathepsin L activity by interfering with endosomal acidification (e.g., baflomycin A1) or by compounds that directly block the proteolytic activity of cathepsin L.

It has also been suggested that the antimalarial drugs chloroquine and hydroxychloroquine might exert a potent antiviral effect by virtue of their ability to increase endosomal pH. Inside cells, chloroquine and hydroxychloroquine are rapidly protonated and concentrated in endosomes. The positive charge of the chloroquine increases the pH of the endosome, which prevents cathepsin-induced priming of the viral S protein. Both chloroquine and hydroxychloroquine decrease SARS-CoV-2 replication in cultured cells; however, hydroxychloroquine is more potent than chloroquine (37). In a small single-arm study of patients with confirmed COVID-19, treatment with hydroxychloroquine was associated with a significant difference in clearing of viral nasopharyngeal carriage of SARS-CoV-2 within 3 to 6 days when compared with that of untreated control subjects that were studied at 3 to 6 days. Azithromycin when added to hydroxychloroquine was significantly more efficient for virus elimination (38). However, both therapies can result in QT prolongation, and as such, caution needs to be exercised when using these therapies together. Chloroquine and hydroxychloroquine can also manifest in cardiotoxicity, including cardiomyopathy, both systolic and diastolic, atrioventricular block, and bundle branch

### Table 3: Select Treatment Trials Targeting Viral Replication

| Drug Name | Mechanism of Action | NCT Number | Title |
|-----------|---------------------|------------|-------|
| Umifenovir | Antiretroviral | NCT04260594 | Clinical Study of Arbidol Hydrochloride Tablets in the Treatment of Pneumonia Caused by Novel Coronavirus |
| ASC09 + ritonavir; lopinavir + ritonavir | Antiretroviral | NCT04261907 | Evaluating and Comparing the Safety and Efficiency of ASC09/Ritonavir and Lopinavir/Ritonavir for Novel Coronavirus Infection |
| Darunavir + cobicistat | Antiretroviral | NCT04252274 | Efficacy and Safety of Darunavir and Cobicistat for Treatment of COVID-19 (DC-COVID-19) |
| Lopinavir + ritonavir; umifenovir | Antiretroviral | NCT04252885 | Efficacy of Lopinavir Plus Ritonavir and Arbidol Against Novel Coronavirus Infection (ELACOI) |
| Lopinavir + ritonavir | Antiretroviral | NCT04330690 | Treatments for COVID-19: Canadian Arm of the SOLIDARITY Trial (CATCO) |
| Remdesivir | Antiretroviral | NCT04280705 | Adaptive COVID-19 Treatment Trial (ACTT) |
| Remdesivir | Antiretroviral | NCT04292899 | Study to Evaluate the Safety and Antiviral Activity of Remdesivir (GS-5734) in Participants With Severe Coronavirus Disease (COVID-19) |
| Remdesivir | Antiretroviral | NCT04292730 | Study to Evaluate the Safety and Antiviral Activity of Remdesivir (GS-5734) in Participants With Moderate Coronavirus Disease (COVID-19) Compared to Standard of Care Treatment |

For an up-to-date listing of trials, search for "COVID-19" at the ClinicalTrials.gov website.

ECMO = extracorporeal membrane oxygenation; FiO₂ = fraction of inspired O₂; nCoV = novel coronavirus; PaO₂ = partial arterial O₂ pressure; PCR = polymerase chain reaction; RR = respiratory rate; other abbreviations as in Tables 1 and 2.
block (39). Hydroxychloroquine will be used as one of the treatment arms in the World Health Organization (WHO) multinational SOLIDARITY (Efficacy of Different Antiviral Drugs in SARS-CoV-2) trial (40) and is also currently being investigated in a number of other studies (Tables 2 and 8). Interestingly, amiodarone, which is a cationic amphiphile, was shown to inhibit Ebola virus infection in vitro in target cells, using concentrations of amiodarone that overlapped those detected in the sera of patients treated for arrhythmias. Both amiodarone and its main metabolite, monodesethyl amiodarone, were shown to interfere with the fusion of the viral envelope with the endosomal membrane, thus blocking viral replication (41). Amiodarone has also been shown to inhibit SARS-CoV infection and spreading in vitro by altering the late compartments of the endocytic pathway by acting after the transit of the virus through endosomes (42).

**REPLICATION OF SARS-CoV IN HOST CELLS.** Because of the exceptionally large size of the CoV RNA genome (~30 kb) and the complexity of CoV-host cell interactions, coupled with the novelty of the SARS-CoV-2 genome, very little is known regarding SARS-CoV-2 replication in cells, let alone how the virus interacts with the host. Given that antiviral strategies are being considered for treatment of patients with COVID-19, here we will review what is generally understood about SARS-CoV replication in mammalian cells, recognizing that this information may change as we learn more about SARS-CoV-2 (see Figure 2).

Once the genomic RNA of SARS-CoV is released into the cytoplasm of the host cell, the positive-strand viral RNA is translated on host ribosomes into a large polypeptide termed the replicase, which undergoes proteolytic cleavage to yield proteins that are required from genome replication, including a viral RNA-dependent RNA polymerase. The viral RNA-dependent RNA polymerase generates a full-length, antisense negative-strand viral RNA template, which is used for replicating positive strand viral genomic RNA, as well as shorter subgenomic negative strand RNAs that serve as templates for synthesizing messenger RNAs that code for structural proteins of the virus, including the S, membrane,

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**TABLE 3 Continued**

| Study Population | Targeted Enrollment | Study Design | Primary Outcome Measure |
|------------------|---------------------|--------------|-------------------------|
| Age ≥18 yrs; subjects with pneumonia diagnosed as 2019-nCoV infection; detection of 2019-nCoV nucleic acid-positive by RT-PCR in respiratory tract or blood samples; virus gene sequence of respiratory tract or blood samples is highly homologous to the known 2019-nCoV | 380 | Randomized, single-arm, open-label umifenovir | Negative viral conversion rate at 7 days |
| Age between 18 to 75 yrs; lab (RT-PCR) and clinically confirmed case of 2019-nCoV pneumonia; hospitalized with a new onset respiratory illness ≤7 days since illness onset | 160 | Randomized, open-label ASC09/ritonavir or lopinavir/ritonavir | The incidence of adverse outcomes, defined by at least 1 of the following: pulse O2 ≤93% without O2 supplementation, PaO2-to-FiO2 ratio ≤300 or RR ≥30 breaths/min assessed at 14 days |
| Pneumonia caused by 2019-nCoV | 30 | Randomized, open-label, single-arm | The viral clearance rate of throat swabs, sputum, or lower respiratory tract secretions at day 7 |
| Age 18–80 yrs; confirmation of SARS-CoV-2 infection by RT-PCR with normal kidney and liver function | 125 | Randomized, open-label (1:1:1) to lopinavir + ritonavir; or umifenovir; or standard care | The rate of viral inhibition, as determined by RT-PCR at days 2, 4, 7, 10, 14, and 21 |
| Age >6 months with confirmed SARS-CoV-2 by RT-PCR, admitted to hospital | 440 | Randomized, open-label (1:1:1) of lopinavir + ritonavir or standard care | Efficacy of intervention at 29 days as determined by 10-point ordinal scale of clinical status |
| Age 18–99 yrs, PCR-confirmed novel coronavirus infection by lab assay; illness as defined by abnormal radiographic imaging, clinical assessment, and pulse O2 ≥94%, requiring O2, or requiring mechanical ventilation | 572 | Adaptive, randomized, double-blind placebo controlled | Time to recovery at day 29 |
| Age ≥18 yrs; confirmation of SARS-CoV-2 infection by RT-PCR ≤4 days before randomization; current hospitalization with fever, pulse O2 ≥94% | 6,000 | Randomized, open-label study of remdesivir 5 days; or remdesivir 10 days | Odds of clinical improvement on a 7-point ordinal scale by day 11 |
| Age ≥18 yrs; confirmation of SARS-CoV-2 infection by RT-PCR ≤4 days before randomization; current hospitalization with fever, pulse O2 ≥94%, radiographic evidence of pulmonary infiltrates | 1,600 | Randomized, open-label study of remdesivir 5 days; or remdesivir 10 days; or standard of care | Odds of clinical improvement on a 7-point ordinal scale by day 11 |
and nucleocapsid proteins. Translation of viral messenger RNAs occurs using the host endoplasmic reticulum. Once the viral structural proteins, S, envelope, and membrane, are translated in the endoplasmic reticulum, they move along the secretory pathway to the endoplasmic reticulum-Golgi intermediate compartment. There, the viral proteins become encapsulated and bud into membranes containing viral structural proteins. Following assembly and maturation, virions are transported to the cell surface in vesicles and released by exocytosis (43,44).

**THERAPEUTICS FOR VIRAL REPLICATION.** There are a number of antiviral drugs that are being repurposed for the treatment of SARS-CoV-2. A partial list of these antiviral drugs are discussed next.

**Nucleoside analogs.** Remdesivir (GS-5734, Gilead Sciences, Inc., Foster City, California) is a nucleoside

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**Table 4: Select Treatment and Prophylaxis Trials Targeting the Immune System**

| Drug Name | Mechanism of Action | NCT Number | Title | Study Population | Targeted Enrollment | Study Design | Primary Outcome Measure |
|-----------|---------------------|------------|-------|-----------------|--------------------|-------------|------------------------|
| IFN-α1β   | Immunomodulatory    | NCT04293887| Efficacy and Safety of IFN-α1β in the Treatment of Novel Coronavirus Patients | Age ≥18 yrs with clinically diagnosed coronavirus pneumonia within 7 days, including RT-PCR evidence of coronavirus and symptoms | 328 | Randomized, open-label, single-arm | Incidence of side effects within 14 days including dyspnea, pulse O₂ ≥94%, and RR ≥24 breaths/min |
| Methylprednisolone | Immunomodulatory | NCT04273321| Efficacy and Safety of Corticosteroids in COVID-19 | Age >18 yrs, diagnosis of novel coronavirus pneumonia (COVID-19) | 400 | Randomized, open-label, single-arm | Incidence of treatment failure in 14 days |
| Methylprednisolone | Immunomodulatory | NCT04244591| Glucocorticoid Therapy for COVID-19 Critically Ill Patients With Severe Acute Respiratory Failure | Age >18 yrs, RT-PCR-confirmed infection, symptoms for >7 days, PaO₂/FiO₂ < 200, positive pressure ventilation or HFNC higher than 45 l/min for <48 h, requiring ICU admission | 80 | Randomized, open-label of glucocorticoid therapy or standard of care | Murray lung injury score at 7 days |
| Sarilumab | Immunomodulatory | NCT04315298| Evaluation of the Efficacy and Safety of Sarilumab in Hospitalized Patients With COVID-19 | Age ≥18 yrs; confirmation of SARS-CoV-2 infection by RT-PCR; current hospitalization with evidence of pneumonia and severe disease, critical disease, or multiorgan system dysfunction | 400 | Adaptive, randomized, double-blind, placebo-controlled with high and low doses | Percent change in C-reactive protein levels at 4 days Percentage of patients reporting clinical severity rated on a 7-point ordinal scale |
| Siltuximab | Immunomodulatory | NCT04329650| Efficacy and Safety of Siltuximab vs. Corticosteroids in Hospitalized Patients With COVID-19 Pneumonia | Age ≥18 yrs; confirmation of SARS-CoV-2 infection by RT-PCR; current hospitalization with evidence of pneumonia; maximum O₂ support of 35% | 100 | Randomized, open-label of siltuximab or methylprednisolone | Proportion of patients requiring ICU admission at 29 days |
| Tocilizumab | Immunomodulatory | NCT04317092| Tocilizumab in COVID-19 Pneumonia (TOCIVID-19) No age or sex limit; SARS-CoV-2 infection by RT-PCR; current hospitalization secondary to pneumonia; pulse O₂ =93%, requiring O₂, or requiring mechanical ventilation (invasive or noninvasive) | 400 | Open-label, single-arm | Mortality at 1 month |

Continued on the next page
Analog that exhibits broad antiviral activity. Remdesivir is a prodrug that is metabolized to its active form GS-441524, which interferes with the action of viral RNA-dependent RNA polymerase, resulting in a decrease in viral RNA production. It is not known, however, whether remdesivir terminates RNA chains or causes mutations in them. Remdesivir was effective against multiple types of CoVs in cell culture and undergone phase III clinical trials for the treatment of influenza, it is not yet approved by the U.S. Food and Drug Administration (FDA). Japan has granted approval for favipiravir for treating viral strains unresponsive to current antivirals. In preliminary studies, favipiravir was shown to have more potent antiviral activity than lopinavir/ritonavir (47).

Favipiravir (Avigan, Fujifilm Toyama Chemical, Tokyo, Japan) is another nucleoside analog antiviral drug that inhibits viral RNA-dependent RNA polymerase. Like remdesivir, it is a prodrug that is metabolized to its active form, favipiravir-ribosyl-5'-triphosphate. Although favipiravir has undergone phase III clinical trials for the treatment of influenza, it is not yet approved by the U.S. Food and Drug Administration (FDA). Japan has granted approval for favipiravir for treating viral strains unresponsive to current antivirals. In preliminary studies, favipiravir was shown to have more potent antiviral activity than lopinavir/ritonavir (47).

Ribavirin (Copegus, Genentech Inc., San Francisco, California) is a prodrug that acts as nucleoside inhibitor. The metabolites of ribavirin resemble adenine or guanosine nucleosides that then become incorporated into viral RNA and inhibit RNA-dependent replication in RNA viruses. Ribavirin is currently FDA-approved for the treatment of chronic hepatitis C virus infection in combination with peginterferon alfa-2a (Pegasys, Genentech).

Protease inhibitors. Lopinavir is a protease inhibitor class that is used in fixed-dose combination with another protease inhibitor, ritonavir (lopinavir/ritonavir [Kaletra, AbbVie Inc., North Chicago, Illinois]) for the treatment of human immunodeficiency virus.

| Drug Name | Mechanism of Action | NCT Number | Title | Study Population | Targeted Enrollment | Study Design | Primary Outcome Measure |
|-----------|---------------------|------------|-------|------------------|--------------------|-------------|------------------------|
| Tocilizumab | Immunomodulatory | NCT04320615 | A Study to Evaluate the Safety and Efficacy of Tocilizumab in Patients With Severe COVID-19 Pneumonia (COVACTA) | Age ≥18 yrs; hospitalized with COVID-19 pneumonia per WHO criteria; pulse O₂ ≥93% or PaO₂/FiO₂ <300 | 330 | Randomized, double-blind placebo controlled | Clinical status using a 7-category ordinal scale at 28 days |
| Anakinra, siltuximab, or tocilizumab | Immunomodulatory | NCT04330638 | Treatment of COVID-19 Patients With Anti-interleukin Drugs (COV-AID) | Age ≥18 yrs; hospitalized with confirmed COVID-19 diagnosis by RT-PCR or other laboratory test, hypoxia defined by PaO₂/FiO₂, CXR or CT scan with bilateral infiltrates | 342 | Randomized, open-label Time to clinical improvement at 15 days |
| Recombinant human IFN-α1b and thymosin α1 | Immunomodulatory | NCT04320238 | Experimental Trial of rhIFNα Nasal Drops to Prevent 2019-nCoV in Medical Staff | Age 18 to 65 yrs, formally serving as medical staff in Taihe Hospital | 2,944 | 2-arm, open-label to IFN-α1b in a low-risk group and IFN-α1b and thymosin α1 in a high-risk group | New COVID-19 diagnosis at 28 days |

For an up-to-date listing of trials, search for “COVID-19” at the ClinicalTrials.gov website.

CT = computed tomography; CXR = chest x-ray; HFNC = high flow nasal cannula; ICU = intensive care unit; IFN = interferon; rhIFN = recombinant human interferon; WHO = World Health Organization; other abbreviations as in Tables 1 to 3.
Results from a randomized, open-label study of 199 hospitalized adult patients with confirmed SARS-CoV-2 infection assigned 1:1 to lopinavir 400 mg–ritonavir 100 mg twice daily for 14 days with standard of care or standard of care alone were recently published (48). All patients had an oxygen saturation of 94% on room air or a ratio of partial pressure of oxygen to the fraction of inspired oxygen <300 mg Hg. The primary endpoint was time to clinical improvement, where clinical improvement was defined based on an ordinal scale or survival from the hospital. The study was designed for 80% power with a 2-sided significance level of α of 0.05 to detect an 8-day difference in median time to clinical improvement. Here, the median time to clinical improvement was 16.0 days (IQR: 13.0 to 17.0 days) in the lopinavir/ritonavir group compared with 16.0 days (IQR: 15.0 to 18.0 days) with standard care. The mortality at 28 days in the treatment group was similar to that observed in the standard care group (19.2% vs. 25%; difference: −5.8%; 95% CI: −17.3% to 5.7%), as was the detectable viral load. However, there were some suggestions of potential benefit with lopinavir/ritonavir with a shorter intensive care unit stay (median: 6 days vs. 11 days) and a shorter time to hospital discharge (median: 12 days vs. 14 days). As noted, the fixed-dose combination of lopinavir/ritonavir is 1 treatment arm in the SOLIDARITY trial (Table 3) (40).

### IMMUNOMODULATORY THERAPIES

Interferons (IFNs) are cytokines that activate the innate immune system in response to viral infection. Type I interferons (IFN-α/β) are synthesized by most cell types in the body response to a viral infection, whereas type II interferon (IFN-γ) is produced by immune cells following antigen stimulation. Both type I and type II IFNs provoke the synthesis of proteins that have antiviral and immunomodulatory effects. Recombinant IFN-β has been shown to inhibit SARS-CoV replication in vitro more effectively than either IFN-α or IFN-γ.
Interestingly, IFN-γ down-regulates the expression of ACE2 on the cell surface and protects type I pneumocytes from SARS-CoV infection (51). The combination of lopinavir/ritonavir and IFN-β1b is being evaluated in the treatment of laboratory-confirmed MERS requiring hospitalization (52) and will also be evaluated in the SOLIDARITY trial (40).

A number of additional immunomodulatory agents are also currently being evaluated, including the interleukin (IL)-6 inhibitor, tocilizumab, and glucocorticosteroids (Tables 4 and 5), given the cytokine storm syndrome that has been observed in subgroups with severe COVID-19 (53) with increased levels of IL-2, IL-6, IL-7, and additional inflammatory cytokines (54). One meta-analysis suggested that the mean IL-6 levels were 2.9-fold (95% CI: 1.17 to 7.19-fold) greater in patients with complicated compared with uncomplicated COVID-19 (54). Tocilizumab (Actemra, Genentech) is FDA-approved for the treatment of severe cytokine release syndrome in patients treated with chimeric antigen receptor T-cell therapy and is also approved for the treatment of rheumatoid arthritis (55–58). Tocilizumab is a monoclonal antibody that binds the IL-6 receptor, both the membrane-bound and soluble forms, thus inhibiting both classic and trans-IL-6 downstream signaling. Similarly, the IL-6 humanized murine chimeric monoclonal antibody siltuximab, although not FDA-approved for the treatment of cytokine release syndrome, has also been used in the treatment of cytokine release syndrome and is also being studied as a potential therapy in severe COVID-19 infections. Siltuximab (Sylvant, Janssen Biotech Inc., Horsham, Pennsylvania) binds directly to IL-6 and prevents the activation of immune effector cells. Sarilumab (Kevzara, Sanofi US, Bridgewater, New Jersey) is a human monoclonal antibody against the IL-6 receptor that was developed for the treatment of rheumatoid
There are no systematically obtained clinical data yet that support a benefit to the use of steroids, and some reports have suggested a possible detriment with delayed viral clearance and increased risk of infection with MERS and SARS, although the role of steroids in COVID-19 is an area of active investigation (Table 4) (59).

COVID-19 AND CARDIOVASCULAR DISEASE

The COVID-19 pandemic has presented innumerable challenges to health care organizations and health care providers. Given that the vast majority of patients with cardiovascular disease are at high risk for SARS-CoV-2 infection, the cardiovascular and cardio-oncology communities will play a major role in caring for patients with COVID-19 now and for the foreseeable future. As a community, we have a long tradition of enrolling patients into clinical trials that evaluate therapeutic agents whose mechanisms of action are familiar, which facilitates reaching clinical equipoise when enrolling patients in clinical trials. In the coming months, our communities will be asked to contribute patients to clinical trials where the mechanisms of action of the therapeutic agents are less familiar and the...
knowledge base required for providing care for COVID-19 is accelerating at a dizzying pace. Here we have tried to provide a foundation for physicians who are on the front line of providing care to patients with COVID-19, so that they can better understand the emerging cardiovascular epidemiology of COVID-19, as well as the biological rationale for the plethora of clinical trials that are either being designed or are currently recruiting patients.
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KEY WORDS: ACE2, clinical trials, COVID-19, renin angiotensin system, SARS-CoV-2
STATE-OF-THE-ART REVIEWS

COVID-19 for the Cardiologist
Basic Virology, Epidemiology, Cardiac Manifestations, and Potential Therapeutic Strategies

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HIGHLIGHTS

- SARS-CoV-2, the infection responsible for COVID-19, has spread globally, leading to a devastating loss of life. In a few short months, the clinical and scientific communities have rallied to rapidly evolve our understanding of the mechanism(s) of disease and potential therapeutics.
- This review discusses the current understanding of the basic virology of SARS-CoV-2 and the epidemiology, clinical manifestations (including cardiovascular), and mortality of COVID-19. A detailed review of the viral life cycle and putative mechanism(s) of injury frames the discussion of possible preventative and therapeutic strategies.
- The ongoing, unprecedented collective effort will, without a doubt, advance our ability to prevent the spread and optimally care for patients suffering from COVID-19.

SUMMARY

Coronavirus disease-2019 (COVID-19), a contagious disease caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), has reached pandemic status. As it spreads across the world, it has overwhelmed health care systems, strangled the global economy, and led to a devastating loss of life. Widespread efforts from regulators, clinicians, and scientists are driving a rapid expansion of knowledge of the SARS-CoV-2 virus and COVID-19. The authors review the most current data, with a focus on the basic understanding of the mechanism(s) of disease and translation to the clinical syndrome and potential therapeutics. The authors discuss the basic virology, epidemiology, clinical manifestation, multiorgan consequences, and outcomes. With a focus on cardiovascular complications, they propose several mechanisms of injury. The virology and potential mechanism of injury form the basis for a discussion of potential disease-modifying therapies. (J Am Coll Cardiol Basic Trans Science 2020;5:518–36) © 2020 Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
of this novel viral infection. Despite being overwhelmed, through conventional and historically unconventional mechanisms, clinicians managing patients with COVID-19 have made a concerted effort to rapidly educate colleagues in expectant regions of the world on lessons learned. The world’s regulatory agencies and pharmaceutical industry are using emergency mechanisms to expedite the access to and study of therapeutic options. These widespread efforts, drawn from many arenas, are driving a rapid expansion of collective experience and understanding of COVID-19.

Here, we review this body of work with a focus on our basic understanding of the mechanism(s) of disease and translation to the clinical syndrome and potential therapeutic options. Specifically, we discuss the basic virology, epidemiology, and clinical manifestations, including presentation, progression, multiorgan consequences, and outcomes. With a focus on the cardiovascular complications, we propose several potential mechanisms of injury. We discuss a range of possible therapeutic options in the context of the viral life cycle and possible mechanisms of injury. Finally, in recognition of the scale of this crisis, we address the ethical considerations around standards of care in the event of resource scarcity.

**BASIC VIROLOGY OF SARS-CoV-2**

**GENETICS AND STRUCTURE.** *Coronaviridae* comprise a family of enveloped, single-stranded, positive-sense, RNA viruses with comparable genomic organization and functional mechanisms. CoVs are canonically divided into alpha, beta, gamma, and delta genera predicated on genetic clustering. The alpha- and beta-CoV are known to cause human diseases, such as common respiratory infections. The SARS-CoV-2 and SARS-CoV-1 are beta-CoVs (1-3). CoV are so named because of the characteristic crown, or corona, of electron density that they exhibit on transmission electron micrographs. This appearance is thought to be caused by the densely packed protein that studs the viral membrane and is responsible for receptor binding to target-cell membranes.

The CoV genome is organized into 2 parts. Highly conserved with the CoV family, the 5’ terminal end, encodes the *replicase* - the nonstructural proteins responsible for viral replication within the cell (1-3). It is translated as 1 peptide (~790 kDa) from which the constituent functional proteins are subsequently cleaved. CoV genomes encode 16 nonstructural proteins, as in SARS-CoV-2, and they exhibit a multitude of functions required for viral replication (2,4,5). Critical proteins for viral replication include the main protease (nsp3), the papain-like protease (nsp5), and the RNA-dependent RNA polymerase (nsp12, RdRp). The other replicase constituent proteins repurpose the cellular machinery to facilitate viral replication and to blunt the intrinsic host immune functions (1,6).

The remaining one-third of the CoV genome encodes the structural proteins and a variety of accessory proteins (the latter not discussed here). The structural proteins are the constituent proteins of the transmissible viral particle, or virion. The key structural CoV proteins are the nucleocapsid protein (N) and 3 transmembrane proteins: the spike protein (S), the membrane protein (M), and the envelope protein (E) (1-5) (Figure 1). The S protein is responsible for virus-cell receptor interactions (7-11) (Figure 1). The E and M proteins are responsible for membrane structure and fusion. The N protein binds viral RNA and mediates its interaction with the S, E, and M proteins for genome encapsulation (1,12).

**LIFE CYCLE.** The life cycle of SARS-CoV-2 has not been rigorously established; however, given the considerable sequence homology, it is presumed to be similar to that of SARS-CoV-1 and other CoVs (4,5). In general, the CoV life cycle consists of a series of steps that begins with viral binding to a target cell and culminates in viral reproduction. Knowledge of this process informs an understanding of viral physiology and also will serve as the basis for discussion of antiviral therapeutics (8) (Figure 1). The aim of evolving therapeutics will be to break the “links in the chain” of the viral life cycle in order to forestall the propagation of infection within the cells of an individual patient.

SARS-CoV-2 is known to bind to cells via the same receptor as SARS-CoV-1, the membrane-bound glycoprotein angiotensin-converting enzyme 2 (ACE2) (4). It has not been observed to bind other CoV receptors, namely dipeptidyl peptidase 4 (DPP4) or aminopeptidase N (APN) (4,13). After binding of ACE2, the virus is internalized via endocytosis without access to the host intracellular compartment until a membrane fusion event occurs (4) (Figure 1). This process is mediated, at least in part, by another membrane bound protease known as transmembrane serine protease 2 (TMPRSS2), which cleaves the S protein as a necessary step of membrane fusion (7). Interestingly, the protease activity of the CoV receptors, ACE2, DPP4, and APN, does not seem necessary for membrane fusion (14).
Upon membrane fusion, the viral RNA genome enters the intracellular compartment. At this point, the viral RNA may be translated into its encoded structural and nonstructural proteins. The translation of the nonstructural proteins, or replicase, results in the production of a single massive polypeptide chain, from which the 16 constituent nonstructural proteins are cleaved. This process is initially mediated by intracellular proteases, and then further propagated by the function of the CoV main protease and papain-like protease (1). Another replicase protein, the RNA-dependent RNA polymerase (RdRp), is responsible for the replication and amplification of the viral genome (15). During this process, mutations may be acquired by errors in replication and recombination events (1).

Upon amplification of the viral RNA, more viral structural and nonstructural proteins may be generated. Viral structural proteins, because of their transmembrane nature (with the exception of the N protein), are targeted to the endoplasmic reticulum (ER) membrane with appropriate signal sequences. Viral RNA, bound by N protein, interacts with the structural proteins on the membrane of the ER and Golgi apparatus before another membrane fusion event on these membranes results in viral budding and exocytosis (1,8,12).

Importantly, the precise molecular differences that account for the important clinical differences between SARS-CoV-2 and SARS-CoV-1 infections, such as prolonged latency, widely variable symptoms, a possible predisposition for individuals with pre-existing cardiovascular conditions, and a predilection for myocardial complications, remain unclear.

**PATHOGENESIS: ACE2.** SARS-CoV-2, SARS-CoV, and HCoV-NL63, a virus that causes a mild respiratory
infection, are all known to employ ACE2 as a receptor (3,4,16,17). Given the functions of ACE2 in the cardiovascular system, the importance of angiotensin-directed pharmacology in cardiovascular disease and the apparent propensity for severe illness among patients with COVID-19 with cardiovascular comorbidity, the ACE2 molecule has been the subject of much attention (18). Indeed, major clinical societies have issued consensus statements on the use of ACE inhibitors and angiotensin receptor blockers (ARBs) in the setting of the COVID-19 pandemic, as discussed subsequently (19).

ACE2 is a single-pass transmembrane protein with protease activity that cleaves the vasoconstrictor angiotensin II into the vasodilator angiotensin 1 to 7 (20–23). In doing so, it functions as a counter-regulatory enzyme to the functions of ACE1, which generates angiotensin II (20). In humans, the protein has a broad pattern of expression and has been found in the lung epithelium (in particular, the type II pneumocyte), the myocardium, the endothelium, the gastrointestinal tract, bone marrow, kidneys, and spleen among other tissues, potentially explaining the multiorgan

ASCVD = atherosclerotic cardiovascular disease; COVID-19 = coronavirus disease-2019; DIC = disseminated intravascular coagulation; MI = myocardial infarction.
injury observed with SARS-CoV-2 infection (24). Another relevant feature of the Ace2 gene expression is its encoding on the X chromosome, which may account for possible sex differences observed in the epidemiology of COVID-19 (25).

In animal models of acute respiratory distress syndrome (ARDS), due to chemical pneumonitis, overwhelming sepsis, endotoxemia, or influenza, Ace2KO mice have more severe acute lung injury (ALI) relative to their wild-type counterparts as evaluated histologically and by measures of elastance (26-28). The phenotype of increased elastance was rescued by administration of recombinant human ACE2, which affirms a causal link between Ace2 deficiency and a more profound state of ALI (26,28). Additionally, the administration of losartan, an angiotensin II type 1 receptor (AT1R) blocker mitigated the exacerbating effects of SARS-CoV S protein in an animal model of ARDS (29). Losartan also abrogated the severity of ALI due to influenza in mice (27,28).

In regard to the counter-regulatory properties of ACE1 and ACE2, the effects of Ace2 deficiency appear to be rescued by Ace1 deficiency in mice. For example, Ace2KO mice demonstrated more severe ALI than did Ace2KO;Ace1−/− mice, with further reduction in severity observed in Ace2KO;Ace1−/− mice (26). This dose-responsiveness also implies causation. Comparable effects were seen with myocardial dysfunction, as Ace2KO;Ace1−/− and Ace2KO;Ace1−/− mice had no evidence of the contractile deficit observed in Ace2KO mice (30). Of note, however, in each of the previous cases, the animal models were constitutive knockout systems (rather than lineage-specific or inducible knockout). Thus, the ACE2-expressing cell that mediates each phenotypic abnormality has not been determined.

SARS-CoV-2 is able to utilize ACE2 isoforms from swine, bats, civets, and humans, suggesting a mechanism whereby the virus may have been initially transmissible from species to species and, with mutation, evolved into a novel pathogen (4). Notably, murine ACE2 is not a functional receptor for SARS-CoV-2, thereby requiring transgenic expression of human ACE2 if mice are to be used as a research model (4).

ACE2 undergoes cleavage by the membrane-bound protease ADAM17, resulting in the release of soluble ACE2 into the bloodstream (31). The effects of soluble ACE2 are unclear in humans; however, it appears to have favorable effects on lung function in models of ARDS, influenza, and respiratory syncytial virus infection (26,28,32). Soluble ACE2 has been studied in a phase II trial of ARDS, but large-scale, well-powered clinical outcomes trials are needed (33). Research is ongoing to determine whether soluble ACE2 may act as a specific therapeutic to SARS-CoV-2 in the role of a decoy receptor, as discussed subsequently (34).

Finally, given the necessity of ACE2 for viral infection, the role of ACE inhibitors or ARBs in COVID-19 has drawn intense attention. Importantly, the ACE2 enzyme itself is not inhibited by ACE inhibitor or ARB use (21). ACE inhibitors or ARB exposure may result in ACE2 protein up-regulation in animal models; however, not all animal models exhibit this effect. The existing epidemiology of COVID-19 among patients taking ACE inhibitors or ARBs is confounded by cardiovascular comorbidities that may alter ACE2 and angiotensin II expression (18). At this time, it is unclear if ACE inhibitors or ARBs use influences receptor expression and whether variable expression impacts the propensity for or severity of SARS-CoV-2 infection.

TRANSMISSION. Exposure to the Huanan seafood market was common among the earliest cases contributing to the SARS-CoV-2 epidemic in China, suggesting that this was a zoonotic disease with an intermediate animal host (nonaquatic animals were sold in the market) (35). Genomic analyses have identified approximately 87% DNA sequence homology between SARS-CoV-2 and 2 SARS-like CoVs isolated from Chinese horseshoe bats, bat-SL-CoVZXC21, in the Zhejiang province in China (36). Notably, no bats are sold in the market, and at the onset of the outbreak in December, most bat species in Wuhan would be hibernating. Thus, similar to SARS-CoV-1 and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), while bats may be the natural reservoir for SARS-CoV-2, there is likely an unidentified intermediate animal host responsible for animal-to-human transmission. Despite closure of the Huanan market on January 1, 2020, the epidemic continued to expand, and case clusters with no exposure to the market were reported, indicating the occurrence of human-to-human transmission (37).

Akin to other respiratory viruses, SARS-CoV-2 spreads primarily through small respiratory droplets that are expelled from infected individuals and can travel approximately 3 to 6 feet. The virus can exist in nature on surfaces and can last for up to 4 h on copper, 24 h on cardboard, and up to 72 h on plastic and stainless steel surfaces, leading to fomite transmission (38). In fact, the Japanese National Institute of Infectious Disease reported detection of SARS-CoV-2 RNA on surfaces in the cabins of symptomatic and asymptomatic passengers on the Diamond Princess up to 17 days after they were vacated (39). Live
virus has also been isolated and cultured from fecal specimens, raising the possibility of orofecal transmission, though corroborating clinical evidence for this method of transmission is lacking (40). Airborne transmission may be facilitated in health care settings in which aerosol-producing interventions are being performed, including endotracheal intubation, bronchoscopy, suctioning, nebulizer treatment, noninvasive positive pressure ventilation, and delivery of oxygen via a high-flow nasal cannula. These transmission data support the clinical recommendations that airborne precautions, including use of N95 respirators, should be implemented in these aerosol-producing settings, whereas standard droplet precautions should be used during all other encounters with infected individuals (41). In a fully susceptible population, reflected by early stages of the epidemic in China, studies have estimated a basic reproductive number ($R_0$) of 2.38 for SARS-CoV-2, meaning that every infected individual is likely on average to spread the virus to 2 to 3 other individuals (42). An outbreak will continue to increase in size if the $R_0 > 1$. For context, seasonal influenza has an $R_0$ of 1.5 (43). Substantial transmission from asymptomatic hosts has facilitated the widespread transmission of SARS-CoV-2 and contributed to its pandemic potential (42). A study from Singapore with extensive contact tracing identified 7 clusters of cases in which secondary spread of the infection occurred 1 to 3 days prior to symptom development in the source patient (44). Thus, containment measures aimed solely at isolating symptomatic individuals are inadequate. Furthermore, contact-tracing efforts should take into account the presymptomatic contagious period to comprehensively capture all potentially exposed individuals. $R_0$ is not a static measure, and interventions including self-quarantine, contact isolation, social distancing, and enhanced hygiene measures have proven to be effective in China. Following implementation of such measures in China, the $R_0$ steadily decreased from 2.38 prior to January 2 to 0.99 during the period of January 24 to February 8, 2020 (42).

**Epidemiology and Clinical Manifestations of SARS-CoV-2**

**Epidemiology.** The burden of the SARS-CoV-2 virus has evolved rapidly since it first appeared in Wuhan, China, in December 2019. What began as a few case reports of atypical pneumonia now spans the globe as a pandemic. At present, most published data come from China and form the basis for our understanding of the epidemiology of COVID-19. In the largest published registry to date, the Chinese Center for Disease Control and Prevention reported high-level details for patient characteristics, severity of manifestations and survival in 72,314 cases of putative (47%) and confirmed (63%) COVID-19 (45). In this population, predominantly identified by the presence of symptoms (~99%), <2% of cases occurred in children <19 years of age, suggesting that children either are either resistant to infection or rarely symptomatic. Of confirmed cases, most (87%) were mild, defined by no or mild pneumonia; 14% were severe with significant infiltrates or signs of respiratory compromise, and 5% were critical, with respiratory failure (e.g., mechanical ventilation), shock, or multiorgan system failure. The first confirmed case of COVID-19 in the United States was identified on January 20, 2020, and the United States has now surpassed all other countries in the absolute number of cases. However, given the rapid and recent onset of the burden, there are few published data reflecting the experience with COVID-19 in the United States. In an early snapshot from the U.S. Centers for Disease Control and Prevention in 4,226 confirmed cases with symptoms or exposure, only 5% occurred in those under 20 years of age (46). Although data are rapidly accumulating, much of the epidemiology of this virus remains unknown. Most publications are small, single-center studies, and detail the clinical characteristics, complications, and outcomes in the subset of patients who were hospitalized. As a result of the limitations on testing and the data suggesting that many infected individuals may be asymptomatic, the true burden of infected individuals is unclear and underestimated (42,47). The variable manifestation of symptoms not only hampers public health initiatives to trace and isolate infected individuals, but also limits our ability to accurately estimate infectivity, symptom burden, and nonfatal and fatal complication rates in the overall population of infected individuals. With that caveat, the published data provide insights into the more vulnerable, at-risk populations who require hospitalization. Although the individual studies are small, the predictors of more severe manifestations and poor outcomes have been generally consistent, as detailed subsequently.

**Clinical Presentation and Syndrome.** In a multicenter case series of 1,099 hospitalized patients from China, the most common symptoms were fever in up to 90%, followed by cough, fatigue, sputum production, and shortness of breath (48). Less common symptoms included headache, myalgias, sore throat,
nausea, vomiting, and diarrhea. The American Association of Otolaryngology has recently highlighted anosmia and dysgeusia as possible symptoms of disease as well (49). The median incubation period, or time from probable exposure to first symptom, was 4 (interquartile range [IQR]: 2 to 7) days (48). Another report detailed that 99% of infected patients develop symptoms within 14 days (50). Common lab derangements on admission included lymphopenia, elevations in C-reactive protein (CRP), lactate dehydrogenase, liver transaminases, and D-dimer (48). Notably, procalcitonin was rarely elevated (48). These data are generally consistent across multiple smaller studies, several of which noted elevations in other inflammatory markers, such as interleukin (IL)-6, ferritin, and erythrocyte sedimentation rate (51–55). Evidence of cardiac or kidney injury at admission was variable across studies but tended to be absent upon hospitalization (48,51–53,56). Chest computed tomography at the time of admission was abnormal in 87% of patients, with ground-glass opacities or local or patchy “shadowing” (48).

**Disease Progression.** Many of the more severe manifestations, such as ARDS, acute kidney injury (AKI), and myocardial injury, tend to occur as many as 8 to 14 days after the onset of symptoms and portend worse outcomes (53). Within a hospitalized population, rates of intensive care unit (ICU) admission range between 26% and 32% across most studies (35,48,51–53,57). Several studies have identified older age and baseline burden of comorbidity, such as diabetes, hypertension, prior coronary disease, and prior lung disease, as predictors of more significant disease progression, with higher rates of ARDS, AKI, cardiac injury, ICU admission, and death (51–53,58,59). Increases in markers of inflammation, coagulation, and cardiac injury also correlate with disease severity and rise throughout the course of the disease (53,54,56). In hospitalized populations, the timing of death occurred at a median of 16 to 19 days after illness onset (53,58). The median time from symptom onset to discharge in survivors was around 3 weeks (53).

**Noncardiovascular Clinical Manifestations.**

**Respiratory Failure.** The most prominent complication of COVID-19 is respiratory failure. As previously described, the majority of patients have no or mild symptoms (45). In hospitalized patients, respiratory symptoms are common and range in severity from cough (60% to 80%) or dyspnea (19% to 40%) to ARDS (17% to 42%) (51–53,56,57). ARDS rates were only 3.2% in the largest case series, but this may be an underestimate due to a short average follow-up time of 12 days, with the vast majority of patients remaining hospitalized at the end of study (48). ARDS tends to occur ~1 to 2 weeks into illness and is often precipitous and protracted (51,53,57). For these reasons, and to avoid risk of provider infection with emergent intubation, professional societies recommend early intubation in the event of respiratory decline (41). Intubation was required in 10% to 33% in the various Chinese series; however, rates of high-flow nasal cannula and noninvasive mechanical ventilation also were high (35,51–53). These therapies are believed to result in aerosolization and are generally not recommended—consequently, more patients will be intubated when unable to be supported by nasal cannula or a nonrebreather mask (41). Older age, baseline hypertension, diabetes, high fever, lymphopenia, injury to other organs (e.g., AKI, acute liver injury [ALI]), and elevated D-dimer and inflammatory markers were predictors of ARDS; advanced age, neutropenia, elevated D-dimer, and inflammation are associated with higher mortality in those with ARDS (51). Development of ARDS, along with acute cardiac injury, was an independent predictor of death (56). Importantly, hypoxicemic respiratory failure is the leading cause of death in COVID-19, contributing to 60% of deaths (58).

**Renal Injury.** Estimates vary as to the incidence of AKI in COVID-19, ranging between 0.5% and 15% (35,48,52,53,56,59). Among hospitalized patients, the rates of proteinuria (43.9%) and hematuria (26.7%) appear to be even higher (59). AKI occurs in the first few days after admission in patients with baseline chronic kidney disease, and after 7 to 10 days in patients with normal baseline renal function (59). Mechanisms of renal injury have been hypothesized to include both acute tubular necrosis, direct cytotoxic effects of the virus itself, and immune-mediated damage (59).

**Liver Injury.** Transaminitis is common, with an incidence of 21% to 37%, and as high as 48% to 62% of patients who are critically ill or who do not survive (35,48,53). ALL, defined as either alanine aminotransferase or aspartate aminotransferase >3 times the upper limit of normal, reported to occur in 19.1% (n = 4 of 21) of patients who were admitted to an ICU in Washington State (55).

**Cardiovascular Manifestations.** Cardiac Injury.

Numerous studies have reported acute cardiac injury as an important manifestation of COVID-19. In studies published to date, acute cardiac injury was variably defined as either cardiac troponin elevation >99th percentile alone or a composite of troponin elevation, electrocardiographic, or echocardiographic abnormalities (52–56,58). Importantly, many aspects of this
The reported rate of cardiac injury varies between studies, from 7% to 28% of hospitalized patients, a number that is likely partially dependent on the definition used and the severity of cases at the hospital from which the data were drawn (52–54,56). Notably, patients with evidence of cardiac injury tend to be older, with more comorbidities, including baseline hypertension, diabetes, coronary heart disease, and heart failure (54,56). Across all studies, cardiac injury is associated with worse outcomes, including ICU admission and death (52–54,56). Based on serial assessment of troponin, researchers in China reported that the median time to the development of acute cardiac injury was 15 (IQR: 10 to 17) days after illness onset, occurring after the development of ARDS (53). Of note, early cardiac injury has been reported, even in the absence of respiratory symptoms (60). In a case series by Shi et al. (56), the mortality rate for those hospitalized with subsequent evidence of cardiac injury was significantly higher than for those without cardiac injury (51.2% vs. 4.5%; p < 0.001) and, along with ARDS, was an independent predictor of death. The magnitude of troponin elevation correlates modestly with the degree of high-sensitivity CRP (hsCRP) elevation (54). Dynamic increases in troponin were associated with a higher mortality rate (54,61). Importantly, the mechanism of cardiac injury may be multifactorial, including demand ischemia, toxicity from direct viral injury, stress, inflammation, microvascular dysfunction, or plaque rupture, as discussed subsequently (Central Illustration).

**Arrhythmia.** Arrhythmias have been noted in several published reports. In a case series of 138 hospitalized patients with COVID-19, 16.7% (n = 23) developed an unspecified arrhythmia during their hospitalization (52); higher rates were noted among patients admitted to the ICU (44.4%, n = 16). A case series of 187 hospitalized patients provided insight into specific arrhythmias, reporting sustained ventricular tachycardia or ventricular fibrillation among 5.9% (n = 11) of the patients (54). These findings are consistent with arrhythmias documented in influenza, which has been known to cause both atrioventricular node dysfunction and ventricular arrhythmias (62).

**Heart failure, cardiogenic shock, and myocarditis.** Heart failure and myocardial dysfunction have been described in COVID-19 (53,55,58,60,63). In a case series of 191 patients, heart failure was noted as a complication of COVID-19 in 23% (n = 44) of all patients and among 52% (n = 28) of nonsurvivors, though the definition of heart failure was not clearly detailed (53). A smaller series of 21 elderly, critically ill patients in Washington State reported incident systolic dysfunction and cardiogenic shock in 7 (33%) patients (55). Outside of this series, the incidence of cardiogenic shock has not been reported. Two case reports have documented cardiogenic shock in the setting of an elevated troponin, ST-segment elevations, a reduction in left ventricular systolic function, and no obstructive coronary disease in patients with COVID-19 (60,63). One report confirmed fulminant myocarditis by cardiac magnetic resonance (60). Neither patient underwent endomyocardial biopsy. Both were treated with inotropes and steroids with recovery of left ventricular function. The potential etiologies of the clinical myocarditis are discussed in detail subsequently (Central Illustration). In 1 case series from China, myocardial damage or heart failure contributed to 40% of deaths overall, with 7% attributed to solely to circulatory failure without respiratory failure (58).

**Thrombosis.** One of the prominent findings replicated across most early studies of COVID-19 includes disarray of the coagulation and fibrinolytic system. Hospitalized patients with moderate and severe COVID-19 and those with poorer outcomes are noted to have prolonged prothrombin time, elevated D-dimer, and activated partial thromboplastin time (35,53,54,64). In the context of a clinical picture that is consistent with disseminated intravascular thrombosis, it is reasonable to speculate that COVID-19 would be associated with venous or arterial thrombi; however, the incidence has not been published. A pathology report from SARS-CoV-1 demonstrated fibrin thrombi in 17 of 20 patients examined with 12 of them showing pulmonary infarcts (65). One preliminary case report, which has not been peer-reviewed, from a COVID-19 patient described autopsy findings of microthrombi in the pulmonary vasculature (66). As there is an absence of published data documenting thrombotic events in COVID-19, routine use of anticoagulation is not recommended without evidence of a thrombotic indication; however, empiric anticoagulation is being used in some centers (Lorenzo Grazioli, Papa Giovanni XXIII hospital in Bergamo, Italy, personal communication, March 2020) (67).

**Mortality.** COVID-19 has a lower estimated case fatality rate (CFR) than its predecessors, SARS-CoV-1 and MERS-CoV, which were 9.4% and 34.4%, respectively (68). However, given the high global burden of infection seen in COVID-19 compared with SARS and MERS, the absolute number of fatalities far surpasses that of SARS and MERS, crossing 70,000 fatalities at the time of this review (69). CFR
estimates have been challenging with SARS-CoV-2, as populations have not been widely screened for infection—leading to an underestimate of the denominator and probable overestimate of the CFR. Crude, unadjusted estimates for the global CFR are ~5% at the time writing with notable variation by country: Italy 11.9% (13,155 deaths), Spain 9.0% (9,387 deaths), South Korea 1.7% (169 deaths), China 4.1% (3312 deaths), Iran 6.4% (3,036 deaths), Germany 1.2% (931 deaths), and the United States 2.3% (5,137 deaths) (69). Regional and national differences in CFR may be a result of multiple factors, including: 1) variable testing of the general and asymptomatic or mildly symptomatic population; 2) differing age across countries; 3) variable health care system resources and preparedness; and 4) widely different public health measures for virus control. Importantly, as health care capacity is exceeded, a large number of deaths may occur because of limited availability of critical care resources, such as mechanical ventilation. When adjusted for underlying demography and underascertainment of cases, the CFR rate was estimated to be 1.4% in China (70).

The general pattern of fatalities across the age groups appears to be consistent throughout the world. In general, greater age is associated with greater risk of severe disease as well as death. According to the Chinese Center for Disease Control and Prevention report of over 70,000 cases, the age-related CFR was as follows: <1% in <50 years of age, 1.3% in 50 to 59 years of age, 3.6% in 60 to 69 years of age, 8% in 70 to 79 years of age, and 14.8% in 80 years of age and older (45). This steep increase in age-related mortality was also observed in Italy, the United States, and South Korea (46,71,72). In fact, age, along with markers of disease severity (D-dimer and sequential organ failure assessment [SOFA] score) were the only independent predictors of mortality in 1 study (53).

Multiple associations have been reported between baseline characteristics and comorbid conditions with mortality in COVID-19. In univariate analyses of predictors of death, Zhou et al. (53) reported that age, coronary heart disease, diabetes, hypertension, respiratory rate, SOFA score, elevated white blood cell count, lymphocyte count, creatinine, lactate dehydrogenase, high-sensitivity troponin I, D-dimer, and elevated inflammatory markers such as ferritin, IL-6, and procalcitonin were associated with death (53). However, in multivariable modeling, only age (per year increase, odds ratio [OR]: 1.10; 95% confidence interval [CI]: 1.03 to 1.17), SOFA score (OR: 5.7; 95% CI: 2.6 to 12.2), and elevated D-dimer (OR: 18.4; 95% CI: 2.6 to 128.6) remained independent predictors of mortality, as described previously (53). In another multivariate analysis of 416 patients from Wuhan, after controlling for age, baseline cardiovascular, pulmonary, and renal disease, only presence of cardiac injury and development of ARDS were significantly associated with mortality (OR: 4.3; 95% CI: 1.9 to 9.5; and OR: 7.9; 95% CI: 3.7 to 16.7, respectively) (56). However, it should be noted that both of these complications tend to occur in older individuals (56,73).

**PUTATIVE MECHANISMS OF CARDIOVASCULAR MANIFESTATIONS IN SARS-CoV-2**

As mentioned in previous sections, COVID-19 patients present with highly variable acuities of disease and disease progression. Cardiac injury is a common feature of the disease process, and 40% of patients die with myocardial injury as a proximate finding (58). Although multiple therapies are currently under development and in trials for treatment of COVID-19, as discussed in a later section, understanding the mechanism(s) of cardiac disease will be vital to effective and timely targeted treatment of this syndrome and its devastating sequelae. Here, we propose several putative mechanisms of COVID-19-induced cardiovascular disease (Central Illustration).

**DIRECT VIRAL MYOCARDIAL INJURY.** The presence of ACE2 receptors on the myocardium and vascular endothelial cells provides a theoretical mechanism for direct viral infection of the heart with resultant myocarditis. Reports have documented clear cases of myocarditis syndromes (60,63). However, to date, there are no reports of biopsy-proven SARS-CoV-2 viral myocarditis with viral inclusions or viral DNA detected in myocardial tissue. The closely related SARS-CoV-1 has been documented to cause a viral myocarditis with detection of viral RNA in autopsyed hearts (74,75). In light of the shared host cell entry receptor between SARS-CoV-2 and CoV-1, a direct viral myocardial entry and resulting injury is plausible with SARS-CoV-2 as well (76).

Another hypothesized mechanism of direct viral injury to the myocardium is through an inflammation-mediated vasculitis. The ACE2 receptor is highly expressed in arterial and venous endothelial cells (24). There are pathologic data from SARS-CoV-1 showing evidence of vasculitis with monocyte and lymphocyte infiltration, vascular endothelial cell injury, and stromal edema in the heart (77). Either direct viral entry into myocardial endothelial cells could trigger a vasculitis or presence of virus could lead to an indirect immunological response and
resulting hypersensitivity reaction (78,79). This insult would be associated with myocardial injury and perhaps even overt myocardial dysfunction in COVID-19.

**MICROVASCULAR INJURY.** Microthromboses and macrothromboses were observed in autopsy evaluations of 3 patients who died of SARS-CoV-1 (80). A prominent finding of SARS-CoV-2 is disarray of the coagulation and fibrinolytic system, with >70% of nonsurvivors meeting criteria for disseminated intravascular coagulation (DIC) (81). It may be hypothesized that myocardial injury is a result of microthrombus formation in the myocardial vasculature in the setting of a hypercoagulable state like DIC.

Infections and sepsis are a leading cause of DIC in general (82). The exact mechanism of DIC in the setting of sepsis and ARDS is complex, but is generally thought to be related to an immune-mediated exhaustion of the coagulation and fibrinolytic systems promoting bleeding and thrombosis in the same patient (83). Endothelial injury and inflammatory cytokines, such as IL-6 and tumor necrosis factor alpha (TNF-α), upregulate tissue factor expression, driving a prothrombotic state (84-87). Dysregulation of antithrombin III, plasminogen activator inhibitor type 1 (PAI-1), and protein C in the setting of significant inflammation and sepsis promote an anticoagulated state (88-90). Furthermore, platelet activation also ensues in the context of sepsis and inflammation, further tipping the fine balance of the coagulation system (91-94). In summary, the immune activation seen in severe COVID-19 infection is likely sufficient to trigger DIC, microvascular dysfunction, and myocardial injury.

**STRESS CARDIOMYOPATHY.** The role of stress (Takotsubo) cardiomyopathy in COVID-19 related cardiac injury is not known, with no published reports at this time, however, the authors have personally observed several cases consistent with stress cardiomyopathy. However, several of the proposed mechanisms of COVID-19-related cardiac injury detailed in this review are also thought to be implicated in the pathophysiology of stress cardiomyopathy, particularly those of microvascular dysfunction, cytokine storm, and sympathetic surge (95).

**ACUTE CORONARY SYNDROME.** Any discussion of myocardial injury would be incomplete without addressing the issue of acute coronary syndrome (ACS) and myocardial infarction (MI). A case series from New York City found that 67% of patients with ST elevations had an obstructive epicardial coronary lesion (96). However, there is historical precedent for an association between infection and an elevated risk of ACS. Epidemiologic studies have shown that hospitalization for pneumonia is associated with a higher risk for atherosclerotic events (97). Influenza infection has been well studied and shown to have a temporal association with cardiovascular complications and ACS (98,99). Annual vaccination against seasonal influenza was associated with a 36% lower rate of major adverse cardiovascular events in a meta-analysis of clinical trials evaluating this question (98). Therefore, viral infection is associated with an increased risk for coronary events and prevention with a reduction in this risk. Therefore, it is plausible that ACS will also be an important cause of acute cardiac injury in patients with COVID-19. Accordingly, international societies have devised pathways and protocols to effectively treat COVID-19 patients with ACS, including appropriate and timely use of resources to ensure the best outcome for the patient while also maintaining provider safety (100).

There are multiple pathophysiologic mechanisms by which systemic viral infection (by influenza or SARS-CoV-2, for example) may lead to a higher risk of plaque destabilization and ACS (101). The role of inflammation in the development and progression of atherosclerosis is well established (102,103). The immune response to acute viral infection and the accompanying surge of cytokines and inflammatory mediators can lead to localized arterial inflammation which is more pronounced within coronary plaques (61,104). Entry of viral products into the systemic circulation, also known as pathogen-associated molecular patterns, can cause innate immune receptor activation which can cascade into activation of immune cells resident in pre-existing atheromata driving plaque rupture (105). Viral pathogen-associated molecular patterns are also believed to activate the inflammasome, resulting in conversion of proinflammatory cytokines into the biologically active cytokines (106). In addition, dysregulation of coronary vascular endothelial function by infection and inflammation may lead to a more vasoconstricted coronary bed (107). All of these changes are putative mechanisms by which COVID-19 infection could lead to destabilization of pre-existing atherosclerotic plaque driving an acute coronary event.

**MYOCARDIAL INJURY SECONDARY TO OXYGEN SUPPLY AND DEMAND MISMATCH.** Periods of severe physiologic stress in the setting of sepsis and respiratory failure can be associated with elevations in biomarkers of myocardial injury and strain in some patients, an entity that confers poorer prognosis...
The mechanism of such myocardial injury is thought to be related to a mismatch between oxygen supply and demand, without acute atherothrombotic plaque disruption, and consistent with a diagnosis of type 2 MI (101,111). Indeed, patients who suffer from type 2 MI compared with type 1 MI have higher mortality rates, which may in part be explained by a higher burden of acute and chronic comorbid conditions in the type 2 MI population (112). Furthermore, type 2 MI on the background of coronary artery disease (CAD) has a worse prognosis than those patients without CAD. Given the age and comorbidity profile of patients hospitalized with severe COVID-19, it is reasonable to assume that this population has a higher risk of underlying nonobstructive CAD; therefore, the presence of type 2 MI in this population is likely a marker of and contributor to the poor outcomes of COVID-19 patients with troponin elevations (56).

SYSTEMIC HYPERINFLAMMATORY RESPONSE WITH RESULTING MYOCARDIAL INJURY. Perhaps 1 of the more intriguing mechanisms for cardiac injury in severe COVID-19 patients stems from the significant systemic inflammatory response. Early reports have demonstrated severely elevated levels of inflammatory biomarkers and cytokines, including IL-6, CRP, TNF-α, IL-2R, and ferritin (113). Higher levels of these biomarkers are associated with more severe COVID-19 manifestations and worse outcomes. A proposed theoretical model of COVID-19 disease progression divides the course into 3 overlapping yet distinct stages. In this staging framework, stage I represents early viral infection with associated constitutional symptoms. In stage II, direct viral cytotoxicity of the pulmonary system with associated inflammatory activation leads to prominent respiratory system compromise, associated with dyspnea and ultimately ARDS and hypoxia. With ACE2 receptors serving as an entry point for viral replication in type II pneumocytes, the pulmonary system becomes the maiden organ of injury. If the host is unable to clear the virus via a productive and protective immune response, COVID-19 progresses to stage III—a hyper-inflammatory state associated with profound elevations in inflammatory biomarkers. Patients who reach stage III have severe COVID-19 manifestations with multiorgan dysfunction and cytokine storm, with immune dysregulating akin to that seen in cytokine release syndrome associated with chimeric antigen receptor T-cell therapy (113-117). This observation is basis for several investigational therapies in COVID-19, including steroids and anti-inflammatory agents, as discussed subsequently.

Prior studies have shown that cardiomyopathy in sepsis is partially mediated by inflammatory cytokines such as TNF-α, IL-6, IL-1β, interferon gamma, and IL-2 (73). Recombinant TNF-α resulted in an early and sustained left ventricular systolic dysfunction in canines (118). Cultured rat cardiomyocytes demonstrated reduced contractility when exposed to IL-6 (119). The mechanism may be through modulation of calcium-channel activity with resultant myocardial dysfunction (120-122). Additionally, nitric oxide is believed to be a mediator of myocardial depression in hyperinflammatory states such as sepsis. Seminal studies found that medium from lipopolysaccharide-activated macrophages suppressed myocyte contractility, a finding reversed with the nitric oxide synthase inhibitor L-N-monomethyl arginine (123). Finally, recent understanding of the key role of mitochondrial dysfunction in septic states has raised questions about the role of this entity in sepsis associated cardiomyopathy (124). Indeed, similar mechanisms are thought to possibly underly the pathophysiology of stress (Takotsubo) cardiomyopathy as well.

POTENTIAL TARGETED OR DISEASE-MODIFYING TREATMENTS IN SARS-CoV-2

The preceding review of the viral physiology of SARS-CoV-2 and the various potential mechanisms of injury to the host serve as the basis for considering specific targeted treatment and prevention. The following section outlines several current candidate classes of agents, including a brief discussion of vaccine development (Figure 1).

NUCLEOTIDE ANALOGS: INHIBITORS OF VIRAL GENOME REPLICATION. The antiviral mechanism of nucleotide analogs is to interfere with RdRp function and viral genome replication and amplification (Figure 1). There are no CoV-specific drugs available at this time, and so ongoing efforts to employ this drug class against SARS-CoV-2 are reliant on pre-existing agents designed for other viral illnesses (125). The most widely applied agent in this class against SARS-CoV-2 has been remdesivir (126). Remdesivir functions as a chain terminator of RNA replication, initially designed for use against Ebola (125). Addition of remdesivir to the growing RNA strand by RdRp blocks the incorporation of additional nucleotides, thereby halting genome replication (127,128). The agent has been shown to have in vitro activity against SARS-CoV2, leading to off-label and investigational use around the world (4,126). Multiple randomized controlled trials are ongoing in China and the United
States for moderate, severe, and critical COVID-19 (NCT04292730, NCT04292899, NCT04252664, NCT04252664).

Another nucleotide analog for the disruption of RdRp-dependent viral replication is favipiravir, which has investigational approval in several countries (129,130). Additional agents that are under study include emtricitabine or tenofovir and ribavirin (129,131).

**PROTEASE INHIBITORS: INHIBITORS OF NONSTRUCTURAL PROTEIN GENERATION.** The antiviral mechanism of action of protease inhibitors is to block viral proteases that cleave the nonstructural proteins from the large, monomeric replicase as detailed previously (Figure 1). As the maturation of nonstructural proteins, such as RdRp, is necessary for viral reproduction, the pharmacologic impairment of the protease should be effective to stop viral replication.

A randomized control trial of lopinavir-ritonavir, a combination protease inhibitor designed for human immunodeficiency virus treatment, in 199 patients with at least moderate COVID-19 did not significantly alter clinical improvement or viral clearance (132). Although the results of this trial were met with disappointment, this negative study should not forestall trials and drug development of protease inhibitors as a therapeutic class, given that this drug was not specifically designed for SARS-CoV-2 (129).

Indeed, the development of inhibitors specific to SARS-CoV-2 main protease is underway. A class of agents identified using structure-based drug design, α-ketamide inhibitors, has demonstrated in vitro efficacy and favorable pharmacokinetics (133). Other candidate protease inhibitors for SARS-CoV-2 include danoprevir, a drug originally intended for hepatitis C virus therapy (134).

**INHIBITORS OF MEMBRANE FUSION.** In order for the viral genome to gain access to cellular machinery for replication, a membrane fusion event must occur between the viral and endosomal membranes, which are noncovalently bound by the interaction between the S protein and ACE2. The exact mechanism of membrane fusion is unknown but appears to be dependent on endosomal maturation and a membrane-bound host protease, TMPRSS2 (7).

**Chloroquine and hydroxychloroquine.** The antiviral properties of chloroquine (CQ) were previously observed in human immunodeficiency virus and other viruses (135,136). CQ and hydroxychloroquine (HCQ) are thought to inhibit endosomal maturation, a process by which endosomes are translocated from the perimembrane regions of the cell to central hubs (137,138) (Figure 1). CQ prevented viral replication of SARS-CoV-1 in vitro (139). A follow-up study demonstrated comparable efficacy of HCQ, a less toxic derivative, and suggested that the mechanism of impaired endosomal maturation indeed applied to SARS-CoV-2 infection in vitro (140). Only poor-quality, nonrandomized, unblinded data exist assessing the benefit of HCQ in COVID-19 (141). Although HCQ is being widely used with an Food and Drug Administration emergency authorization, more data are needed to prove efficacy against SARS-CoV-2 in humans. Notably, CQ and HCQ prolong the QT interval and may induce arrhythmia; significant caution should be used in starting these agents in patients with a QTc interval >500 ms. Concomitant use of other QT interval-prolonging agents is not recommended.

**Camostat.** Camostat is a protease inhibitor approved for the treatment of chronic pancreatitis. Camostat appears to inhibit TMPRSS2 in proteomic and in vitro studies (7,142). A randomized, placebo-controlled trial is underway for this agent in COVID-19 (NCT04321096) (Figure 1).

**NEUTRALIZING ANTIBODIES AND DECOY PROTEINS.** Neutralizing antibodies are designed to bind virions, preventing viral exposure or binding to host cells (Figure 1). Plasma from patients who have recovered from SARS-CoV-2 may contain anti-SARS-CoV-2 IgG antibodies. In a small, single-arm trial of convalescent plasma in COVID-19 patients with ARDS, all had clinical improvement, with 3 of 5 patients weaning from the ventilator (143). Additional trials are ongoing to better define the safety and efficacy of this strategy.

Isolation of SARS-CoV-2-specific neutralizing antibodies with clonal techniques is an appealing strategy to provide targeted therapy, potentially with lower risk of adverse events. Strategies currently under investigation include antibodies cloned from convalescent serum of individuals recovered from SARS-CoV-2 or SARS-CoV-1 and synthetic antibodies. It is unclear whether differences in the S proteins of SARS-CoV-1 and SARS-CoV-2 may limit the effectiveness of antibodies cloned from patients convalescent to SARS-CoV-1 (9). Synthetic antibodies represent an exciting, novel therapeutic avenue. One strategy being explored is to fuse ACE2 to fragment crystallizable region immunoglobulin G, with the hypothesis that this synthetic antibody would serve as a decoy receptor, preventing cellular binding of the virion (144).

In a similar vein, studies are ongoing of decoy proteins that are designed to act as viral “sinks.”
There is preliminary success with this strategy using soluble human ACE2 (34) (Central Illustration).

**ANTI-INFLAMMATORY THERAPY.** Advanced stages of COVID-19 have been likened to cytokine storm syndromes with nonspecific widespread immune activation (115). Elevated levels of inflammatory biomarkers, such as IL-6 and hsCRP, identify patients at high risk of progressing to severe disease and death (53). Immunomodulatory and anti-inflammatory therapy have been used, despite limited data, in patients with evidence of hyperinflammation in an effort to curb pathologic immune activation.

**Corticosteroids.** Corticosteroids have been used in several, severe viral respiratory infections including influenza, SARS-CoV, and MERS-CoV with limited benefit and, in some instances, evidence of delayed viral clearance and increased rates of secondary infection and mortality (145). A retrospective analysis of 84 patients with ARDS secondary to SARS-CoV-2 observed an association with improved survival in patients who received solumedrol (51). In the absence of robust evidence, major professional society guidelines do not recommend routine use of corticosteroids in treatment of COVID-19 but rather restricting its use to patients with other indications for steroids, such as refractory shock or advanced ARDS (41). Clinical trials are ongoing to examine the safety and efficacy of corticosteroids in hospitalized non-critically ill COVID-19 patients (NCT04273321) and in those with ARDS (NCT04323592).

**IL-6 inhibitors.** Elevation of IL-6 in patients with severe COVID-19 has prompted consideration of use of IL-6 inhibitors (tocilizumab, siltuximab) extrapolating from treatment of cytokine release syndrome (146). Tocilizumab, a recombinant humanized monoclonal antibody, and siltuximab, a chimeric monoclonal antibody, both bind soluble and membrane bound IL-6 receptors resulting in inhibition of IL-6-mediated signaling. In 1 preprint case series from China, 21 patients with severe or critical COVID-19 treated with tocilizumab experienced a salutary effect with resolution of fever, improved oxygenation, improvement in lung opacities on chest computed tomography, resolution of lymphopenia, and a reduction in CRP levels within a few days of therapy in the absence of any significant reported adverse events (147). In this preliminary report, 19 patients were discharged alive, and 2 remained hospitalized at the time the case series was published. Several randomized clinical trials of tocilizumab in treatment of severe COVID-19 infection are ongoing (NCT04317092, NCT04306705).

**Azithromycin.** Azithromycin, a macrolide antibiotic, has long been touted for its anti-inflammatory effect and has been used as adjunctive therapy in treatment of community acquired pneumonia and chronic obstructive pulmonary disease exacerbations (148). Limited data suggest that adjunctive azithromycin in moderate-to-severe ARDS is associated with improved outcomes (149). A small nonrandomized study showed that combination azithromycin and HCQ was associated with more effective SARS-CoV-2 clearance in COVID-19 patients compared with either monotherapy with HCQ or standard of care; however, numerous limitations of this study render the data uninterpretable (141). QT interval monitoring is prudent, especially when used in combination with HCQ. Several randomized clinical trials assessing the combination of HCQ and CQ with azithromycin across the severity spectrum of COVID-19 are ongoing or about to be launched (NCT04321278, NCT04322396, NCT04322123, NCT04324469).

**Other anti-inflammatory therapies.** JAK-2 inhibitors inhibit receptor mediated-endocytosis, leading to the hypothesis that it might prevent cellular entry of the SARS-CoV-2 (Figure 1). Additionally, this class of agents have anti-inflammatory effects by inhibiting cytokine release (150). An agent in the class, baricitinib, is being studied in an open-label nonrandomized pilot study in patients with COVID-19 (NCT04320777). Currently, a 3-arm randomized control trial is being launched to compare anakinra monotherapy, emapalumab monotherapy, and standard of care (NCT04324021). Anakinra is a recombinant monoclonal antibody that blocks IL-1 receptors. It has been used to treat autoimmune conditions including juvenile idiopathic arthritis as well as recurrent pericarditis. Emapalumab is a fully human anti-interferon-gamma monoclonal antibody that has been approved by the Food and Drug Administration for treatment of primary hemophagocytic lymphohistiocytosis, a disease reminiscent of the hyperinflammatory state seen in advanced COVID-19. Finally, colchicine, a microtubule polymerization inhibitor and anti-inflammatory drug, is being tested in a large randomized clinical trial of ambulatory COVID-19 patients (NCT0432682).

**OTHER THERAPIES. ACE inhibitors and ARBs.** ACE2 receptor-mediated endocytosis of SARS-CoV-2 is central to the viral life cycle. Conflicting data exist regarding the effect of renin-angiotensinaldosterone-inhibitors, including ACE inhibitors and ARB, on ACE2 activity and levels in various human tissues and the resultant susceptibility to infection with SARS-CoV-2 (18). The totality of the available
data is insufficient to recommend cessation of ACE inhibitors or ARBs in individuals with an existing indication for life-prolonging therapy with these drugs, and major societies have strongly recommended continuation of ACE inhibitor and ARB therapy. An open label randomized trial is on the way to examine the effect of prophylactic ACE inhibitor and ARB withdrawal in COVID-19-naive individuals with essential hypertension as the sole indication for treatment on the risk of infection and subsequent complications (NCT04330300). Based on the preclinical data described earlier in this review, 2 paired trials are currently underway examining losartan therapy in patients with COVID-19 who are ambulatory (NCT04311177) and hospitalized (NCT04312009).

**Statins.** The anti-inflammatory pleiotropic effects of statins have been cultivated in different pathologic states. Statins have been shown in murine models of acute lung injury and in humans to attenuate the inflammatory component of acute lung injury (151,152). A multicenter randomized trial of simvastatin in patients with various causes of ARDS showed no difference as compared with placebo in ventilator-free days, multiorgan failure, and mortality (153). A subsequent study, subphenotyping the trial population in to hyperinflammatory versus hypoinflammatory ARDS, found a statistically significant improvement in survival with simvastatin in the hyperinflammatory group (154). A post hoc analysis of the JUPITER (Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin) trial observed a reduction in incident pneumonia with rosuvastatin (155). The benefit of statin therapy in the hyperinflammatory state in advanced COVID-19 is unknown.

**VACCINES AGAINST SARS-CoV-2.** As discovery of a safe and efficacious vaccine against SARS-CoV-2 is clearly the aspiration for preventative strategies, intense efforts are ongoing employing numerous approaches with accelerated testing. It is believed that all 4 structural proteins (E, M, N, and S) may serve as antigens for neutralizing antibody and CD4+CD8+ T cell responses (156). Based on the experience with SARS-CoV-1 vaccine development, it seems that the most promising candidates target the S protein, which induces humoral and protective cellular immunity (8). Encouragingly, administration of full-length or the ACE2 receptor-binding domain of the S protein of SARS-CoV-1 induced highly potent neutralizing antibodies that conveyed protective immunity in animal models (157,158).

Potential delivery strategies include inactivated or attenuated virus, subunit vaccines, viral vectors, and DNA- or RNA-based vaccines (159). Live attenuated viral vaccines are likely to induce significant immune response but may carry risk of disease, particularly in immunosuppressed individuals. Inactivated “whole” viral or subunit vaccines are relatively easy to develop but do not induce immediate or complete immunity, typically requiring multiple doses to promote humoral, but often not cellular, immunity. Immunity may also wane over time, requiring booster dosing. Viral vector-based vaccines would employ other viruses, such as the vaccinia virus (a poxvirus used for the smallpox vaccine) or adenovirus, to display SARS-CoV-2 antigens. This strategy can promote robust cytotoxic T cell responses but may fail in the face of the pre-existing immunity to or toxicity of the viral vector (160). Nucleic acid-based strategies, which work through delivery of DNA or RNA that are translated by host machinery to produce viral protein antigens, are relatively simple to design but may be limited by toxicity or stability concerns. Of note, at this time, there are no approved DNA or RNA vaccines for humans. Most approaches to SARS-CoV-2 are in preclinical development, with several early trials of RNA (NCT04283461) and viral vector (NCT04299724, NCT04313127, NCT04276896) vaccine strategies ongoing.

**CRISIS STANDARDS OF CARE AND ETHICAL RESOURCE ALLOCATION**

Estimates suggest that, as has happened in Italy and Spain, the burden of COVID-19 will far outstrip the health care capacity in the United States and globally with insufficient availability of hospital and ICU bed capacity, health care providers, and specific therapeutic or supportive interventions, such as mechanical ventilation and renal replacement (161). For this reason, organizations, such as the Italian Society of Anesthesia, Analgesia, Resuscitation and Intensive Care and individual health care institutions are developing guidance for allocation of resources in the event that adequate, additional resources cannot be obtained (162). These efforts are building off of a set of principles established in the wake of the 2009 H1N1 pandemic.

At that time, the U.S. Department of Health and Human Services commissioned the Institute of Medicine to provide expert guidance on implementing alternative standards of health care in the setting of a disaster. In their report, the Institute of Medicine defined the principles of “crisis standards of care,” defined as a substantial change in usual health care operations, including the level of care possible to deliver, in the setting of a pervasive or catastrophic
disaster (163). Notably, this framework recognizes that “the formal declaration that crisis standards of care are in operation enables specific legal/regulatory powers and protection for health care providers in the necessary task of allocating and using scarce medical resources.” Appreciating the distress associated with allocation of scarce medical resources, the Institute of Medicine recommends that the process be guided by 7 ethical principles: fairness, duty to care, duty to steward resources, transparency, consistency, proportionality, and accountability (163).

Working with these principles, ethicists have come to a general consensus that the goal is to maximize benefit while maintaining equity, objectivity, and transparency (161,164). Maximizing benefit ideally involves preserving the most lives as well as the most life-years, acknowledging the importance of prognosis. Although the practical application of these principles is challenging, there appears to be general agreement across the literature on a number of concepts (161,164,165). Most recommend development of a triage or scoring system that accounts for acute and premorbid prognosis in order to allocate scarce resources to those who are most likely to benefit. The scoring system should utilize objective clinical information, in order to minimize the need for clinical judgment and the risk of introducing inconsistency and bias. The use of the system—and the determination that stems from it—should be transparent to providers, patients, and families. Triage should be applied broadly to all patients requiring a particular resource, not just those suffering from the pandemic disease (e.g., applies to decision to use venoarterial extracorporeal membrane oxygenation in patients with myocarditis due to COVID-19 and cardiogenic shock from a non-COVID-19 etiology). A random system (e.g., lottery) should be used to break “ties” in cases with a similar estimated prognosis, rather than a first come, first serve approach. Importantly, many advocate that an independent triage physician make the determination to remove the burden from the bedside health care team. The triage physician may be supported, as necessary, by a triage committee, comprising experts in the area of ethics and relevant medical fields.

Areas of controversy include whether there should be priority allowed for health care providers. Some ethicists argue that they should not be prioritized as that are unlikely to recovery in a time frame that would allow them to continue their professional responsibilities (164). Others argue that granting priority recognizes the assumption of risk and also encourages ongoing participation in patient care (161). Along the same line, an argument has also been made to prioritize research participation (161).

The optimal tool for prognostication also remains elusive. The SOFA score has been suggested as quantitative assessment of acute illness severity; however, there is a recognition that this tool may not be well calibrated to all populations and could lead to inaccurate assessments of prognosis (166,167).

The value of predetermination of this framework with community and provider engagement, establishment of legal authority, and logistic and operational preparedness is clear. Nevertheless, acknowledging the prospect of large-scale rationing of health care is heartbreaking and foreign to most civilian health care providers in developed countries.

**SUMMARY**

In just a few short months, SARS-CoV-2 has spread across the world with distressing speed, threatening global economic and individual health and well-being. Many regional health care systems are overwhelmed and under-resourced, forcing clinicians and administrators to make previously unthinkable decisions regarding allocation of medical care. However, in the wake of this devastation, clinicians and scientists have rallied together to rapidly evolve our understanding of all aspects of SARS-CoV-2 infection, from the basic virology, to the human manifestations to therapeutic and preventative strategies. This unprecedented collective effort will, without a doubt, advance our ability to prevent the spread and optimally care for patients suffering from COVID-19.

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**KEY WORDS** cardiovascular, COVID-19, SARS-CoV-2, treatments, virology
The Heart in COVID-19
Primary Target or Secondary Bystander?

Peter Libby, MD

SUMMARY

In the throes of the current coronavirus disease-2019 (COVID-19) pandemic, interest has burgeoned in the cardiovascular complications of this virulent viral infection. As troponin, a biomarker of cardiac injury, often rises in hospitalized patients, its interpretation and actionability require careful consideration. Fulminant myocarditis due to direct viral infection can certainly occur, but in patients with increased oxygen demands due to tachycardia and fever and reduced oxygen delivery due to hypotension and hypoxemia, COVID-19 disease can cause myocardial injury indirectly. Cytokines released during the acute infection can elicit activation of cells within pre-existing atherosclerotic lesions, augmenting thrombotic risk and risk of ischemic syndromes. Moreover, microvascular activation by cytokines can cause not only myocardial injury but can also harm other organ systems commonly involved in COVID-19 infections including the kidneys. Dealing with the immense challenge of COVID-19, confronted with severely ill patients in dire straits with virtually no rigorous evidence base to guide our therapy, we must call on our clinical skills and judgment. These touchstones can help guide us in selecting patients who might benefit from the advanced imaging and invasive procedures that present enormous logistical challenges in the current context. Lacking a robust evidence base, pathophysiologic reasoning can help guide our choices of therapy for individual clinical scenarios. We must exercise caution and extreme humility, as often plausible interventions fail when tested rigorously. But act today we must, and understanding the multiplicity of mechanisms of myocardial injury in COVID-19 infection will help us meet our mission unsupported by the comfort of strong data.

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contain clear-cut descriptions of fulminant myocarditis in certain individuals (1,2), as ably reviewed in the State-of-the-Art Review paper on cardiac involvement in COVID-19 by Atri et al. (3) in this issue of JACC: Basic to Translational Science. Indeed, the human myocardium can express the receptor that COVID-19 uses to infect host cells, angiotensin-converting enzyme-2, which is the counter-regulatory cousin of the more familiar angiotensin-converting enzyme-1. Thus, no doubt, in some cases, a viral myocarditis due to this agent can occur (Figure 1, far left). Yet, troponin rise seems nearly ubiquitous in patients requiring intensive care, an indication of cardiac involvement in many cases and a marker of poor prognosis as in many other circumstances. But can we, and should we, attribute all rises in troponin to direct myocardial infection by this virus?

To approach this question, we need to distinguish myocarditis due to infection of cardiac cells from myocardial ischemic injury. Flow embarrassment to the heart muscle can result from lesions in epicardial coronary arteries or in the heart’s microvasculature. Cardiac ischemia can also arise from an imbalance between oxygen supply and demand, a type 2 acute coronary syndrome, a situation that can prevail in acute infections, particularly those that affect the lungs like COVID-19 does. Several of these pathophysiologic pathways to myocardial ischemia may affect those without substantial or obstructive coronary artery atherosclerosis. Hence, the distinction between these various mechanisms has important clinical consequences. The need for arduous imaging studies and invasive evaluation may vary considerably in these different scenarios, an issue of great import in acute care facilities stretched to or beyond their limits during a pandemic with a readily contagious and virulent infectious agent such as COVID-19. Considering the pathophysiologic paths to cardiac injury can inform judgment regarding the necessity of transport of severely ill patients and the performance-invasive procedures.

A panel convened by the National Heart, Lung, and Blood Institute in 1997 considered the roles of infectious agents in cardiovascular disease. The summary report of this panel explicitly considered systemic infection and the triggering of acute coronary events, and it reviewed some of the possible mechanisms (4). These considerations included cytokine responses to infection as activators of vascular cells and as inducers of the acute phase response with consequent heightened production of fibrinogen, the precursor of clots, and of endogenous inhibitors of fibrinolysis. More recent panels convened in conjunction with the National Heart, Lung, and Blood Institute re-examined this issue and highlighted the differences between direct infection and secondary responses (5).

The COVID-19 pandemic elevates these pathophysiologic considerations from theoretically interesting to a level of vital clinical importance. The paper by Atri et al. (3) deals comprehensively with cardiac involvement. This commentary and the accompanying illustrations place these considerations in the context of inflammation and vascular biology.

**INFECTION AT REMOTE SITES CAN ELICIT “ECHOES” IN THE PRE-EXISTING ATHEROSCLEROTIC LESION**

The demographic most often affected by life-threatening and fatal COVID-19 has a high prior probability of established atherosclerotic lesions: they are elderly persons, predominantly male, and have pre-existing lung disease, including that associated with cigarette smoking, a risk factor for atherosclerosis. Remote infections such as the severe pneumonitis that too commonly complicates COVID-19 can elicit an acute exacerbation of the chronic smoldering inflammation that characterizes coronary atherosclerotic lesions (Figure 2). The inflammatory cells at a site of regional infection such as the lungs in COVID-19 pneumonitis can produce cytokines such as interleukin-1 and -6 and tumor necrosis factor, mediators that not only propagate local inflammation but can enter the systemic circulation. Such circulating cytokines can stimulate macrophages within the plaque to augment local cytokine production and provoke an increase in tissue factor.
expression that renders lesions more thrombogenic. We have referred to this local response to systemic stimuli as an “echo” phenomenon (4,6). These same systemic cytokines can stimulate leukocyte adhesion molecule expression on the endothelial cells overlying established atheroma, boosting local recruitment of these inflammatory cells. These alterations in pre-existing plaques can enhance their propensity to disrupt, be it by fibrous cap fissure or by superficial erosion, and provoke an acute coronary syndrome.

**INFECTION AT REMOTE SITES CAN ALSO ACTIVATE THE CORONARY MICROVASCULATURE**

Even in individuals without pre-existing epicardial coronary artery disease, systemic cytokines released from sites of local infections such as in pneumonitis can affect intermural coronary vessels. They can also activate the microvascular endothelium, predisposing to vasomotor abnormalities, augmented thrombosis, reduced fibrinolysis, increased leukocyte
adhesion, and other aspects of dysfunction of the microvessels of the coronary circulation. These effects wrought by distant infection can contribute to myocardial ischemia, even in the absence of epicardial atherosclerosis, and could compound cardiac injury in those with flow limitation due to plaque in the larger coronary arteries.

INFECTION AND PNEUMONIA CAN WORSEN THE BALANCE BETWEEN MYOCARDIAL OXYGEN SUPPLY AND DEMAND

The cardinal signs of infection include fever and tachycardia, circumstances that increase the oxygen requirements of the myocardium (Figure 3). Hypoxemia produced by pneumonitis can decrease oxygen delivery to the myocardium. Hypotension in sepsis and in cytokine storm can impair coronary perfusion. Together these systemic effects of infection conspire to limit blood flow in the coronary arteries and reduce oxygen supply while augmenting myocardial oxygen demand. These consequences of infection predispose to myocardial ischemia. They may aggravate the peril of plaques that would not limit flow or provoke ischemia under usual conditions and could produce ischemic injury even in those with little or no coronary artery atherosclerosis.

MULTIPLE MECHANISMS MAY CONTRIBUTE TO CARDIAC COMPLICATIONS OF COVID-19 IN DIFFERENT DEGREES

At one end of the spectrum, a young individual with pristine coronary arteries might suffer severe myocardial injury due to a fulminant myocarditis caused by direct infection with COVID-19 (Figure 1, left). At the other extreme, a person with advanced coronary atherosclerosis could suffer a type 1 or type 2 acute myocardial infarction without direct viral infection of cardiac cells (Figure 1, right). Although we are early in our experience with this novel coronavirus disease, most patients affected by COVID-19 encountered by cardiologists may have more secondary cardiac involvement than primary infective
myocarditis. Thus, many of our patients may fall into the zone between the bookends depicted in Figure 1.

It behooves us to consider the multiple mechanisms of cardiac injury in patients with COVID-19 (Figure 4). As in the BC (before COVID-19) era, interpretation of rises in cardiac troponin requires consideration of the context of the clinical situation. Not all rises in this biomarker of cardiac injury will
result from coronary artery disease requiring invasive assessment or intervention. We urgently need randomized clinical trials to assess the value of interventions including anti-inflammatory therapies ranging from glucocorticoids to cytokine antagonism in addition to antiviral agents as outlined in the elegant exposition of Atri et al. (3).

Dealing with the immense challenge of COVID-19, and confronted with severely ill patients in dire straits with virtually no rigorous evidence base to guide our therapy, we need to call on our clinical skills and judgment. These touchstones can help guide us in selecting patients who might benefit from the advanced imaging and invasive procedures that present enormous logistical challenges in the current context. In the absence of a robust evidence base, we will also need to invoke pathophysiologic reasoning to guide our choices of therapy for each individual clinical scenario. We must do so with caution and extreme humility, recognizing how often plausible interventions fail when tested rigorously. But act today we must, and the overview of Atri et al. (3) and other recent compendia will help us meet our mission unsupported by the comfort of strong data.

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KEY WORDS atherosclerosis, cytokines, endothelial cells, inflammation, sepsis, vascular biology
Precision Medicine in COVID-19: IL-1β a Potential Target

As of April 4, 2020, coronavirus disease-2019 (COVID-19) has been confirmed in 1,395,136 people worldwide, with a mortality of approximately 5.8%.

Since the COVID-19 outbreak, there has been an urgent need for effective treatments. A wide spectrum of disease severity has been described, ranging from asymptomatic, to mildly symptomatic, to severe symptomatic requiring hospitalization, to respiratory failure from acute respiratory distress syndrome. Furthermore, it has been widely reported that the prevalence of the disease is almost 3 times higher in male patients. Overall, this evidence suggests that the prognosis appears to be more conditioned by the host’s response than by the infection itself; thus, a precision medicine approach is highly desirable.

Inflammatory responses and the entity of the cytokine storm produced by the infection seem to be variables potentially related to this huge clinical variability. In this view, a multicenter trial on tocilizumab, TOCIVID-19 (Tocilizumab in COVID-19 Pneumonia), a monoclonal antibody that competitively inhibits the binding of interleukin (IL)-6 to its receptor (IL-6R), is ongoing on patients with COVID-19 with pneumonia. To our knowledge, there are no ongoing studies on the inhibition of IL-1.

Evidence suggests that COVID-19 may have originated in bats. Bats, the only flying mammals, have the ability to asymptotically host a large number of high-profile viruses, such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome coronaviruses. It has been reported that fruit bats can be infected with bat SARS coronavirus without showing any sign of infection (1).

In the pre–COVID-19 era, Ahn et al. (2) demonstrated that the ability of bats to tolerate viral disease, even during a transient phase of high viral load, could be explained by a dampened NLR family pyrin domain containing 3 (NLRP3)-mediated inflammatory response. This supports the hypothesis that an enhanced innate immune tolerance rather than an enhanced antiviral defense can be the key point explaining different clinical scenarios in COVID-19. NLRP3 is a critical component of the innate immune system that detects a broad range of microbial motifs, endogenous danger signals, and environmental irritants, mediating caspase 1 activation and secretion of proinflammatory cytokines IL-1β/IL-18 in response to microbial infection and cellular damage (3).

Aberrant activation of the NLRP3 inflammasome is involved in the pathophysiology of various diseases including diabetes, atherosclerosis, and metabolic syndrome, all of which have been shown to be comorbidities associated with worse outcome in COVID-19. In this regard, canakinumab, a therapeutic monoclonal antibody targeting IL-1β, significantly lowers the rate of recurrent cardiovascular events in patients with a previous of myocardial infarction. In cardiovascular diseases, an important source of inflammatory mediators, including IL-1β, is the visceral adipose tissue (4). Of note, activation of NLRP3 inflammasome in macrophages attenuates uncoupling protein 1 (UCP1) induction and mitochondrial respiration in cultures of primary adipocytes, and the NLRP3 inflammasome activation appears to link obesity and dysfunctional thermogenesis (5).

In conclusion, there are many open questions that merit exploration: 1) verifying the role of NLRP3 in the clinical variability of COVID-19; 2) testing the potential therapeutic effect in COVID-19 of IL-1β inhibition (canakinumab, anakinra); and 3) exploring the role of visceral adipose tissue in the inflammatory response to SARS-COV-2 infection.

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more information, visit the JACC: Basic to Translational Science author instructions page.

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Bats have been getting a lot of bad press lately, following the revelation that the genomic sequence of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) shared 96.2% sequence identity with Bat CoV RaTG13 (1). If one can move beyond the zoonotic public relations nightmare caused by the coronavirus disease-2019 (COVID-19) epidemic, bats present an amazing evolutionary story that may provide insights into the development of new therapeutic approaches for treating COVID-19 patients.

Bats (order Chiroptera) represent one of the largest (>1,300 species) mammalian species, accounting for ~20% of all mammals. What makes bats unique is that they are the only mammals capable of self-powered flight, which likely explains why bats inhabit every continent except for Antarctica. The earliest known bats likely arose during the Eocene era (49 to 53 million years ago); however, their actual evolutionary history is partially obscured by the paucity of intact fossil records. There have been a number of speculative theories with respect to whether bats evolved the ability to fly first or whether they first developed the ability to listen to the reflected echoes from sounds that they emitted to map out their environment (echolocation). Recent information gleaned from a rare 52-million-year-old bat fossil suggests that flight evolved before echolocation (2). Regardless of which came first, bats have a remarkable capacity for flight, and can achieve speeds of up to 160 km/h (99 miles/h), as well as remain in flight continuously for hours at a time. Germane to this discussion, the evolutionary pressures imposed by flight in bats have selected for a novel suite of antiviral immune responses that control viral propagation, while also limiting self-damaging inflammatory responses. This evolutionary step may have allowed bats to emerge as zoonotic hosts for viruses, including SARS-CoV-1 and -2.

Because bats have high metabolic rates and high body temperatures (often >41°C), they are prone to developing oxidative stress during flight, which can lead to deoxyribonucleic acid (DNA) damage, and initiate brisk inflammatory responses if the damaged DNA leaks into the cytosolic compartment. Selective evolutionary pressures related to flying appear to have positively selected for antioxidant and DNA repair pathways that allow bats to effectively engage tissue repair mechanisms without excessive inflammatory responses. As 1 example, bat cells appear to have a diminished ability to detect endogenous damaged DNA because of mutations in stimulator of interferon genes (STING), which is an endoplasmic reticulum adaptor protein that regulates the expression of type 1 interferon (IFN) host response genes (3). Although this adaptation would not be important in terms of directly preventing pathological immune responses to SARS-CoV-2 (a single-stranded RNA), it is likely important in terms of preventing pathological immune responses to DNA damage after prolonged flight. The STING pathway might also represent a novel therapeutic target in COVID-19 patients, wherein SARS-CoV-2–induced cell death might lead to release of damaged DNA fragments in alveolar cells, resulting in a brisk inflammatory response and collateral tissue damage. Perhaps not surprisingly, biotech companies are targeting small molecule inhibitors of the STING pathway to dampen inflammatory signaling in autoimmune diseases (4). Bats also appear to have developed ways to directly limit viral replication, by enhancing autophagic repair mechanisms (5).

Bats have also developed unique mechanisms that allow them to mount and maintain a strong type I IFN
response, which is the critical first line of antiviral defense in mammalian cells (3). The initiation of antiviral immune responses begins with the engagement of germ-line encoded pattern recognition receptors that are ubiquitously expressed in mammalian cells. Single-stranded RNA coronaviruses that enter the endosomal compartment of cells initiate antiviral responses by binding to toll-like receptor 7, an endosomal pattern recognition receptor. In the cytoplasmic compartment of the cell, retinoic acid-inducible gene-1 and mitochondrial antiviral signaling proteins are capable of detecting double-strand viral RNA moieties. Following recognition and engagement with viral genomic material, the aforementioned pattern recognition receptors induce the expression of hundreds of type 1 IFN genes that up-regulate cell-intrinsic and -extrinsic antiviral responses. Intriguingly, certain species of bats constitutively express IFNα genes in the absence of stimulation by genomic viral RNA or DNA. In other mammalian species, chronic inflammation is associated with a poor prognosis; however, bats have evolved novel mechanisms that limit inflammation-mediated cell damage through up-regulation of inhibitory proteins such as c-Rel, which prevents the activation of nuclear factor-kappa B, a central mediator of cellular inflammatory responses (3). The immune cells of bats have also evolved mechanisms to decrease NLRP3 (nod-like receptor pyrin domain containing 3) inflammasome activation (3). Relevant to this discussion, there are 3 ongoing clinical trials that are evaluating colchicine (which inhibits inflammasome activation) in COVID-19 patients. The largest of these trials is the randomized open-label controlled trial to study the benefit of colchicine in patients with COVID-19 (COL-COVID; NCT04350320). There are also ongoing trials using interleukin-1β antagonists (NCT04330638, NCT04324021), which is 1 of the pro-inflammatory cytokines released secreted by cells following inflammasome activation.

The increasing recognition that bats serve as flying resorts for zoonotic diseases that become lethal when they jump to humans has prompted scientists to embark on a deeper understanding of exactly how bats are able to tolerate viral infections without experiencing disease. While some of the same antiviral strategies that bats employ to modulate viral infections are also being tested in COVID-19 clinical trials (e.g., interferons), we still have a lot to learn with respect to how bats are able to mount brisk antiviral responses without also developing collateral tissue damage secondary to sustained chronic inflammatory signaling. Perhaps one day this type of knowledge might move beyond treating COVID-19 patients and could also be utilized to treat cardiovascular diseases, wherein chronic inflammation results in collateral damage and untoward patient outcomes (e.g., heart failure).

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In Figure 3D, the MW of FoxO3a was incorrectly marked as 45kd, whereas the correct MW of FoxO3a is 97kd.

In Figure 6H, a duplicate image was used for the 5% US+Macrophages Exo group and the 5% US+Macrophages Exo+miR-Ctrl.

The corrected figure is below.

The authors apologize for these errors.

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