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Design of the β3-Adrenergic Agonist Treatment in Chronic Pulmonary Hypertension Secondary to Heart Failure Trial

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HIGHLIGHTS

- CpcPH is a relatively common complication of chronic HF, is associated with poor survival, and has no specific pharmacological treatment.
- β3AR stimulation has shown improvement in pulmonary hemodynamics and RV performance in a translational large animal model mimicking this condition.
- The SPHERE-HF trial is a Phase II randomized, double-blind clinical trial designed to evaluate the efficacy and safety of mirabegron (oral β3 AR agonist) in patients with CpcPH secondary to HF.
- The SPHERE-HF trial will include 80 patients treated with mirabegron or placebo for 16 weeks.
- The main outcome is the change in PVR. Secondary outcomes include changes in RV performance, clinical status, NT-proBNP levels, and additional pulmonary hemodynamic parameters.
Combined pre-and post-capillary hypertension (CpcPH) is a relatively common complication of heart failure (HF) associated with a poor prognosis. Currently, there is no specific therapy approved for this entity. Recently, treatment with beta-3 adrenergic receptor (β3AR) agonists was able to improve pulmonary hemodynamics and right ventricular (RV) performance in a translational, large animal model of chronic PH. The authors present the design of a phase II randomized clinical trial that tests the benefits of mirabegron (a clinically available β3AR agonist) in patients with CpcPH due to HF. The effect of β3AR treatment will be evaluated on pulmonary hemodynamics, as well as clinical, biochemical, and advanced cardiac imaging parameters. (Beta3 Agonist Treatment in Chronic Pulmonary Hypertension Secondary to Heart Failure [SPHERE-HF]; NCT02775539) (J Am Coll Cardiol Basic Trans Sci 2020;5:317–27) © 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/).

Pulmonary hypertension (PH) is a common complication of heart failure (HF) (1,2) that results in more severe symptoms, worse exercise tolerance, and increased risk of death (3–5). Initially, this isolated post-capillary PH (IpcPH) is purely passive but has the potential to progress to combined pre- and post-capillary PH (CpcPH), a progressive disease characterized by significant vasoconstriction and vascular remodeling with a worse prognosis than IpcPH (6,7). Although IpcPH can be treated by focusing only on the underlying condition (8), CpcPH requires treatment of both pulmonary vascular remodeling and the primary heart disease.

Currently, there are no specific pharmacological therapies approved for patients with CpcPH (8,9). Clinical studies performed with specific pulmonary vasodilators [i.e., prostanooids (10) and endothelin receptor blockers (11,12)] in cohorts with HF or PH secondary to HF have not shown positive results, primarily because of concomitant systemic hypotension and hepatic toxicity. Although preliminary data from small single-center studies (13,14) that tested phosphodiesterase type 5 inhibitors in PH secondary to HF were promising, more recent evidence (15–17) strongly discourages their use in this setting. In addition, neutral findings have been reported for cyclic guanosine monophosphate (cGMP) stimulation in PH secondary to HF, either with preserved or reduced left ventricular ejection fraction (LVEF) (18,19). Therefore, new treatments are needed for CpcPH.

The sympathetic nervous system is central to the neurohumoral regulation of cardiovascular function and is implicated in many cardiopulmonary diseases. Beta-3 adrenoreceptor (β3AR) expression has been

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demonstrated in the human myocardium and vessels, and it has been described to be upregulated in left heart disease (LHD) (20,21). Like other adrenoceptors, β3ARs are coupled to G proteins, and the downstream activated pathway includes nitric oxide synthase, nitric oxide-activated guanylyl cyclase, and cGMP synthesis, as well as increased cyclic adenosine monophosphate synthesis (22). Loss of cGMP and cyclic adenosine monophosphate signaling represents a hallmark in PH. It is known that within the pulmonary circulation, cyclic nucleotides exert several favorable effects, including vasodilatation, inhibition of smooth muscle cell proliferation, and prevention of platelet aggregation (23).

In recent years, several publications have demonstrated the cardioprotective effect of β3AR stimulation in different experimental models of ischemia-reperfusion injury (24-26) and HF (27-29). Therefore, β3ARs have emerged as a potential therapeutic target in cardiovascular diseases. Recent experimental research has demonstrated that treatment with β3AR agonists produces a beneficial effect on hemodynamics, right ventricular (RV) remodeling, and pulmonary vascular proliferation in a translational porcine model of post-capillary chronic PH (30). In addition, several Phase II and III randomized clinical trials (31-37) have already confirmed the good safety profile of the oral β3AR agonist mirabegron in healthy subjects and in patients with overactive bladder syndrome. Mirabegron, the selective oral β3AR agonist tested in the present trial, is currently approved for the treatment of overactive bladder syndrome in Europe, Japan, and America. A recent study has also demonstrated a good safety profile of mirabegron in patients with HF and reduced LVEF (38).

Based on the previously described concepts and evidence and the positive results of pre-clinical research, we designed a multicenter placebo-controlled Phase II randomized clinical trial to evaluate the efficacy and safety of mirabegron in patients with chronic CpcPH secondary to HF.

**METHODS**

**STUDY HYPOTHESIS.** The main hypothesis of the SPHERE-HF (β3 Adrenergic Agonist Treatment in Chronic Pulmonary Hypertension Secondary to Heart Failure) trial is that maintenance treatment with a selective β3AR agonist (mirabegron) in patients with PH secondary to HF compared with placebo will result in a beneficial effect due to: 1) a reduction in pulmonary vascular resistance (PVR); 2) an increase in RV performance; 3) improvement in clinical status; and 4) no increase in adverse events.

**STUDY ENDPOINTS. Efficacy measures.** The primary outcome is the change in PVR on right heart catheterization, calculated in Wood units as: (mean pulmonary artery pressure (PAP) [mm Hg] – pulmonary capillary wedge pressure [mm Hg]) / cardiac output [l/min]) from baseline to week 16. Secondary outcomes are change from baseline to week 16 in clinical status (measured by 6-min walk distance, New York Heart Association [NYHA] functional class, quality of life evaluated with the Spanish version of the Kansas City Cardiomyopathy Questionnaire, and the dyspnea Borg scale score), other hemodynamic variables assessed by right heart catheterization (mean PAP, transpulmonary gradient, diastolic pressure gradient, and cardiac output), RV performance (RV ejection fraction and cardiac output by cardiac magnetic resonance [CMR] or cardiac computed tomography [CCT]), as well as plasmatic levels of N-terminal pro-hormone of brain natriuretic peptide (NT-proBNP).

Safety measures include hospital admissions for HF or respiratory failure, death, urgent heart transplantation, initiation of intravenous therapy due to worsening HF (diuretics or inotropic drugs), adverse events, and adverse drug reactions, as well as monitoring of heart rate and the QTc interval on electrocardiography (ECG) (by Framingham method). External monitoring of all clinical events and ECG acquisitions will be performed.

**STUDY POPULATION AND INCLUSION/EXCLUSION CRITERIA.** Patients have been identified and recruited from 4 different tertiary hospitals across Spain, all of which have a reference HF Unit. Inclusion and exclusion criteria are presented on Table 1. At the first protocol, an exclusion criterion of QTc interval on ECG >430 ms in men and >450 in women was included as a general safety standard. This criterion was later modified by an amendment to QTc >480 ms, based on the high percentage of patients with CpcPH who were excluded at baseline and the absence of data that suggested that mirabegron significantly increased QTc.

**STUDY DESIGN.** This trial is a Phase II double-blind multicenter, placebo-controlled randomized controlled trial. It consists of a screening phase and the randomized double-blind 16-week period (main study), which includes a dose titration phase and a maintenance phase (Figure 1).

During the screening phase, eligible patients, usually identified after a right heart catheterization, are invited to participate in the study. After providing...
written informed consent, patients undergo the following baseline procedures and assessments (all must be completed within 4 weeks before random allocation): demographic and medical history data collection; physical examination (including blood pressure, heart rate, and pulse oximetry); NYHA functional class; blood sample analysis including NT-proBNP; ECG; echocardiography; right heart catheterization; 6-min walking test, and CMR. Those patients with formal contraindications for CMR can undergo a dedicated CCT examination to measure RV volumes and function (Table 2, Figure 2).

Patients who fulfill all inclusion criteria and none of the exclusion criteria are randomized to receive either mirabegron (50 mg) or placebo once daily in a blinded fashion (visit 0). Visit 1 represents the day of the first study medication dose and must take place in the 5 days after visit 0. A safety visit is performed 1 week after initiation of therapy (visit 2) because, according to the medication technical sheet, steady-state concentrations are achieved at 7 days of once-daily dosing with mirabegron. Thereafter, medication dose is titrated every 2 weeks for 8 weeks (visits 3, 4, and 5) based on patients’ monitoring of blood pressure, heart rate, QTc interval, blood analysis, and clinical status assessed at that visit (Table 3 shows the recommended titration algorithm). At the end of the dose titration phase (visit 6), all patients are expected to have reached their optimal dose. To assure blinding of the treatment arms, patients allocated to the placebo group undergo titration from visit 3 onwards, following the same algorithm.

During the maintenance phase (8 weeks), patients continue receiving the same dose assigned at visit 6, unless a decrease in dose is required for safety purposes. An intermediate clinical visit is performed halfway through the maintenance phase (visit 7). At visit 8 (end of the study), patients undergo all study examinations performed at baseline and stop the study medication. The last visit (visit 9) takes place 30 days after the last study medication dose for

### Table 1: Inclusion and Exclusion Criteria

| Inclusion Criteria | Exclusion Criteria |
|--------------------|--------------------|
| Written informed consent | Noncoronary cardiac surgery (e.g., valvular surgery) or noncoronary structural percutaneous procedure (e.g., Mitraclip) within the 12 months preceding recruitment or scheduled. |
| Age ≥18 years of age | Myocardial infarction or coronary revascularization within the 3 months preceding recruitment. |
| HF with reduced, intermediate or preserved LVEF, according to the definition of the European Society of Cardiology guidelines. | CRT implantation within the 6 months preceding recruitment. |
| Combined pre- and post-capillary PH determined by RHC showing the following: PCWP or LVEDP ≥15 mm Hg. Mean PAP ≥25 mm Hg, and: PVR ≥3 WU and/or diastolic gradient ≥7 mm Hg, or transpulmonary gradient ≥12 mm Hg | Sinus tachycardia or uncontrolled atrial fibrillation (HR >100 beats/min). |
| NYHA functional class II–IV | Uncontrolled systemic hypertension (systolic BP >180 mm Hg or diastolic BP >110 mm Hg) or symptomatic hypotension (systolic BP <90 mm Hg). |
| On optimized evidence-based pharmacological treatment | Diagnosis of infiltrative cardiomyopathy. |
| Stable clinical condition defined as no changes in therapeutic regimen for HF or hospitalization in the 30 days preceding recruitment and no current plan for changing therapy.† | Pre-menopausal women who have not undergone total hysterectomy |
| Expected survival <1 yr due to a disease other than HF. | |
| Severe renal failure (GFR <30 ml/min/1.73 m²). | |
| Severe hepatic impairment (transamnise elevation >3 times ULN). | |
| Prolonged QTc interval on the ECG (<430 ms in men or >450 ms in women). | |
| Concomitant use with specific pulmonary vasodilators (sildenafil, bosentan, macitentan, riociguat, or other endothelin receptor blockers, phosphodiesterase 5 inhibitors or guanylate cyclase stimulators). | |
| Treatment with digoxin, flecainide, propafenone, dabigatran, tricyclic antidepressants or other CYP2D6 inhibitors (other than β-blockers). | |
| Severe COPD (FEV1/FVC ratio <0.7 together with FEV1 <50% predicted value). | |
| Severe restrictive lung disease (TLC <50%). | |
| Participation in another clinical trial | |
| Known allergy to mirabegron or any of the excipients | |

†In the case of heart failure (HF) with preserved left ventricular ejection fraction (LVEF) it refers to an adequate control of comorbidity and optimized fluid status. †This criterion was changed by an amendment to QTc >480 ms.

BP = blood pressure; COPD = chronic obstructive pulmonary disease; CRT = cardiac resynchronization therapy; FEV1 = forced expiratory volume in the first second; FVC = forced vital capacity; GFR = glomerular filtration rate; HR = heart rate; LVEDP = left ventricular end-diastolic pressure; NYHA = New York Heart Association; PAP = pulmonary artery pressure; PCWP = pulmonary capillary wedge pressure; PVR = pulmonary vascular resistance; RHC = right heart catheterization; TLC = total lung capacity; ULN = upper limit of normal; WU = Wood units.
security monitoring, according to clinical trials regulation.

Table 2 shows a detailed calendar of the procedures that are performed at each study visit.

**SAMPLE SIZE.** A sample size sample size of 31 subjects per group achieves 80% power to reject the null hypothesis of zero treatment difference when the 2-sided significance level (alpha) is 0.05 and 2.1 Wood units, which are the minimal clinically important difference between groups. We assumed a SD of 3.2 Wood units in PVR (39) and a correlation between baseline and 16 weeks of 0.6. Assuming a 20% dropout rate, the estimated number of patients needed is 40 per group (80 total). Calculations were made using the power.t.test function available in R software (R Foundation, Vienna, Austria).

**Manufacturing of study medication.** Manufacturing of mirabegron and placebo is carried out in the pharmaceutical area of the Hospital Clinic Pharmacy department. The excipient for placebo capsules (mixture of microcrystalline cellulose and colloidal silica) is received with the corresponding certificate of analysis from the manufacturer (Fagron Iberica S.A.U, Tarrasa, Spain). The material the lot number and expiration date are verified for each entry, and the corresponding certificate of analysis is archived. Similarly, at the reception of mirabegron (Betmiga; bought from Astelas Pharma [Chuo, Tokyo, Japan] for 28.9€/box containing 30 pills of 50 mg of Betmiga), the batch and expiration date are checked and recorded. Betmiga pills are taken out of the blisters and automatically encapsulated (a single pill into a capsule). Similarly, the excipient is included in the capsules. Immediately, capsules including mirabegron or excipient are introduced in polyethylene bottles (30 capsules each) that are labeled as study medication, including the batch number and expiration. The bottles are kept in the clinical trials area in conservation conditions (atmosphere temperature <25 °C).

**STATISTICAL METHODS.** Randomization procedure. Participants will be assigned, on an individual basis, to mirabegron or placebo using randomly selected block sizes stratified by center using the Blockrand package (R Foundation). The randomization list will be provided exclusively to the pharmacy department responsible for the medication preparation, which

| TABLE 2 Overview of Study Visits and Examinations |
|-----------------------------------------------|
| V0   | V1   | V2   | V3   | V4   | V5   | V6   | V7   | V8   | V9   |
| Informed consent | X |
| Inclusion/exclusion criteria | X |
| Demographic variables, prior medical history and medication | X |
| Anamnnesis | X | X | X | X | X | X | X | X | X |
| Physical examination | X | X | X | X | X | X | X | X | X |
| Laboratory | X | X | X | X | X | X | X | X |
| ECG | X | X | X | X | X | X | X | X |
| Kansas City Cardiomyopathy Questionnaire | X | X |
| Echocardiogram | X |
| Right heart catheterization | X | X |
| NT-proBNP | X | X |
| 6-min walking test | X | X |
| CMR | X | X |

CMR = cardiac magnetic resonance; ECG = electrocardiogram; NT-proBNP = N-terminal pro-hormone of brain natriuretic peptide; V = visit.
will prepare the medication kits by identifying them with a unique sequential number for the entire study and will provide the kits to the centers. Researchers will assign received medication kits in a sequential order and record the kit number provided to each patient in the data collection system. **Statistical analysis.** The following analysis populations are pre-defined for this study: intention to treat (ITT) population—all randomized patients; per protocol (PP) population—patients from the ITT group who have the final (16-week) measurement of the primary outcome and who have taken at least 80% of all the medication doses; and safety population—all patients who have taken ≥1 doses of the assigned treatment.

Baseline descriptive statistics will be calculated according to standard methods on the ITT and PP populations. Qualitative variables will be described as absolute number (n) and frequency (%), whereas quantitative variables will be described by mean ± SD.

**TABLE 3** Recommended Medication Dose Titration Algorithm

| Clinical Assessment | Recommended Action |
|---------------------|--------------------|
| Normal BP (systolic BP ≥95 and ≤135 mm Hg) AND HR ≤90 beats/min AND QTc interval* <430 ms in men or ≤450 ms in women AND blood analysis within normality AND patient asymptomatic | Up titrate study medication in 50 mg/day |
| If the patient develops any of the following: significant hypotension (systolic BP <80 mm Hg) or hypertension (systolic BP >145 mm Hg) OR tachycardia (HR >100 beat/min) OR prolonged QTc interval* (>430 ms in men or >450 ms in women) OR worsening of renal function/transaminitis elevation on blood analysis OR symptoms associated with medication | Reduce or stop study medication dose |
| If the patient presents with mild hypotension (systolic BP >80 and <90 mm Hg) OR mild hypertension (systolic BP >135 and ≤145 mm Hg) AND/OR HR 90–100 beats/min (with a QTc interval* <430 ms in men and ≤450 ms in women) AND blood analysis is within normality | Maintain study medication dose |

*This criterion was changed by an amendment to QTc >480 ms for both sexes.

Abbreviations as in Tables 1 and 2.
or median (interquartile range), depending on normality (assessed using the Shapiro-Wilk test). Efficacy measures will be calculated on the ITT and PP analyses. The treatment effect on the primary outcome measure will be analyzed using a linear regression model that includes the baseline value, treatment, baseline × treatment interaction, center, and center × treatment interaction. Interaction terms will be removed if the Wald test is not statistically significant (p > 0.10). The efficacy analyses done on the PP set will be considered confirmatory (main analyses). For the ITT population, in those cases with missing final values of the primary outcome measure, these values will be replaced by the baseline data (last observation carried forward).

The following exploratory analyses of the primary outcome measure will be contemplated: LVEF (LVEF <40% vs. ≥40%); and the maximum tolerated dose (mg/d) subgroups and their interaction with treatment. Secondary outcome measures will be analyzed similarly using linear models for quantitative normal variables or generalized models for quantitative non-normal variables or logistic regression model that includes the baseline value, treatment, baseline × treatment interaction, center, and center × treatment interaction. The analysis of the secondary variables will be carried out using the available information, without missing imputation techniques.

A descriptive analysis will be performed to evaluate safety of the treatment using the safety population. No interim analyses are planned. A 2-tailed p value < 0.05 will be considered statistically significant, unless otherwise specified.

**DESCRIPTION OF THE STUDY PROCEDURES AND EXAMINATIONS. Right heart catheterization.** The procedure will be performed using a Swan-Ganz catheter introduced via the internal jugular vein using a standard methodology. Hemodynamic measurements will include right atrial pressure, systolic, diastolic, and mean PAP, as well as pulmonary capillary wedge pressure at end-expiration. Zero level will be set at the level of the anterior axillary line while lying flat; 2 different mean PAP values will be registered over a 5- to 10-min period, and the average calculated. Cardiac output will be quantified by the thermodilution method (5 measurements, average of 3, excluding the highest and lowest values).

**6-min walking test.** Tests will be carried out following the recommendations of the American and European Respiratory Societies (40). Patients will be instructed to walk along a 30-m long corridor and to walk as far as possible for 6 min. Tests will be supervised by a nurse, and heart rate and oxygen saturation will be recorded.

**Quality of life questionnaire.** The Kansas City Cardiomyopathy Questionnaire, which has been validated in the Spanish population, will be used to assess quality of life.

**Physical examination.** Blood pressure will be measured at every visit using an automated sphygmomanometer in a quiet room. Three measurements will be performed over a 10-min period, and the average between the second and third measurement will be registered. Heart rate and oxygen saturation (using a pulse oximeter) will be also entered into the electronic case report form. Patients will be weighed on every study visit, and functional class (following the NYHA classification), as well as HF signs and symptoms will be recorded.

**Blood samples.** A venous sample will be obtained at visits 0, 3, 4, 5, 6, and 8 and tested for complete blood count, serum glucose, as well as renal and liver function. NT-proBNP measurement will also be performed at visits 0 and 8.

**ECG.** Heart rhythm, heart rate, QT, and QTc interval will be measured.

**Echocardiography.** Studies will be performed according to standard American Society of Echocardiography and European Association of Cardiovascular Imaging guidelines, with special dedication to the evaluation of RV performance (41,42). The acquisition protocol is standardized across all centers, and studies are digitally stored for dedicated offline analysis at the Imaging Core Laboratory located at Centro Nacional de Investigaciones Cardiovasculares. Left ventricular (LV) volumes and ejection fractions will be measured using the modified Simpson rule (biplane method). Left and right atrial areas will be obtained at ventricular end-systole, excluding the pulmonary vein confluence and the left atrial appendage. RV dimensions, end-systolic and end-diastolic RV areas, and the tricuspid annular plane systolic excursion will be measured from a RV-focused apical 4-chamber view. Fractional area change will be calculated as the difference in RV end-diastolic area and RV end-systolic area divided by the RV end-systolic area. RV index of myocardial performance (Tei index) and the tricuspid lateral annular systolic velocity wave (S’) will be measured using Doppler tissue imaging velocity of the lateral tricuspid annulus. Valvular disease and diastolic function will be graded according to the American Society of Echocardiography and European Association of Cardiovascular Imaging standards (43–45). A 3-beat 2-dimensional digital clip of a RV-focused, 4-chamber view will be acquired for quantification of RV strain by speckle tracking echocardiography (Figure 2A). All analyses will be performed using the
EchoPAC v112 (GE Healthcare, Chicago, Illinois) or Xcelera R4.1 and QLAB 10.2 (Philips, Amsterdam, the Netherlands) software.

**CMR.** CMR studies will be performed with either 1.5- or 3.0-T magnets, using dedicated surface coils for cardiac studies and retrospective electrocardiographic gating. The acquisition protocol is standardized across all centers, and all studies are digitally stored for dedicated offline analysis at the Imaging Core Laboratory located at Centro Nacional de Investigaciones Cardiovasculares (CNIC) (Madrid, Spain).

The CMR examination includes the following sequences. A steady-state free precession cine sequence is used to acquire 15 contiguous short-axis slices covering both ventricles from base to apex and reconstructed into 25 cardiac phases each for the evaluation of biventricular volumes and function. Two-dimensional flow imaging (phase contrast) is performed perpendicular to the main pulmonary artery with a velocity-encoded gradient echo sequence and an upper velocity limit of 100 cm/s (with further increases for any signal aliasing). For this purpose, 2 double-oblique orthogonal views oriented along the main axis of the pulmonary artery trunk are acquired with a standard steady-state free precession cine sequence and used as the reference to prescribe a plane truly perpendicular to the main pulmonary artery for the acquisition of phase contrast images. Care is taken to ensure that the imaging plane remains between the pulmonary valve and pulmonary artery bifurcation throughout the cardiac cycle. In addition, aortic velocity and LV cardiac output are systematically measured (46). For delayed gadolinium enhancement imaging, patients receive an intravenous bolus of 0.125 mmol/kg of gadolinium followed by 20 ml of saline. After 5 to 10 min, contiguous short-axis views matching the cine images are acquired using a phase-sensitive inversion–recovery fast gradient echo sequence for the evaluation of delayed gadolinium enhancement. Typical breath-hold times range from 8 to 14 s. In patients with an inability to perform such breath-holds, delayed gadolinium enhancement images are repeated with a single-shot inversion–recovery steady-state free precession sequence during free breathing. T1 mapping sequences will be acquired just before contrast administration and 15 min after contrast administration in a short-axis view at the level of the papillary muscles for estimation of the extracellular volume fraction.

**CMR analysis.** Cine, 2-dimensional flow imaging and delayed enhancement will be analyzed using specialized software (IntelliSpacePortal v9.0, Philips) by blinded investigators in the Imaging Core Laboratory at CNIC. On cine images, end-diastolic and end-systolic frames will be selected based on visual assessment of largest and smallest LV volumes, respectively, and on the opening or closing of atrioventricular and semilunar valves. Biventricular endocardial contours will be manually traced in end-diastole and end-systole, and Simpson’s method will be used to automatically calculate end-diastolic volumes, end-systolic volumes, and ejection fractions. RV trabeculations will be adjudicated to the blood pool (Figures 2B and 2C). Similarly, the inner contours of the main pulmonary artery cross section will be outlined in each cardiac phase. Through integration of pulmonary artery areas and flow, the following parameters will be quantified: peak velocity; average velocity during the complete cardiac cycle; minimum and maximum areas; and pulmonary artery net forward volume. RV-arterial coupling (the ratio of pulmonary artery effective elastance to RV maximal end-systolic elastance) will be estimated as (end-systolic volume/stroke volume) (47). Regions of interest will be drawn on T1 maps in the myocardial anterior and inferior RV insertion points, interventricular septum, LV lateral wall and LV cavity blood pool before and after contrast administration for estimation of the extracellular volume, as previously described (Figures 2D and 2E) (48).

**CCT.** CCT studies are performed using at least a 64-slice scanner with retrospective electrocardiographic gating mode acquisition (ideally with modulation dose). CCT images are acquired from the level of the aortic arch to the dome of the diaphragm in a breath-hold mode. Nonionic iodinated contrast agent (approximately 80 ml) is injected at a rate of 5 ml/s with a power injector, followed by a saline 40 ml flush. A bolus tracking technique is used to trigger image acquisition once attenuation in a region of interest placed in the descending aorta has reached a pre-set threshold of 100 HU.

Axial images are reconstructed with an image matrix of $512 \times 512$ pixels and a slice thickness of 1.25 mm from 0% to 90% of the cardiac cycle to calculate RV function. Post-processing of CCT images will be performed on a dedicated workstation with specific RV function quantification package (IntelliSpacePortal v9.0, Philips) (Figures 2F and 2G).

**DURATION OF THE STUDY.** The SPHERE-HF clinical trial started recruitment in June 2017, and it is expected that the inclusion phase will end by June 2020. As of December 18, 2019, a total of 61 patients have been included in the study.
DISCUSSION

PH associated with HF has repeatedly been shown to decrease survival, particularly in patients with CpcPH (6, 29). Although IpcPH is entirely reversible when pulmonary arterial wedge pressure is normalized by medical or surgical interventions, this is not the case for CpcPH, due to associated pulmonary arterial remodeling. The latter correlates with pulmonary vascular gradients and increased PVR (49). Approximately 12% to 14% of patients with PH and HF present with CpcPH, a prevalence similarly distributed in reduced or preserved HF (6). As underlined by Vanderpool and Naeije (50), knowledge of the specific phenotype of CpcPH is of great importance to the design of future trials of targeted therapies for PH and HF.

Several randomized controlled trials tested the effect of pulmonary vasodilators in HF but few of them focused in patients with confirmed PH and only a minority of studies focused on CpcPH. Studies that evaluated the effect of prostanoids (epoprostenol) (10) and endothelin-1 receptor antagonists (bosentan) (11, 51) in patients with advanced HF and severe LV systolic dysfunction were terminated early due to an increased rate of adverse events in the investigational drug group compared with the control group. In the recent MELODY (Macitentan in Pulmonary Hypertension due to Left Ventricular Dysfunction) trial (12), which randomized 63 patients with CpcPH to macitentan or placebo, there were no differences in the main endpoint (composite of fluid retention or worsening in NYHA functional class) between groups. Phosphodiesterase-type 5 inhibitors also failed to show a consistent effect in PH secondary to HF (13–17). Moreover, in the recent Spanish multicenter SIOVAC (Sildenafil for Improving Outcomes After Valvular Correction) trial that included 200 patients with residual PH after surgically treated valvular heart disease, sildenafil was associated with worse outcomes than placebo (17). Regarding soluble guanylate cyclase stimulators (riociguat), neutral findings were reported in PH secondary to HF with preserved and reduced LVEFs (18, 19). Several limitations of the previous trials should be noted: 1) in most of them, the target population was not selected based on the pulmonary hemodynamic status (confirmed diagnosis of PH) but included the full spectrum of patients with LHD, and only a minority were targeted at patients with CpcPH; 2) most studies were single center, which limited the extrapolation of results; 3) few studies were conducted in patients with CpcPH; and 4) most lacked a comprehensive evaluation of hemodynamics, exercise capacity, and imaging of the RV. Based on this evidence, targeted treatment using pulmonary arterial hypertension-approved therapies on PH secondary to LHD is discouraged by the current PH guidelines (8).

The SPHERE-HF trial addresses a new approach to treat PH secondary to HF and overcomes most of the previously mentioned limitations. SPHERE-HF is based on solid large animal experimentation data (30). SPHERE-HF is a multicenter randomized controlled trial that targets patients with LHD and confirmed PH with an established pre-capillary component (PVR >3 Wood units and/or diastolic gradient ≥7 mm Hg or transpulmonary gradient ≥12 mmHg, criteria based on PH guidelines valid at the time of the trial design). In this way, SPHERE-HF targets those patients who are most likely to develop pulmonary vascular remodeling and impaired RV function, and therefore, may potentially benefit most from β3AR stimulation. As opposed to other trials, the primary outcome measure in SPHERE-HF is the change in PVR rather than mean PAP. We consider PVR a more robust endpoint because it recapitulates pulmonary pressures and the degree of RV dysfunction and has demonstrated prognostic relevance in PH (52), and particularly in PH secondary to LHD (3, 8, 9, 53–55). In addition, cardiac imaging is a critical part of the SPHERE-HF clinical trial. The effect of mirabegron on RV performance will be evaluated using state-of-the art imaging techniques (CMR, CCT, and echocardiography) and several markers of subclinical myocardial disease (e.g., speckle tracking–derived strain and extracellular volume quantified by T1-mapping CMR) will be assessed to quantify subtle changes in RV function under treatment. Finally, despite the limitations derived from the sample size, important patient-centric outcomes (e.g., functional class or quality of life) have been prespecified as secondary outcomes of the current SPHERE-HF.

STUDY LIMITATIONS. First, the trial focuses in patients with CpcPH by including patients with reduced or preserved LVEF based on a common hemodynamic and biological phenotype (56). Because we recognize that it may include some heterogeneity, a pre-specified subanalysis regarding LVEF (<40% vs. ≥40%) will be considered. Second, this is a proof-of-concept trial with a hemodynamic
endpoint; if positive, a Phase III trial with clinical patient-center endpoints will be performed. The dose selection for this study was based on previously reported safety data, and particularly, cardiovascular safety for mirabegron up to 200 mg/day has been established (31–33,38).

CONCLUSIONS

SPHERE-HF will test the hypothesis that in patients with CpcPH secondary to HF, therapy with a β3AR agonist improves pulmonary hemodynamics, RV performance, and functional status with a good safety profile.

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**KEY WORDS** adrenoceptors, imaging, pulmonary hypertension, treatment
In Situ Immune Profiling of Heart Transplant Biopsies Improves Diagnostic Accuracy and Rejection Risk Stratification

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**HIGHLIGHTS**

- Quantitative multiplexed immune-phenotyping of cardiac allograft biopsies provides novel diagnostic and prognostic information about allograft health.
- Reduced proportions of cells expressing PD-L1 and FoxP3 are associated with clinical evidence of serious allograft injury, even when conventional histologic analysis provides falsely reassuring histologic rejection grades.
- The proportions of PD-L1- and FoxP3-expressing cells are dynamic within cardiac allografts, and reduced levels can precede future rejection.
Recognizing that guideline-directed histologic grading of endomyocardial biopsy tissue samples for rejection surveillance has limited diagnostic accuracy, quantitative, in situ characterization was performed of several important immune cell types in a retrospective cohort of clinical endomyocardial tissue samples. Differences between cases were identified and were grouped by histologic grade versus clinical rejection trajectory, with significantly increased programmed death ligand 1+ , forkhead box P3+ , and cluster of differentiation 68+ cells suppressed in clinically evident rejection, especially cases with marked clinical-histologic discordance. Programmed death ligand 1+ , forkhead box P3+ , and cluster of differentiation 68+ cell proportions are also significantly higher in “never-rejection” when compared with “future-rejection.” These findings suggest that in situ immune modulators regulate the severity of cardiac allograft rejection. (J Am Coll Cardiol Basic Trans Science 2020;5:328–40) © 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/).

Cardiac allograft rejection (CAR) is a serious concern among transplant recipients, occurring in roughly one-third of patients in the first year post-transplantation and conferring an increased risk of short- and long-term graft failure. Because of the high morbidity and mortality associated with CAR, the International Society for Heart and Lung Transplantation (ISHLT) has recommended regular surveillance endomyocardial biopsy (EMB) with standardized histologic grading for CAR since 1990.

Unfortunately, the guideline-recommended ISHLT grading scheme suffers from a lack of prognostic accuracy, correlating poorly with the clinical trajectory of a current rejection event and lacking the ability to stratify patients by future CAR risk (1–4). A failure of histologic grade to correlate with a patient’s imminent clinical trajectory can cause false reassurance in some cases and false alarm in others, with resultant treatment delays or overtreatment posing serious risks to patients. Because conventional ISHLT histologic grade never provides longer-term risk stratification, surveillance testing and immunosuppression-weaning protocols cannot be tailored to individual CAR risk. As a result, low-risk patients undergo more EMB procedures and more aggressive immunosuppression than are needed with attendant risks of infection and procedural complications. On the other hand, high-risk patients undergo premature weaning of immunosuppression, resulting in potentially avoidable rejection episodes. Both of these scenarios represent opportunities for better outcomes through improved diagnostic accuracy and greater prognostic insight.

The traditional approach to morphologic CAR detection involves the identification and rough quantification of basophilic immune cells on hematoxylin and eosin-stained slides. This approach provides few insights into the specific types (and therefore, functions) of the immune cells present within EMB tissue. Expert panels have explicitly acknowledged the need for “further characterization of the nature of the
Inflammatory infiltrate” to extract additional clinically relevant information from EMB samples (5). Despite this 15-year-old call to action, there has been very limited application of tissue-level immune phenotyping in human heart transplant tissues. This stands in contrast to oncologic and rheumatologic medicine, where deeper phenotyping of immune populations and immune effector pathways have resulted in improved risk stratification and better targeting of therapeutic strategies (6–12).

In this paper, we report novel proof-of-concept studies applying a state-of-the-art, fully quantitative, multiplex immunofluorescence (QmIF) methodology on archived clinical EMB tissue samples, with a focus on uncovering immune phenotypes that can improve the diagnostic and prognostic performance of histologic analysis for CAR. Using a custom panel of IF markers focused on cell-mediated immune responses and modified from commercially available panels validated in multiple oncologic publications (13–17), we performed in situ identification and quantification of cluster of differentiation 3 (CD3), CD8, CD68, forkhead box P3 (FoxP3), and programmed death ligand 1 (PD-L1). Due to limited research in human heart transplant tissues, the rationale for the markers selected for this cell-mediated rejection panel comes predominantly from animal models and renal transplantation (18–33). T cells are implicated in most cases of CAR, but are in actuality a heterogeneous group of cells capable of exerting pro-inflammatory and anti-inflammatory effects under different conditions. As a result, quantification of the pan-T-cell marker CD3 in addition to markers of T-cell subtypes and effectors was the objective of this proof-of-concept work. Within this context, our novel application of QmIF demonstrated striking new diagnostic and prognostic insights beyond the standard ISHLT grade, supporting potential benefits from further application and refinement of the immune-phenotyping panel.

**METHODS**

**COHORT CONSIDERATIONS.** The study cohort was selected from the transplant records at the Hospital of the University of Pennsylvania and consisted of biopsy events that occurred between 2007 and 2013. Cases were manually selected to allow for exploration of the potential diagnostic and prognostic implications of performing immune phenotyping within EMB tissue. Specifically, the cohort of 46 transplant EMB described in this paper was selected to permit assessments of how immune cell populations differ between tissues with low versus high ISHLT grades, between tissues corresponding to clinically silent versus clinically evident rejection trajectories, and between tissues from patients who will go on to experience serious rejection (future rejection) versus those who will not (never rejection). The retrospective chart review and analysis of archived tissue specimens employed in this research were approved by the Institutional Review Board at the University of Pennsylvania.

For these retrospective cases, the ISHLT histologic grade assigned by the attending pathologist at the time of the EMB procedure was used as the reference standard for rejection diagnosis. These grades were further simplified by assigning a binary histologic grade label: “low-grade” rejection was defined as ISHLT 2004 consensus criteria histologic grades 0R or 1R and “high-grade” rejection was defined as ISHLT histologic grade 2R or 3R. This grouping typically defines the distinction used to determine whether augmented immunosuppressive therapy is prescribed (34).

For the same 46 EMB events, the distinction between “clinically silent” and “clinically evident” rejection was made based on a set of major and minor criteria to determine whether allograft injury was present. Clinical metadata from within 7 days of each EMB event were collected to allow for determination of clinical trajectory. These data were derived from electronic health record–documented symptoms, physical exam findings, lab results, echocardiographic parameters, electrocardiogram findings, and invasive hemodynamic data. The major criteria in Table 1 for differentiating clinically evident from clinically silent rejection trajectories are based on definitions of “hemodynamic compromise” in previous prospective investigations of allograft rejection (35–37) and provide high specificity for clinically significant rejection. The minor criteria in Table 1 permit identification of clinically significant cases with greater sensitivity. By design, a subset of each clinical trajectory category represented an EMB with clinical-histologic discordance (low-histologic grade meeting criteria for clinically evident rejection, or high-histologic grade with none of the criteria met for clinically evident rejection). All determinations of clinically evident versus clinically silent rejection were completed prior to the performance of immunostaining.

Finally, the 19 EMB in this cohort that were categorized as low ISHLT grade and clinically silent were further classified by the patient-level incidence of future serious CAR events. These EMB were assigned a binary label as either “future rejection” or “never rejection,” based on whether a serious CAR event occurred within the first 3 years post-transplantation. These cases allow for assessment of whether...
TABLE 1  Criteria for Determining Clinically Evident Rejection

| Major criteria                                                                 | Minor criteria                                                                 |
|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Cardiac index $\geq 2.0$ and use of inotropes                                   | Absolute decrease in LVEF of $\geq 20\%$                                        |
| Admission to hospital for rejection treatment, along with 1 major or 2 minor criteria: | Absolute decrease in LVEF of $\geq 10\%$ and to a level of $\leq 50\%$            |
| Cardiac index $\geq 2.3$, provided this represents a $\geq 20\%$ decrease in cardiac index from baseline | New arrhythmia—atrial fibrillation, flutter, or ventricular arrhythmia           |
| Right atrial pressure $>10$ mm Hg or pulmonary capillary wedge pressure $>18$ mm Hg provided this represents a $\geq 40\%$ increase from baseline | New low voltage ECG not due to pericardial effusion or pulmonary disease         |
| Absolute decrease in LVEF of $\geq 10\%$ and to a level of $\leq 50\%$          | Cardiac troponin elevated $\geq 3\times$ the upper limit of normal and $\leq 3\times$ the patient's baseline, not due to coronary artery disease/graft vasculopathy |
| Documented diagnosis of increased LV wall thickness and an LV wall thickness increase of $\leq 2$ mm from baseline value | Documented new or worsened right ventricular dysfunction by echo                 |
| Documented new or worsened right ventricular dysfunction by echo               | Documented clinical signs or symptoms of rejection or heart failure:             |
| Signs — new gallop, new low pulse volumes, new rales.                          | Symptoms — new or worsened dyspnea, orthopnea, exercise intolerance documented by provider as likely due to cardiac cause. |

EGC = electrocardiogram; LV = left ventricular; LVEF = left ventricular ejection fraction.

TABLE 1  Criteria for Determining Clinically Evident Rejection

| Admission to hospital for rejection treatment, along with 1 major or 2 minor criteria: |
|-------------------------------------------------------------------------------------|
| Major criteria                                                                 |
| Cardiac index $\geq 2.0$ and use of inotropes                                       |
| Absolute decrease in LVEF of $\geq 20\%$                                           |
| Minor criteria                                                                 |
| Absolute decrease in LVEF of $\geq 10\%$ and to a level of $\leq 50\%$             |
| Documented new or worsened right ventricular dysfunction by echo                   |
| Documented clinical signs or symptoms of rejection or heart failure:               |
| Signs — new gallop, new low pulse volumes, new rales.                              |
| Symptoms — new or worsened dyspnea, orthopnea, exercise intolerance documented by provider as likely due to cardiac cause. |

meaningful differences occur in IF markers in advance of a serious CAR event.

RETROSPECTIVE SAMPLE ACQUISITION AND PREPARATION. All EMB tissues analyzed for this study had been sampled using a standard percutaneous method, fixed in 4% paraformaldehyde, and embedded in paraffin wax as per usual post-transplantation clinical care and pathology laboratory workflows at the Hospital of the University of Pennsylvania. Five, 4-μm thick serial sections were cut from formalin-fixed paraffin-embedded blocks and mounted onto positively charged glass microscope slides (48382-119; VWR Corp., Radnor, Pennsylvania) used for immunohistochemistry, with 1 section per slide. All cut, unstained tissue sections were stored in a nitrogen chamber to minimize oxidation and degradation of tissue epitopes. In addition to EMB slides, 2 slides from native (nontransplant) heart tissue obtained from cadaveric organ donors and 1 slide from human lymph node tissue were used as “negative” and “positive” staining controls, respectively.

TARGET SELECTION FOR MULTIPLEX IMMUNOFLUORESCENCE. The cytotoxic T-cell marker CD8 was selected due to recognition that this cell population is a primary effector of myocyte injury during cellular rejection (18-20). Regulatory T-cell transcription factor FoxP3 was selected due to the immune-modulatory, anti-inflammatory effects these interleukin-10 and transforming growth factor-β secreting cells are thought to exert in renal allografts (21-23) and animal models of heart transplantation (24,25). The monocyte lineage marker CD68 is used in the diagnosis of antibody-mediated rejection, but it has also been implicated in cellular rejection, albeit with conflicting results on the effects these cells exert (26,27). Finally, PD-L1 is 1 component of the PD-L1/PD1 immune “checkpoint” molecules that interact to suppress cytotoxic actions of activated T cells. Though checkpoint inhibitors used in cancer immunotherapy have been implicated in myocarditis, a role for checkpoint molecules in human heart transplantation has included only case reports of severe rejection following oncologic PD1/PD-L1 inhibitor treatment (28-30). However, the checkpoint pathway has been recognized for its role in abrogating T-cell responses and promoting tolerance in animal models of organ transplantation (31-33).

QUANTITATIVE MULTIPLEX IMMUNOFLUORESCENCE METHODS. All study tissue samples were assigned deidentified study identifications and sent to Akoya Biosciences (Hopkinton, Massachusetts) for multiplex staining, slide digitization, and per-slide image quantification. The scientific team performing staining and image quantification was blinded to patient outcomes. Details of deparaffinization, reagent preparation, and automated staining workflows are described in the Supplemental Appendix. Details on antibodies utilized for multiplex staining are summarized in Supplemental Table 1.

Whole slide multispectral image acquisition. High-throughput whole slide multispectral (MOTIF) image scans were acquired on the Vectra Polaris (Akoya Biosciences) at 20× using narrow, multiband filters. Exposure times were set to avoid saturation.

Image quality control and annotation selection. Following image acquisition, scans were imported into Phenochart (Akoya Biosciences) to assess the image quality. Up to 5 1×1 regions of interest were
annotated for each sample to conduct quantitative image analysis. Some samples were unable to yield 5 regions of interest due to confounding factors, such as tissue folds, small tissue biopsy size, or large areas without myocardium (e.g., vessels, fibrosis, fat).

**Quantitative multispectral image analysis.** A spectral library was created from the stained library slides, and the autofluorescence spectrum was isolated using the autofluorescence slide. All annotated regions of interest were imported into a new project in inForm Tissue Finder (Akoya Biosciences) and were spectrally unmixed using the generated spectral library. Of note, the image analyst was blinded to the slide cohort categories to avoid bias.

**Staining quality control.** Individual fluorophore signal intensities, in normalized counts, were assessed on the multiplex imagery following spectral unmixing to confirm proper staining intensity levels and absence of residual cross talk.

**InForm analysis workflow.** The Trainable Tissue Segmentation module was used to automatically segment all samples into total cardiac tissue and background based on hand-drawn training regions. Cell segmentation was conducted using the Adaptive Cell Segmentation, which uses algorithms to account for variations in staining and background levels within and across images to identify cellular compartments from multiple image planes. The algorithm first identifies the cell nuclei (via 4′,6-diamidino-2-phenylindole staining), followed by the cell membrane and cytoplasm for each, and has further settings for cell splitting and staining quality. The Phenotyping module, which utilizes a user-trainable random forest classifier, learned and phenotyped all markers, except for PD-L1, in which the staining intensity was scored per cell on a 0 to 3+ scale using thresholding to enable histology score (H-score) calculations. All cell segmentation and scoring data and imagery were exported for further analysis.

**Statistical methods.** Raw cell count (and PD-L1 intensity) data generated by inForm quantitative image analysis were sent to the University of Pennsylvania for further analysis. Cases were analyzed by pre-specified groups as described in “cohort considerations.” For CD3+, CD8+, CD68+, and PD-L1 cell count data, values were normalized by total cell counts to account for differences in tissue size and myocardial area. FoxP3+ cells were normalized by the total T-cell (CD3+) cell count. Groupwise chi-squared testing was performed on the normalized cell count data. Comparisons included cases grouped by high versus low ISHLT histologic grade, by clinically evident versus clinically silent rejection determination, and by patient-level future-rejection versus never-rejection label. Cases were also analyzed by
Of the 19 cases with low ISHLT grades and clinically silent rejection, 6 would go on to have a future rejection within ~1 year and 13 would never reject over the next 3 years. Of the 4 discordant high ISHLT grade EMB associated with a clinically silent course, none manifested evidence of clinically evident allograft rejection in the subsequent 3 months.

**Tissue Immunophenotyping Results.** For the 33 EMB cases that completed QmIF staining, there were a total of 191,331 cells in the regions that underwent quantitative analysis, with CD3+ cells representing the most common immune cell type, as summarized in Table 2.

Table 3 shows the study results grouped by ISHLT grade (high vs. low) and by clinical rejection syndrome (clinically silent vs. clinically evident). High-grade EMB have significantly higher proportions of CD3+ and CD8+ cells than do low-grade EMB (p < 0.001). Grade-agnostic grouping by rejection syndrome severity demonstrates analogous results, with serious, clinically evident rejection events having higher proportions of CD3+ and CD8+ cells than clinically silent rejection events (p < 0.001). Figure 2A provides a compelling visual demonstration of how these conventional T-cell markers correlate predominantly with grade classification and provide little help for discriminating between cases with versus without serious clinical rejection syndromes.

High-grade EMB also have significantly higher proportions of macrophage marker CD68 when compared with low-grade EMB (p < 0.001), but unlike the traditional T-cell markers CD3 and CD8, the opposite relationship is seen when cases are grouped by the severity of the clinical rejection syndrome. Clinically silent rejection events have a significantly higher proportion of CD68+ cells than do clinically evident rejection events (p < 0.001). An examination of the results from EMB with clinical-histologic discordance provides the explanation for these opposing results (Table 4, Figure 2B), with discordant high-grade cases manifesting by far the highest proportion of CD68+ cells (5.45%; p < 0.001) whereas discordant low-grade cases manifest the lowest (0.14%; p < 0.001).

Cells expressing the regulatory T-cell marker FoxP3 are twice as abundant in clinically silent rejection events, as compared with clinically evident rejection both within the low-histologic grade and the high-histologic grade cohort (p < 0.001 for both). Even more striking, PD-L1+ cells were more than 4-fold more abundant in clinically silent cases compared with clinically evident cases, regardless of ISHLT grade. Table 4 and Figure 2B highlight the strong, grade-independent correlation of FoxP3 and

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**RESULTS**

A total of 46 EMB episodes plus 3 additional control tissues were selected for this analysis, and their characteristics are detailed in Supplemental Table 2. In total, 36 of 49 (74%) of the formalin-fixed paraffin-embedded tissue slides (including the 3 non-EMB control samples) submitted for QmIF underwent successful staining, quality control assessment, and comprehensive quantitative analysis. Of the 33 transplantation EMB cases that completed study staining and analysis (see Figure 1), 22 had low ISHLT grades (1R or 0R) and 11 had high ISHLT grades (2R and 3R). Of the cases with low ISHLT grades, 19 had clinically silent rejection and 3 had clinically evident rejection. Of the cases with high ISHLT grades, 7 had clinically evident rejection and 4 had clinically silent rejection. None of the instances of clinically evident rejections corresponding to low ISHLT grades met ISHLT criteria for antibody-mediated rejection (39).

Specifically, none had new donor-specific antibodies or significant C4d deposition by clinical IF staining.
PD-L1, with similarly high proportions found either in low- or high-grade clinically silent EMB and similarly low proportions found in either low- or high-grade clinically evident EMB.

The per-patient PD-L1 H-score, a composite measure incorporating both PD-L1 staining prevalence and signal intensity, is strongly associated with clinical rejection syndrome but not with histologic grade (Tables 3 and 4, Figure 3B). Patients with clinically silent rejection syndromes, whether the EMB received a high or low grade, manifest PD-L1 H-scores that are at least 4 × higher than those seen for clinically evident rejection events of either grade class. Illustrative hematoxylin and eosin- and QmIF-stained digital slides for high and low ISHLT grades with both concordant and discordant clinical rejection syndromes are shown in Figure 3.

**Immunophenotyping future- and never-rejection cases.** To assess whether immune phenotypes differ between patients who will suffer important rejection events in the future and those who will not, we analyzed the subset of cases in which serial, concordant, low-grade EMB were available for individual patients. These patients were assigned a binary label as either future rejection or never rejection. For the pre-specified future-rejection subgroup, 6 EMB preceding a high-grade, clinically evident rejection event within 6 weeks before clinically evident rejection, there is a dramatic and statistically significant drop-off to extremely low levels for FoxP3+ and CD68+ and the PD-L1+ cells (Table 5, Figure 4).

**DISCUSSION**

These proof-of-concept studies applying QmIF technology within the cardiac transplantation population provide compelling evidence for the feasibility and utility of immune phenotyping to improve the diagnostic and prognostic value of allograft EMB specimens. First, using archived formalin-fixed, paraffin-embedded tissue blocks—some more than 10

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**TABLE 2** QmIF Total Cell Count Results

| Cell Type | Cell Count | % of Total |
|-----------|------------|------------|
| CD8+      | 8,646      | 4.52       |
| CD3+      | 15,571     | 8.14       |
| CD68+     | 2,814      | 1.47       |
| FoxP3+    | 382        | 0.20       |
| PD-L1+    | 8,932      | 4.67       |
| DAPI (total cells) | 191,331 | 100.00 |

CD3+ = cluster of differentiation 3+; DAPI = 4′,6-diamidino-2-phenylindole; FoxP3+ = forkhead box P3+; PD-L1+ = programmed death ligand 1; QmIF = quantitative multiplex immunofluorescence.

**TABLE 3** QmIF Results by Immune Cell Type With Cases Grouped by Clinical Rejection Trajectory and Conventional Histologic Grade

| Marker | Low Grade | High Grade | p Value | Clinically Silent | Clinically Evident | p Value |
|--------|-----------|------------|---------|------------------|-------------------|---------|
| CD8+   | 1.16 ± 0.04 (1,049/90,731) | 7.55 ± 0.08 (7,597/100,600) | <0.001 | 2.93 ± 0.05 (2,754/94,098) | 6.06 ± 0.07 (5,892/97,233) | <0.001 |
| CD3+   | 3.3 ± 0.06 (2,995/90,731) | 12.5 ± 0.1 (12,576/100,600) | <0.001 | 5.6 ± 0.07 (5,266/94,098) | 10.6 ± 0.09 (10,305/97,233) | <0.001 |
| CD68+  | 0.83 ± 0.03 (757/90,731) | 2.04 ± 0.04 (2,057/100,600) | <0.001 | 1.91 ± 0.04 (1,797/94,098) | 1.05 ± 0.03 (1,017/97,233) | <0.001 |
| FoxP3+ | 3.21 ± 0.32 (96/2,995) | 2.27 ± 0.13 (286/12,576) | 0.039 | 3.61 ± 0.26 (190/5,266) | 1.86 ± 0.13 (192/10,305) | <0.001 |
| PD-L1+ | 6.48 ± 0.08 (5,876/90,731) | 3.04 ± 0.05 (3,056/100,600) | <0.001 | 7.86 ± 0.09 (7,392/94,098) | 1.58 ± 0.04 (1,540/97,233) | <0.001 |

PD-L1 H-score per EMB

|        |        |        |        |        |        |
|--------|--------|--------|--------|--------|--------|
| 3 (1-16) | 4 (1-11) | 0.88 | 5 (2-16) | 1 (0-2) | 0.018 |

Values are percentage of total cells ± SE (n/N) or median (interquartile range). *Percentage of CD3+ cells ± SE.

EMB = endomyocardial biopsy; H-score = histology score; other abbreviations as Table 1.
FIGURE 2  QmIF Results Grouped by ISHLT Grade and Clinical Status

(A) Quantitative multiplex immunofluorescence (QmIF) results for cluster of differentiation $3^+$ (CD3$^+$) and CD8$^+$ cells, grouped by ISHLT grade and subgrouped based on concordance or discordance between ISHLT grade and clinical evidence of rejection (see Table 1). Proportions of CD3$^+$ and CD8$^+$ cells correlate well with ISHLT grade and do not help discriminate between cases classified by clinical rejection trajectory. (B) QmIF results for CD68$^+$, forkhead box P3$^+$ (FoxP3$^+$), and programmed death ligand 1$^+$ (PD-L1$^+$) cells, grouped by ISHLT grade and subgrouped based on concordance or discordance between ISHLT grade and clinical evidence of rejection (Table 1). Within low ISHLT grades, there are significant differences between cases with clinically silent versus evident rejection (CD68$^+$: 0.99% vs. 0.14%; $p < 0.001$; FoxP3$^+$: 3.68% vs. 1.60%; $p < 0.001$; PD-L1$^+$: 7.59 vs. 1.3; $p < 0.001$). Within the high ISHLT grades, there are also significant differences between silent and evident rejection (CD68$^+$: 5.45% vs. 1.23%; $p < 0.001$; FoxP3$^+$: 3.55% vs. 1.88%; $p < 0.001$; PD-L1$^+$: 8.85 vs. 1.64; $p < 0.001$). Overall, these QmIF markers correlate much better with clinical rejection severity than they do with traditional ISHLT grade and may provide additional diagnostic value for assessing rejection on EMB. Error bars = SE. $p < 0.001$ versus clinically evident within the *low histologic grade cohort and †high histologic grade cohort. Abbreviations as in Figure 1.
years old—successful application of Opal multiplex IF staining technology sufficient for advanced quantitative analysis was achieved in the sizable majority of cases. In designing this experiment, we incorporated a “grade-agnostic” framework using the clinically evident and clinically silent labels to classify EMB by the contemporary clinical status of the patient. We hypothesized that QmIF results might suggest mechanisms underlying the frequent discordance between patients’ clinical status and the ISHLT grade given to EMB samples. For conventional T-cell markers CD3 and CD8, the QmIF results largely conformed to prior evidence and accepted pathophysiology and do not significantly help discriminate between patients with and without serious rejection syndromes. High ISHLT grades had markedly higher proportions of CD3+ and CD8+ cells, consistent with the higher overall quantity of basophilic lymphocytes seen in standard hematoxylin and eosin slides with high ISHLT histologic grades. Cases grouped by clinical rejection severity followed a similar pattern, with clinically evident rejection having a higher proportion of these T-cell markers. Because ISHLT grading is primarily predicated on rough quantification of lymphocyte foci (within which CD3+ and CD8+ cells predominate), these findings suggest that misgrading is not the primary reason for clinical-histologic disagreement in our discordant case subgroups.

The proportion of CD68+ cells was also significantly higher in high ISHLT grade rejection, consistent with several prior reports in renal transplantation that have suggested that macrophages represent a major (and occasionally predominant) cell line in some cases of cellular rejection (26,27). In contrast, when cases are grouped by clinical status, the proportion of CD68+ cells is significantly lower in EMB associated with more serious, clinically evident rejection syndromes. This finding is driven almost entirely by the cases with clinical-histologic discordance—clinically silent/high-grade EMB have by far the highest proportion of CD68+ cells with a prevalence that is ~40× higher than that seen in clinically evident/low-grade cases (which had by far the lowest proportion). In these clinically silent/high ISHLT grade cases, inspection of the QmIF slide (Figure 3C4) reveals robust CD68-staining within the dense cellular infiltrates, which stands in stark contrast to the modest CD68 staining within the cellular infiltrate of the discordant high ISHLT grade case (Figure 3D4). Because discordant high ISHLT grade cases have comparable (or greater) numbers of CD3+ and CD8+ cells, this visual observation makes it tempting to speculate that CD68+ cells may be part of an important “late” protective response, mitigating serious injury to an allograft after lymphocytic infiltration occurs.

The proportion of FoxP3+ cells and cells staining strongly for PD-L1 also differ significantly when cases are grouped by clinical trajectory. Clinically silent rejection events, regardless of ISHLT grade, have significantly higher proportions of both of these anti-inflammatory markers than do clinically evident rejection events. These results raise the possibility that the interactions of immune-modulating cell subtypes and pathways may play a role in determining whether an initial allo-immune response becomes a clinically serious one, though this study was notably not designed to demonstrate such causal relationships. It is worthwhile to highlight that the discordant

### Table 4: QmIF Results by Immune Cell Type, With Cases Grouped by Contingency Table Designation Based on Concordance Versus Discordance of Clinical Rejection Severity and ISHLT Histologic Grade

| Marker | Contingency Table Groups | p Values for Intergroup Comparisons |
|--------|--------------------------|-----------------------------------|
|        | C-Low vs. D-Low | C-Low vs. C-High | C-Low vs. D-High | C-High vs. D-High | C-High vs. C-High |
| CD8+   | 1.17 ± 0.04 (871/74,616) | 9.67 ± 0.09 (1,883/19,482) | 1.1 ± 0.03 (178/16,115) | 7.04 ± 0.08 (5,714/81,118) | 0.5 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| CD3+   | 3.09 ± 0.06 (2,309/74,616) | 15.18 ± 0.11 (2,957/19,482) | 4.26 ± 0.07 (686/16,115) | 11.86 ± 0.1 (9,619/81,118) | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| CD68+  | 0.99 ± 0.03 (735/74,616) | 5.45 ± 0.07 (1,062/19,482) | 0.14 ± 0.01 (22/16,115) | 1.23 ± 0.03 (995/81,118) | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| FoxP3+ | 3.68 ± 0.39 (85/2,309) | 3.55 ± 0.34 (105/2,957) | 1.6 ± 0.48 (11/686) | 1.88 ± 0.14 (181/9,619) | <0.001 | <0.001 | 0.80 | 0.60 | 0.001 | <0.001 |
| PD-L1+ | 7.59 ± 0.09 (5,667/74,616) | 8.85 ± 0.09 (1,725/19,482) | 1.3 ± 0.04 (209/16,115) | 1.64 ± 0.04 (1,331/81,118) | <0.001 | <0.001 | 0.002 | 0.001 | <0.001 | <0.001 |
| PD-L1 H-score per EMB | 4 (2-16) | 9 (6-19) | 1 (0-2) | 1 (0-4) | 0.09 | 0.13 | 0.24 | 0.64 | 0.034 | 0.037 |

Values are percentage of total cells ± SE (n/N) or median (interquartile range). *Percentage of CD3+ cells ± SE.

C = concordance; D = discordance; High = high grade; ISHLT = International Society of Heart and Lung Transplantation; Low = low grade; other abbreviations as in Tables 2 and 3.
cases manifest the most extreme proportions of these potentially allograft-protective markers, with the discordant high-grade cases containing the highest proportions and the discordant low-grade cases containing the lowest. Visual inspection of QmIF slides further highlights the potential importance of PD-L1, with a diffuse PD-L1⁺ (green) staining pattern seen throughout the myocardium in clinically silent biopsies (Figures 3A5 and 3C5) that is conspicuously absent in clinically evident rejection cases (Figures 3B5 and 3D5). It is also worth highlighting the particularly dense PD-L1 staining seen within the cellular infiltrate of the discordant high ISHLT grade slide (Figure 3C5), which may suggest an active and dynamic PD-L1 presence at the site of an acute immune response.

Overall, the QmIF results of EMB cases grouped by clinical trajectory support the hypothesis that to accurately assess the threat to allograft function,

| Marker       | Never Rejection | Future Rejection | p Value |
|--------------|-----------------|------------------|---------|
| CD8⁺         | 0.48 ± 0.02 (230/48,245) | 2.43 ± 0.05 (641/26,371) | <0.001 |
| CD3⁺         | 1.19 ± 0.04 (573/48,245) | 6.58 ± 0.08 (1,736/26,371) | <0.001 |
| CD68⁺        | 1.01 ± 0.03 (488/48,245) | 0.94 ± 0.03 (247/26,371) | 0.32    |
| FoxP3⁺*      | 10.82 ± 1.30 (62/573) | 1.32 ± 0.27 (23/1,736) | <0.001 |
| PD-L1⁺       | 10.7 ± 0.1 (5,160/48,245) | 1.92 ± 0.04 (507/26,371) | <0.001 |
| PD-L1 H-score per EMB | 5 (3-16) | 1.5 (0-3) | 0.034 |

Values are percentage of total cells ± SE (n/N) or median (interquartile range). *Percentage of CD3⁺ cells ± SE. Abbreviations as in Tables 2 and 3.
consideration of more than just a rough count of basophilic immune cells as performed in conventional ISHLT grading is required. This hypothesis is reinforced when the concordant low ISHLT grade EMB are differentiated from one another as future-rejection cases versus never-rejection cases, based on whether the patient experiences a serious rejection event in the first 3 years post-transplantation. The immune profiles of never-rejection EMB are defined by particularly high proportions of PD-L1+ and FoxP3+ cells. In contrast, future-rejection EMB as a whole exhibit lower levels of these alloprotective markers, with striking reductions observed by 3 to 6 weeks prior to an upcoming clinically evident rejection. The nearly complete loss of FoxP3+ and PD-L1+ cells in the weeks preceding clinically evident rejection supports both their potential utility as biomarkers for identifying patients at high risk for significant rejection events, as well as the need to further investigate potential alloprotective role of these markers in heart transplantation.

Mechanistically, the interplay between monocyte lineage cells (CD68+), regulatory T cells (FoxP3+), and PD-L1-expressing cells suggested by our results has been the focus of significant biomedical research, though rarely in the context of organ transplantation (40–47). The binding of PD-L1 to PD1 on helper T cells, beyond simple “inactivation” with consequent decreases in inflammatory cytokine production, has been shown to stimulate FoxP3+ expression and effector T-cell differentiation into regulatory T cells (41,42,44,45). Macrophages and regulatory T cells can interact in a synergistic fashion, with cytokines produced by regulatory T cells encouraging macrophages to differentiate into M2 anti-inflammatory macrophages. In turn, M2 macrophages secrete specific cytokines and express PD-L1, both of which can facilitate further helper T-cell differentiation into regulatory T cells. These established mechanisms lend immunologic plausibility to our findings, though confirmation of these mechanisms within allograft tissues is beyond the scope of the present work.

**STUDY LIMITATIONS.** The results we have presented must be considered in the context of the limited sample size both in terms of patients and EMB events, and generalizing these findings should be done with caution. Nevertheless, the high numbers of cells analyzed along with the unbiased computational methods used to analyze them provide reassurance that the phenomena uncovered in this cohort are valid within this cohort. We also recognize that the discordant high ISHLT grade biopsy designation is an area of some uncertainty, because it is standard practice for all high ISHLT grade biopsy events to receive some form of altered immunosuppression regardless of the presence (or absence) of abnormal clinical diagnostic data. Thus, a more serious clinical trajectory could potentially have been averted by early alterations in therapy in these false-positive cases, with no way to know for certain whether the rejection event was destined to remain clinically silent. However, these discordant high ISHLT grade cases met none of the clinical criteria for clinically evident rejection, and our study findings seem to support the existence of different immunobiologies in comparison to concordant high ISHLT grade cases. It is also worth noting that prior case series demonstrating benign clinical courses with certain untreated high ISHLT grade EMB support the theory that there are distinct phenotypes with distinct fates existing within the traditional high ISHLT grade grouping (1). Finally, the relatively high rate of technical failure of the QmIF analysis (26% of EMB) is a concern, but it may reflect our dependence on residual material following routine clinical processing and the 6- to 12-year interval between EMB sampling and QmIF analysis in this experiment.

**FUTURE DIRECTIONS.** Considering future opportunities, it should be noted that the present QmIF
Although there is indeed important additional work to be performed, the present research represents an important step toward precision diagnosis and risk stratification in cardiac transplantation. Our results demonstrate the feasibility and translational potential of the QmIF methodology, while identifying several key, and potentially targetable, mechanisms involved in determining the severity of an alloimmune response.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Clinically, these proof-of-concept studies support the feasibility and clinical benefits of applying immune-phenotyping for characterization of cardiac allograft biopsies. Specifically, the findings that the abundance of regulatory T cells and PD-L1 expression is linked to clinical allograft status suggests that native rejection suppressing processes may contribute to the discordance frequently observed between patients’ clinical trajectory and ISHLT biopsy grade. The findings that suppressed PD-L1 is linked to clinically significant allo-immunity is a relevant extension of recent reports that checkpoint molecule inhibitors can induce severe myocarditis.

TRANSLATIONAL OUTLOOK: From a translational perspective, the implicated allo-protective actions of CD68-expressing macrophages, FoxP3-expressing regulatory T cells, and PD-L1 expression merit both further mechanistic investigation in preclinical animal models and clinical investigation in larger retrospective and prospective cohorts, including a greater emphasis on time-course studies. Enhanced mechanistic clarity will ultimately enable more precise immune-suppressive strategies with superior performance for recipients of solid organ transplants.

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Improving Diagnostic Accuracy and Rejection Risk Stratification

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KEY WORDS

allograft rejection, immune checkpoint molecules, immune regulation, quantitative immunohistochemistry

APPENDIX

For supplemental tables, please see the online version of this paper.
Immune Cell Profiling and Risk Stratification
Cast a Wider Net*

M. Elizabeth H. Hammond, MDa,b,c

Long-term graft failure is the major hurdle in human cardiac transplantation. Advanced immunosuppressive therapies have substantially decreased the risk of acute graft failure but have limited efficacy in preventing graft failure from allograft coronary artery disease or chronic heart failure. In this proof of concept study in this issue of JACC: Basic to Translational Science, Peyster et al. (1) have applied quantitative multiplexed immunofluorescence (QmIF) microscopy for immunophenotyping of mononuclear immune cell types in a selective retrospective cohort of cardiac transplant biopsies. Three-year outcomes based on histologic classification were compared to clinical rejection trajectories by using multiple parameters of clinical worsening of cardiac performance. Immune modulators of CD8-positive cytotoxic T cells (regulatory T-cell transcription factor FoxP3 and programmed death ligand [PD-L1]) and a marker of macrophage lineage (CD68) were chosen as test markers. QmIF identified discrepancies between histologic and clinical predictions of long-term cardiac failure based on those markers. The proportion of PD-L1- and FoxP3-expressing cells were dynamic within cardiac allografts, and reduced levels of these cells predicted future allograft failure better than histologic grading.

The QmIF methodology applied is robust and may hold promise for prognosticating outcomes based on endomyocardial biopsy. The study design could be a useful model to understand the failings of histologic grading in predicting outcome. As a proof of concept study, the statistical design was adequate. Unfortunately, there was a high failure rate (26%) of application of the method to retrospective biopsies which may have produced a serious sampling error. No table was provided to rule out this potential source of error based on comparison of the included and excluded cases.

For future comparisons, it will be important to use the most recent validated International Society of Heart Lung Transplantation (ISHLT) histologic grading schema (established in 2013), so that antibody-mediated rejection (AMR) can be carefully evaluated in such investigations (2). The current study was based on the ISHLT schema from 2005, in which AMR was only loosely defined. Studies of long-term outcomes of cardiac allograft recipients have shown that AMR, detected by routine surveillance biopsies, even when clinically silent, strongly predicted adverse long-term outcomes for the recipients (3). The most recent schema emphasizes the histologic features of AMR including endothelial activation, adherence of macrophages to capillary walls, and the presence of interstitial edema. In fact, recent publications have emphasized the value of histologic features as diagnostic criteria for AMR. A validation study showed that chronic cardiovascular mortality after cardiac transplant was predicted equally by either immunopathologic or histologic features of...
AMR as well as by the simultaneous presence of both features on surveillance biopsies (4).

Careful histologic assessment correlated with the QmIF studies would enhance the diagnostic value. For example, the high-grade lesions illustrated (1) are histologically different and may be from very different post-transplantation time points. The infiltrate in the clinically silent high-grade rejection (case C1) consisted mostly of activated lymphocytes and macrophages in what appeared to be an early acute cellular rejection (1). The second case (D1), from an endomyocardial biopsy with significant longstanding myocyte injury and repair, is likely from a later post-transplantation time point (1). The infiltrate in the latter biopsy is composed of a more pleomorphic population of cells and appears to also involve capillary injury and repair. Based only on histologic features, the immunophenotypes would be expected to vary. Comparing cases serially and at similar post-transplantation intervals would add precision to the QmIF study.

In the 2013 ISHLT schema, the role of macrophages in AMR is highlighted by the inclusion of CD68-positive macrophages within capillaries as part of the grading schema. In this pilot study (1), there is no mention of the location of the CD68-positive cells. The study found discordance of CD68-positive cells in a comparison between clinically silent and clinically evident rejection and in cases with clinical pathologic discordance. Macrophages are pivotal cells in innate immune responses and adapt phenotypically and functionally based on local circumstances. It is likely that the macrophages detected in this study represent diverse populations. In view of recent reports of the heterogeneity of macrophage populations in allograft injury, future studies should include markers of both pro-inflammatory (case M1) and anti-inflammatory pro-fibrogenic (case M2) subtypes of macrophages, both of which were detected by the CD68 marker (1). A recent report in a mouse cardiac transplant model demonstrated that M2 cells were critical components of the response to chronic allograft injury. Such cells, when exposed to mTOR deletion, express PD-L1 and exert potent immune regulatory functions, mediating long-term graft survival rather than graft loss (5).

Although immune mechanisms in mouse models with knock-out population designs are not exactly analogous to human allograft immune processes, several important elements should be considered. mTOR deletion in macrophages and T lymphocytes is likely commonly operative in human cardiac transplantation because of the widespread use of rapamycin, a potent mTOR inhibitor. Using QmIF, the impact of mTOR deletion on macrophage subpopulations and PD-L1 expression could be explored. PD-L1 was investigated in the current study only by quantification and not cellular localization. Double-labeling methods would shed important light on which cells are PD-L1-positive, some of which are likely macrophages or dendritic cells. Another critical addition to QmIF studies will be markers to detect innate immune cell contributions, such as the role of complement components, inhibitors of complement activation, and NK cells. Innate immune responses likely mediate some aspects of chronic allograft failure (6). Investigation of these elements, along with those of adaptive immune responses, especially if carried out in time-course studies, could shed light on these various mechanisms. Antibody-mediated and cell-mediated rejection often occur together, and it is unknown if this co-occurrence is related to mere association or to different mechanisms at play in such circumstances. Innate immune mechanisms may be operative in different ways in these situations. In addition, studies using QmIF should also include comparison with molecular studies of endomyocardial biopsies, which are now being increasingly investigated to predict outcome as complements to histologic testing (7). Exploration of molecular pathways in addition to histologic and immunopathologic features will help to clarify the mechanisms underlying chronic allograft failure, especially if correlated with histologic and immunopathologic features.

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KEY WORDS allograft rejection, immune checkpoint molecules, immune regulation, quantitative immunohistochemistry
In Vivo Imaging of Venous Thrombus and Pulmonary Embolism Using Novel Murine Venous Thromboembolism Model

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**Summary**

This work established a new murine venous thromboembolism (VTE) model. This model has multiple novel features representing clinical VTE that include the following: 1) deep venous thrombosis (DVT) was formed and extended in the long axis of femoral/saphenous vein; 2) thrombus was formed in a venous valve pocket; 3) deligation of suture-induced spontaneous pulmonary emboli of fibrin-rich DVT; and 4) cardiac motion-free femoral/saphenous vein allowed high-resolution intravital microscopic imaging of fibrin-rich DVT. This new model requires only commercially available epifluorescence microscopy. Therefore, this model has significant potential for better understanding of VTE pathophysiology. (J Am Coll Cardiol Basic Trans Science 2020;5:344–56) © 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/).

**Highlights**

- We established a novel clinically relevant murine DVT model at femoral/saphenous vein induced by flow restriction and light illumination.
- Our model newly succeeded in inducing DVT in a valve pocket and enabled spontaneous pulmonary embolism of fibrin-rich thrombus from lower extremity vein, reproducing the clinical VTE scenario.
- This model is suitable for motion-free in vivo high-resolution imaging of fibrin-rich DVT development and organization using 2-photon microscopy, enabling the real-time imaging of migration of platelets and leukocytes into the erythrocyte-rich DVT.

**Abbreviations and Acronyms**

- **DVT** = deep venous thrombosis
- **FITC** = fluorescein isothiocyanate
- **IQR** = interquartile range
- **IVC** = inferior vena cava
- **PE** = pulmonary embolism
- **ROS** = reactive oxygen species
- **VTE** = venous thromboembolism

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Venous thromboembolism (VTE), including the following 2 sequential clinical situations, deep venous thrombosis (DVT) and pulmonary embolism (PE), is responsible for a significant number of cardiovascular deaths (1).

Studies have shown that leukocytes and platelets play a major role in the pathogenesis of DVT (2–4). Recent advances in molecular imaging techniques allowed visualization of dynamic behavior of inflammatory cells at a single-cell level in living organs; however, the dynamic movement of inflammatory cells in fibrin-rich DVT has not been visualized due to the lack of appropriate animal models suitable for in vivo imaging. There are several issues associated with the already established murine DVT models that they are inappropriate for in vivo imaging and unable to mimic clinical VTE features including spontaneous PE from lower extremity vein and common site of DVT (valvular pocket) (5,6).

Ligature-based inferior vena cava (IVC) models, which include stasis and stenosis models, are most commonly used and have clinical features of DVT (2,7); however, these models are not suitable for in vivo imaging owing to their location in the body and their large thrombus size for microscopic visualization.
imaging. Also, they are incapable of spontaneous embolization due to complete ligation. Models used in past in vivo thrombus imaging studies were mainly ferric chloride (8–10) or laser-induced thrombus (11–13), which form non-DVT-like platelet-rich thrombus.

In a previous study, we succeeded in designing a venous ligation DVT model in the jugular vein, allowing us to monitor inflamed activity of DVT in the same animal using fluorodeoxyglucose–positron emission tomography (14). However, owing to its large thrombus size and pulsatile artifact, high-resolution in vivo imaging at a single-cell level using intravital fluorescence microscopy was not feasible in jugular DVT.

Establishment of novel VTE imaging model is critical to overcome these issues. In this study, we established a novel murine VTE model, which is suitable for real-time in vivo imaging of thrombus formation in the large lower extremity veins using

![FIGURE 1](image)

(A) Surgical ligation of the femoral vein induced flow restriction. Dotted white line indicates the femoral vein. Bars = 5 mm. (B) Venous flow velocity at the saphenous vein in ligated mice showed significant decrease compared with nonligated mice (median: 2.27 [interquartile range (IQR): 1.86 to 2.59] vs. 0.35 [IQR: 0.20 to 0.61] mm/s; p < 0.001; n = 8 to 16 per group). ***p < 0.001. Box-and-whisker plot: middle line represents median value, box indicates IQR (25th, 75th percentiles), and range bars show maximum and minimum. (C) Even after ligation of the femoral vein (V), venous flow in the saphenous vein persisted at a low level as flow diverted (white arrows) into its side branches. Bars = 500 μm. (D) Imaging of real-time thrombus formation (visualized as a filling defect [*] in fluorescein isothiocyanate [FITC]-dextran-based venography) in the flow-restricted saphenous vein following the light irradiation through excitation filter in FITC channel (475/35 nm). Thrombus formation was initiated from the downstream of blood flow within seconds after the irradiation and extended distally in the long-axis direction of the vessel. See also Video 1. Without ligation, thrombus was not formed during the observation. Bars = 500 μm. (C,D) White arrows indicate venous flow direction. Prox = proximal.
intravital fluorescence microscopy and is capable of inducing spontaneous PE.

METHODS

Detailed methodology for all protocols, antibodies, labeling agents, and imaging systems used in this study are provided in the Supplemental Methods.

SURGICAL LIGATION OF THE FEMORAL VEIN. The anesthetized mice were immobilized on a plastic board in a dorsal position. Simple skin incisions permitted vessel exposures of femoral/saphenous vein and artery. The femoral vein at the proximal site of venous bifurcation was separated carefully from the femoral artery, and surgically ligated with 7-0 nylon sutures to induce complete vessel occlusion as shown in Figure 1A, resulting in significant decrease of venous flow at saphenous vein (median: 2.27 [interquartile range (IQR): 1.86 to 2.59] vs. 0.35 [IQR: 0.20 to 0.61] mm/s; p < 0.001). Unlike the IVC, venous flow was restored even after the complete ligation at femoral vein (Figure 1B) through the backflow into the side branch (Figure 1C). All mice with bleedings of the femoral vein during surgery were excluded from further experiments.

STATISTICAL ANALYSIS. All statistical analyses were performed using GraphPad Prism 7 software (La Jolla, California). Results were expressed as median with IQR (25th, 75th percentiles). The statistical significance of differences between 2 groups were assessed using Mann-Whitney U test. Differences between more than 2 groups were evaluated using Kruskal-Wallis test followed by the Dunn multiple comparison post hoc test. A p value of <0.05 was considered statistically significant.

RESULTS

VISUALIZATION OF REAL-TIME THROMBUS FORMATION AT LIGATED FEMORAL/SAPHENOUS VEIN BY FLUORESCENCE MICROSCOPY. We initially tried to establish the stasis DVT model at the saphenous vein to allow in vivo imaging with fluorescence microscopy. However, due to multiple side branches and collateral formation within days, we failed to induce thrombus formation by ligation alone. We then tried to monitor the behavior of leukocytes and platelets after the ligation as they are reported to attach to the vein wall within several hours after the IVC ligation (2). Surprisingly, we could reproducibly observe real-time thrombus formation within minutes without any treatment with chemical agent for thrombus induction (Figure 1D, Video 1), indicating that observation by fluorescence microscopy triggered thrombus formation at ligated femoral/saphenous vein. We also confirmed thrombus was formed in both female C57BL/6J mice and ICR mice (a strain of albino mice) in the same manner (n = 5, respectively), indicating thrombus induction was sex-, strain-, and melamine-independent (data not shown).

We hypothesized that thrombus formation in this model requires both flow restriction and light irradiation by excitation light from fluorescence microscopy.

Thrombus formation in this model requires flow restriction and light irradiation by filtered excitation light. We assessed the relationship between venous flow velocity and thrombus area. We measured thrombus area in nonligated, partially ligated (to achieve middle range of flow velocity), and totally ligated femoral vein after exposure of fluorescein isothiocyanate (FITC)-channel filtered light (475/35 nm) for 60 s. The results showed that reduction of blood flow increased thrombus area and formation rate (Supplemental Figure 1A). Without ligation to reduce blood flow, thrombus never formed during the observation for several minutes (Figure 1D, bottom).

Next, to clarify the required conditions of light irradiation of thrombus formation in this model, we assessed the light intensity, light exposure time, and wavelength of excitation light in the thrombus formation. Higher light intensity and longer light exposure time induced larger thrombus area, indicating that thrombus formation is light flux irradiation-dependent (Supplemental Figures 1B and 1C). We generally use the FITC filter to observe thrombus as a negative area of FITC dextran; however, other wavelengths such as 350/50 nm (4′,6-diamidino-2-phenylindole channel) and 542/20 nm (tetramethyl rhodamine iso-thiocyanate channel) could also induce thrombus formation, suggesting that wavelength of irradiation light did not affect thrombus formation (Supplemental Figure 1D). To rule out the effect of fluorescence reaction between FITC-filtered excitation light and FITC dextran, we confirmed that thrombus could be induced with other blood flow imaging dyes including Evans blue dye (Wako Pure Chemical Industries, Osaka, Japan) and Tracer-653 probe (Molecular Targeting Technologies, Inc., West Chester, Pennsylvania) (Supplemental Figure 1E). We also tested FITC-filtered light exposure without FITC dextran. Although real-time thrombus formation was not visualized, injection of FITC dextran after light
FIGURE 2  Formed Thrombus in Our New DVT Model Has Common Features With Clinical DVT

A

![Image of FITC-dex (WBC, Platelet) merged with RBCs]

B

![Image of FITC-dextran and fibrinogen merged with platelets]

C

![Bar graph showing thrombus area comparison between control and anemia]

D

![Macroscopic and in vivo FM images]

E

![Images of HE and Carstairs staining]

F

![Zoom-in images of FITC-dextran]

G

![Images showing time progression of FITC-dextran and DVT formation]

H

![Images showing time progression of FITC-dextran and thrombus formation with 0s, 10s, 12s, 14s, 16s, 18s intervals]

I

![Graph showing thrombus area over time with control and heparin treatment]

Continued on the next page
irradiation showed thrombus as a negative contrast, indicating that FITC dextran and fluorescence reaction are not necessary as a trigger of thrombus formation (data not shown).

**Formed thrombus in our new DVT model has common features with clinical DVT.** Notably, formed thrombus in our model exhibited multiple common features with human DVT in morphology, histology, and common sites. Unlike the conventional thrombus imaging models such as ferric chloride and rose bengal models, formed thrombus in our model showed a large mass of elliptical shape that extended and elongated in a long-axis direction of the vessel without occlusion, similar to clinical DVT (5). Both ferric chloride and rose bengal models exhibited a small dot pattern that mainly consisted of platelets and few erythrocytes, similar to arterial thrombosis (Figure 2A). Although excitation light-irradiation alone without ligation failed DVT formation within minutes, longer (>10 min) continuous light illumination resulted in platelet-rich thrombus, similar to a previous laser-induced thrombus model (Figure 2A, bottom).

It is well recognized that a clinical DVT mainly consists of erythrocytes and fibrin network with less platelet aggregation. Thrombus in our model allowed in vivo multicolor imaging to visualize thrombus components such as, for example, fibrin, erythrocytes, and platelets. Mice were injected with FITC dextran, rhodamine 6G (platelets and leukocytes) and human fibrinogen or anti-glycophorin A antibody (erythrocytes) before light irradiation. The multicolor imaging revealed the fibrin- and erythrocyte-rich thrombus with low accumulation of platelets and leukocytes (Figure 2B), recapitulating clinical DVT components. Real-time imaging of thrombus formation using FITC-labeled red blood cells also supports the formation of erythrocyte-rich thrombus (Video 2). Anemic mice induced by phlebotomy exhibited a significant decrease in thrombus size (median: 0.31 [IQR: 0.22, 0.48] vs. 0.19 [IQR: 0.14, 0.31] mm²; p = 0.017) (Figure 2C), indicating a significant role of erythrocytes in this DVT model. In vivo photomicrograph of thrombus represented red-white thrombus, similar to clinical DVT. Intravital microscopy of the same thrombus precisely visualized the localization of platelet-rich white thrombus lesion as a rhodamine 6G-positive area (Figure 2D). Pathological analyses of formed thrombus in our model confirmed erythrocyte- and fibrin-rich thrombus with infiltrating leukocytes, similar to clinical DVT (5) (Figure 2E).

Furthermore, our DVT model could reproduce several features of clinical DVT for the first time. DVT was formed at a frequent site, such as the venous valve pocket and venous confluences, indicating the similarity of rheological effects in human DVT (15,16) (Figures 2F and 2G). Additionally, DVT in our model enabled spontaneous embolization, allowing real-time visualization during thromboembolism (Figure 2H, Video 3). Lastly, this model enabled monitoring of pharmacological effects of antithrombotic drugs. Similar to clinical DVT, thrombus development in our model was prevented by pre-injection of unfractionated heparin (Figure 2I).

**Spontaneous DVT embolization to pulmonary artery, as a novel PE model.** Because the current DVT model allows spontaneous detachment of lower-extremity DVT, we tested whether this model can generate acute PE. After DVT formation in the femoral vein, we deligated the suture to allow embolization of thrombi. Alexa Fluor 647 conjugated fibrinogen (fibrinogen-AF647) was pre-injected to visualize thrombi. A large fibrin-rich DVT was visualized by in vivo fluorescence microscopy (Figure 3A). Although DVT was not embolized just after the deligation, most
of the femoral DVT had disappeared within 30 min after the deligation, indicating that thrombi had spontaneously embolized to the pulmonary artery. Resected lungs exhibited strong fibrinogen-AF647–based fluorescence (Figure 3B, top). Images of the resected lungs from fibrinogen-AF647–injected mice without DVT were also taken to exclude the post-mortem clot formation at pulmonary artery, and these mice showed no evidence of fluorescence signal (Figure 3B, bottom). To confirm that fluorescence signal is produced by embolized thrombus, histological assessment was performed. Hematoxylin and eosin staining of resected lungs showed fibrin-rich thrombus with inflammatory cells in the pulmonary artery. Carstairs staining showed that fibrinogen-AF647–positive structures imaged by fluorescence microscopy are fibrin-rich thrombus (Figure 3C). These data indicate that our DVT model can be used as a novel PE model.

PLATELET–, NEUTROPHIL–, AND OXIDATIVE STRESS–INDEPENDENT THROMBUS FORMATION. A
recent study demonstrated the significant role of platelets and neutrophils in the initiation of DVT formation in the IVC-ligature model (2). To elucidate the role of these cells in the DVT formation in our model, we imaged leukocytes and platelets during DVT formation. Multicolor real-time imaging of DVT formation revealed that rhodamine 6G-labeled leukocytes and platelets did not clearly accumulate and attach to the venous wall prior to DVT formation (arrows). Pre-treatment with an antiplatelet antibody (right panel) neither prevented thrombus formation nor changed thrombus size (median: 0.42 [IQR: 0.21 to 0.62] vs. 0.38 [IQR: 0.26 to 0.61]; p = not significant [NS]; n = 10 per group for platelet depletion; and median: 0.34 [IQR: 0.26, 0.45] vs. 0.34 [IQR: 0.26, 0.55]; p = not significant [NS]; n = 6 per group for neutrophil depletion, respectively). Pre-treatment with deoxyribonuclease also did not affect the DVT formation in our model (median: 0.39 [IQR: 0.30, 0.60] vs. 0.45 [IQR: 0.33, 0.61]; p = NS; n = 5 per group). Reactive oxygen species (ROS) did not localize within the thrombus area in our model (upper panel), whereas laser/ROS model using hematoporphyrin (lower panel) showed ROS-positive platelet-rich thrombus. Both platelet- and neutrophil-depletion did not prevent thrombus formation and did not change thrombus size acutely (p = NS, respectively) (Figures 4B and 4C). Neutrophil extracellular traps are also reported as a key player of DVT initiation (2,17); however, pre-treatment with deoxyribonuclease also did not affect the DVT size in our model (Figure 4D). As oxidative stress is considered as a major contributor to the formation of DVT in models such as the laser-induced thrombus model and the rose bengal model, we then assessed the role of oxidative stress in our model. To visualize reactive oxygen species (ROS) during DVT formation, 3′-(p-aminophenyl) fluorescein was administered before DVT initiation. However, ROS signal was not shown in thrombus area in our model, whereas laser/ROS model using hematoporphyrin (12) showed ROS-positive platelet.
Thrombus (Figure 4E). In addition, an antioxidant and ROS scavenger, N-acetylcysteine failed to block DVT formation in our model, indicating the mechanism of DVT formation in our model is ROS-independent (Figure 4F). These results collectively suggest that DVT formation in our model is platelet-, neutrophil-, and ROS-independent, unlike previous animal thrombosis models.

**TWO-PHOTON IMAGING OF FORMED DVT.** Recent advances in the 2-photon microscopy enabled fluorescence imaging of living deeper tissue such as, for example, brain, skin, bone, and heart (12,18,19). To the best of our knowledge, this is the first model visualizing erythrocyte-rich DVT in murine femoral vein with a single-cell level resolution. High-resolution imaging with 2-photon microscopy allows for a clear distinction between rhodamine 6G-labeled leukocytes and platelets by size (Figures 5A and 5B). Second harmonic generation can visualize collagen fibers; therefore, the vein wall and venous valve can be imaged without an exogenous labeling agent. DVT formed within venous valve pocket and surrounding platelets accumulation can be clearly imaged (Figures 5C to 5E).
DYNAMICS OF PLATELETS AND LEUKOCYTES IN DVT ORGANIZATION PROCESS. Serial imaging of DVT by epifluorescence microscopy easily allows visualization of rhodamine 6G-positive area in whole thrombus; however, distinction between leukocytes and platelets is not possible because of insufficient resolution (Figure 6A). High-resolution 4-dimensional imaging by multiphoton microscopy enables clear discrimination of and visualizes dynamics of both leukocytes and platelets during the thrombus organization process (Figure 6B). Although recent studies suggest the role of platelets in the initiation of DVT (2), the comprehensive role and dynamics of platelets in the DVT developing and organization process are unknown. Whole thrombus imaging with multiphoton microscopy showed that platelet accumulation within DVT is proximal side-dominant, and resolution is distal side-dominant (Supplemental Figure 2A). We newly observed the platelets’ accumulation and deposition on the surface of the DVT, resulting in changing the rheology of blood flow and showing multiple layers of platelet-rich and erythrocyte-rich layers (Figure 6C). This might explain why DVT exhibits an annual ringlike structure. We also observed the leukocytes migrating into the DVT accompanied with platelets. As
neutrophil-platelet interaction is recently reported as facilitators of inflammation (20,21), the accumulation of platelets on the formed DVT might play a role in the initiation of inflammation process, which is not a hemostatic role. Leukocyte migration can also be imaged and analyzed via ImageJ (National Institutes of Health, Bethesda, Maryland) (Figures 6D and 6E, Video 4). Tracking of rhodamine 6G-positive leukocytes shows directional migration of leukocytes toward the center of DVT, suggesting that leukocytes are actively recruited to DVT (Figure 6E). Those migrating leukocytes were confirmed as mainly Ly-6G-positive neutrophils (Supplemental Figure 2B), which has been reported in past histological analyses (14).

DISCUSSION

Coagulation factors and platelet activation have been thought to be key elements of venous thrombosis until recent discoveries using a murine DVT model, which showed that activation of immune responses strongly influence blood coagulation and pathological thrombus formation (2,22). Although murine ligation-based models at IVC are widely used, an opposite scenario is seen in clinical DVT. Clinical DVT forms mainly in flow-maintained veins, followed by flow restriction after initial thrombus development, whereas ligation-based models induced flow-restriction first. The other limitation of the IVC-based model is the lack of venous valve. Venous valve pockets and venous confluence in the lower extremity veins are reported as frequent sites of DVT initiation (15,16,23).

Recent studies reported in vivo thrombosis imaging systems available for high-resolution microscopic imaging (2,11,12,24). However, these thrombus models are injury-induced (laser-induced or electrolytic) platelet thrombus-based, not a fibrin- and erythrocyte-rich thrombus, which is visualized in our study. Furthermore, most of these models use small vessels such as cremaster muscle and mesentery vessels. These microvascular thrombosis models are related with pathological thrombus that occurs during sepsis and other inflammatory disorders, but that is not relevant to large vessels such as jugular and femoral vein, as observed in human VTE (25).

In terms of thrombus induction on the stage of microscopy, our model is similar with the conventional laser-induced in vivo imaging models (11-13,26); however, we succeeded in thrombus induction in femoral/saphenous vein, where is literally a deep vein, as opposed to cremaster and mesenteric arterioles in the previous laser-induced model. Second, we used filtered white light (mainly through FITC filter in the microscope) produced from a mercury lamp, not a nitrogen or helium-neon laser as used in past studies (11-13,26). We believe that weaker light stimulation and larger vein size are the reasons that thrombus could not be formed by light irradiation alone and that the formed thrombus was not a platelet-rich thrombus.

A recent study succeeded in applying the IVC stenosis model for in vivo imaging using 2-photon microscopy (2). The study elegantly showed the recruitment of leukocytes and platelets at venous vessel wall as an initiation of DVT. However, formed DVT was too large to visualize the immune cell recruitment from circulating blood flow by intravital fluorescence microscopy. Therefore, visualization of immune cell behavior after DVT formation has been technically limited. To the best of our knowledge, our model, for the first time, enabled visualization of real-time imaging of erythrocyte-rich thrombus formation and organization process in vivo.

Furthermore, our model enabled us to visualize the initiation of thromboembolism and induce pulmonary embolization after DVT formation. PE is a common and fatal complication of DVT. Repeated PE triggers the pulmonary hypertension, which is known as chronic thromboembolic pulmonary hypertension. Although there are murine models of PE in the past, the pathophysiology is quite different from the clinical PE. Systemic administration of coagulation factors such as thrombin (27) and thromboplastin (28) and exogenous clot injection (29) are commonly used to induce PE, but the occurrence of pulmonary thrombus is not clearly related and embolized from pre-existing DVT in the lower extremity. The photochemical injury model using rose bengal (30) and the ferric chloride model (31) could induce PE after DVT formation in large vessels, but the main components of thrombus are platelets. Therefore, to the best of our knowledge, this is the first clinically relevant PE model as a consequence of erythrocyte- and fibrin-rich DVT formed in the femoral/saphenous vein, which is the common source of clinical PE. Because our model allows repeated small DVT induction and embolization, our PE model has the potential to be a novel chronic thromboembolic pulmonary hypertension model and further evaluation is desired.

STUDY LIMITATIONS. The intravascular infusion of rhodamine 6G labels both platelets and leukocytes;
CONCLUSIONS

Our newly established DVT model enables real-time in vivo imaging of erythrocyte-rich DVT formation and organization process in the murine femoral vein. The formed DVT in this model reproduces several common features of clinical DVT. We propose the utility of our model for better understanding of the pathophysiology of clinical DVT using the high-resolution visualization of the DVT organization process such as the dynamic recruitment of platelets and leukocytes to erythrocyte-rich thrombus.

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COMPETENCY IN MEDICAL KNOWLEDGE: There are several animal models of thrombosis. However, all of the models have advantages and disadvantages. For instance, the ferric chloride model does not need complicated surgical techniques and is applicable for in vivo microscopic imaging. However, it induces platelet-rich thrombus, thereby the ferric chloride model is not appropriate for DVT experimental study. The IVC-ligation model is currently the most used fibrin- and erythrocyte-rich DVT model. However, its location and large thrombus size are inappropriate for in vivo microscopic imaging and inducing pulmonary emboli.

TRANSLATIONAL OUTLOOK: Thromboembolic events mostly occur only once throughout the 70 to 90 years of human life, and it only takes several seconds from the initiation of thrombus detachment to complete embolization. Therefore, understanding and visualizing such a quick event has been technically challenging even in animal models. Here, we newly succeeded in inducing and visualizing thrombus formation in vivo, and furthermore, we visualized the moment of thromboembolic event. Our model has significant potential for better understanding of VTE pathophysiology.

PERSPECTIVES

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KEY WORDS 2-photon microscopy, deep vein thrombosis, imaging, pulmonary embolism, venous thromboembolism

APPENDIX For an expanded Methods section, and supplemental figures, a table, and videos, please see the online version of this paper.
Shining a Light on Venous Thromboembolism*

Yogendra Kanthi, MD,a Aditya Sharma, MDb

Prefrontal studies are important grounds to develop and test hypotheses and novel approaches for translation to human disease. A number of distinct pathologic processes can result in venous thromboembolism. Studies using murine models have shed light on contributions from multiple cell types. Roles have been identified for endothelial and platelet activation, neutrophil extracellular trap generation, myeloid-derived tissue factor release, and erythrocyte-mediated thrombus stabilization. Critical roles have also been described for activation of the inflammasome resulting in maturation of the paradigmatic inflammatory cytokine, interleukin-1β, in venous thrombogenesis (1).

Several models to examine venous thrombogenesis and resolution have been described, each with strengths and limitations. To help investigators select an appropriate model to test their hypotheses, several professional societies recently published a consensus statement with detailed methodologies, advantages, and disadvantages of the most widely used models of deep vein thrombosis (DVT) (2). In this issue of JACC: Basic to Translational Science, Okano et al. (3) describe an intriguing new model of venous thromboembolism in the femoral and saphenous veins. The most commonly used murine models of DVT involve manipulation of the infrarenal inferior vena cava (IVC) by stopping or altering blood flow or inducing free radical generation. Studying thrombosis in IVC provides sufficient tissue to examine both the vein wall and thrombus itself. However, the IVC lacks valves and does not replicate flow dynamics seen in human superficial and deep veins with valves where thrombosis often occurs.

Initial DVT models involve interrupting blood flow in the infrarenal IVC by complete ligation of the vein and all branch vessels (1,2). The static column of blood generated coagulates upstream of the ligation site. Thrombi generated by this “stasis” model are rich in erythrocytes and fibrin and are reproducible in size. This model can be used to study coagulation in gene-modified mice and for following pharmacologic interventions for thrombus resolution days-to-weeks after surgery. Although this model of stasis DVT in mice does not typically result in pulmonary thromboembolism, rats subjected to IVC ligation under hypoxic, hyperbaric conditions developed thrombi downstream of the ligation in the direction of flow with embolism to the lungs. Specific cell-depletion experiments to date suggest that platelets, neutrophils, and monocytes may not play significant roles in thrombogenesis in this model of DVT. Therefore, investigators interested in these cellular inputs to thrombosis should consider an alternate approach.

A modification of this model involves restricting but not occluding the IVC to reduce vein diameter to approximately 10% of its normal size while...
minimizing endothelial injury (1,2). Narrowing the IVC lumen generates turbulent blood flow patterns and activation of platelets, neutrophils, and monocytes as they interact with the endothelium (4). Platelets play essential roles in DVT under these conditions, monocytes release tissue factor–bearing microvesicles, whereas neutrophils contribute to thrombogenesis through several mechanisms including formation of extracellular chromatin traps and reactive oxygen species generation. This "stenosis" model of DVT has inherent variability in thrombus frequency and size, recapitulating the clinical condition. It is typically used to study thrombus accretion and propagation beyond the first several hours of thrombogenesis. Thrombus and vessel tissue is sufficiently sized for analysis. Notably, these DVTs have histologic features characteristic of human thrombi, with alternating layers of erythrocyte-rich "red" and fibrin- and platelet-rich "white," typically described as "lines of Zahn." Intravital microscopy following IVC restriction can help shed light on cellular recruitment and interactions during initial thrombogenesis. Significant limitations include the large number of animals needed for studies due to variability in thrombus incidence and size and the need for sophisticated equipment for image acquisition due to the depth of the IVC. This model has also recently been suggested to result in pulmonary artery thrombi, although it is unclear whether this is a result of thromboembolism from the IVC or in situ pulmonary arterial coagulation, which may result from procoagulant microvesicles or soluble mediators.

Free radical–induced thrombosis models using chemical or electrical triggers have been used in large and small veins. Application of iron(III) chloride to the adventitial surface of the vein can be used to trigger thrombosis, although this may cause vessel wall tissue damage. Alternate models deliver a small 1.5 to 3 volt current directly to the external surface of the saphenous or femoral veins for short periods of time. This rapidly induces thrombosis in these valve-containing veins, which can be used for acute or chronic thrombus formation and resolution. Another advantage of this model is the small vein diameter that makes these vessels well-suited for application of real-time intravital microscopy to study cellular and thrombus dynamics. A limitation of this method of DVT formation is that the thrombi generated are rich in platelets and fibrin, reminiscent of "white clot" observed in arterial thrombi rather than those seen in DVT. Another recently developed model involving electrolytic injury involves running a current through a needle inserted into the IVC to generate intraluminal free radicals and subsequent thrombosis. Side branches are ligated for consistent flow dynamics. This model has several advantages, including thrombus formation in all experiments, which reduces the number of animals required for each experiment. In addition, thrombus size can be controlled by altering current and time of exposure, which gives investigators significant flexibility to test prothrombotic conditions and antithrombotic interventions. As with other surgical models, the electrolytic IVC injury model requires technical proficiency for consistent results.

Each model carries strengths and weaknesses, making model selection critical to test specific hypotheses. Okano et al. (3) describe a new method of venous thrombosis that starts with ligation of the femoral vein to create disturbed flow. The investigators discovered that whereas disturbed flow alone did not generate a thrombus, intravital imaging with fluorescein isothiocyanate–dextran and filtered epifluorescent light triggered coagulation in the imaged segments within minutes. They also found no contribution to thrombogenesis from fluorescent dyes with differing quantum efficiencies as thrombi were seen even if dye was added after light exposure. Clots formed in vein valves, where disturbed flow dominated after downstream ligation. These thrombi were predominantly composed of erythrocytes and fibrin. Although neutrophils and platelets were also recruited to the thrombotic milieu, their role is unclear. Neutrophils were recruited to the center of the thrombus. Treatment with deoxyribonuclease did not alter thrombus size, leading the investigators to conclude that neutrophil extracellular traps are not pathogenic in this model of DVT. However, it is unclear whether deoxyribonuclease can passively penetrate thrombi, and the granulocyte and antimicrobial proteins decorating neutrophil extracellular traps may still be local effectors. Platelet deposition likely alters local flow dynamics and increases thrombus accretion. The presented data also suggest DVT size can be modified by varying light intensity and duration. Notably, thrombosis in the veins could be triggered by longer light exposure even in the absence of flow disturbance. Although the mechanism for filtered light–induced thrombosis in this model is unknown, it has several advantages. First, DVT can be induced in limb veins without chemical or laser-induced injury of the vessel wall. Second, intravital visualization of thrombus dynamics in the earliest stages of DVT is an important strength of this model. Although thrombosis in the mesenteric and cremasteric venules is an excellent tool for intravital
microscopy, differences in gene expression and flow dynamic patterns in these microvascular beds limit extrapolation of observations to larger veins of interest. Third, investigators can use the “tunability” of thrombus size by light exposure to study prothrombotic conditions or therapeutic interventions. Fourth, deligation of the femoral vein led to restored venous return and embolization of the thrombi to the lungs providing a much-needed model to study acute pulmonary embolism. Several questions remain about this platform that investigators will note, including the lack of mechanism by which filtered light induces thrombosis. Light at many spectral wavelengths is known to induce changes in cellular processes, which have not yet been elucidated in this model. Photocoagulation with lasers has been used to treat retinal and other diseases, presumably by thermal injury. Erythrocytes have been found to contribute to venous thromboembolism in several ways, including supporting thrombin generation, expression of surface phosphatidylserine, interacting with other cells, and altering fibrin structure and stability (3). It is unclear whether these cellular processes are enrolled in this erythrocyte-dependent model. Furthermore, low-resolution imaging with epifluorescence precludes real-time individual cell tracking although these can be defined ex vivo. Finally, the significance of engagement by neutrophils and platelets remain unknown in this model of thrombosis.

The new platform developed by Okano et al. (3) to study venous thrombosis offers distinct strengths to gain new insights into the earliest stages of thrombogenesis. Careful selection of the most appropriate preclinical tool among the available models will greatly help investigators generate important insights to develop new approaches for this highly prevalent and morbid disease.

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KEY WORDS: animal models, coagulation, deep vein thrombosis, pulmonary embolism, venous thrombosis.
Therapeutic Antibody Against Phosphorylcholine Preserves Coronary Function and Attenuates Vascular $^{18}$F-FDG Uptake in Atherosclerotic Mice

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HIGHLIGHTS

- Phosphorylcholine is a pro-inflammatory epitope in atherogenic oxidized phospholipids.
- This study investigated effects of a novel monoclonal IgG1 antibody against PC on vascular function and atherosclerotic inflammation.
- Treatment with phosphorylcholine antibody preserved coronary flow reserve and decreased uptake of $^{18}$F-FDG in atherosclerotic lesions in hypercholesterolemic mice.
- Noninvasive imaging techniques represent translational tools to assess the efficacy of phosphorylcholine-targeted therapy on coronary artery function and atherosclerosis.
SUMMARY

This study showed that treatment with a therapeutic monoclonal immunoglobulin-G1 antibody against phosphorylcholine on oxidized phospholipids preserves coronary flow reserve and attenuates atherosclerotic inflammation as determined by the uptake of 18F-fluorodeoxyglucose in atherosclerotic mice. The noninvasive imaging techniques represent translational tools to assess the efficacy of phosphorylcholine-targeted therapy on coronary artery function and atherosclerosis in clinical studies. (Am Coll Cardiol Basic Trans Sci 2020;5:360-73) © 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/).

Oxidized phospholipids (OxPLs) mediate many atherogenic processes, including endothelial dysfunction, the accumulation of inflammatory cells into the vessel wall, and the uptake of oxidized low-density lipoprotein (OxLDL) cholesterol by macrophages, thereby promoting foam cell formation and atheroma growth (1–3).

Phosphorylcholine (PC) is the polar headgroup of the membrane phospholipid phosphatidylcholine. It is a pro-inflammatory epitope in OxPLs that is recognized as a danger-associated molecular pattern by the innate immune system (1). Human serum contains endogenous antibodies against oxidized epitopes, with the predominant antibody being immunoglobulin (Ig)-M antibody against PC (4–6). Low levels of IgM anti-PC are associated with an increased risk of atherosclerotic cardiovascular events in different patient populations (4,7–10). In subjects with elevated lipoprotein(a) [Lp(a)] and increased inflammatory activity in the arterial wall, activation of circulating monocytes by OxPLs can be inhibited by the IgM E06 antibody, the prototypic murine antibody against PC (11). Experimental studies have indicated that this antibody blocks the scavenger receptor-mediated uptake of OxLDL on macrophages in vitro (5), and that induction of the PC antibody formation by immunization (12,13) or direct infusion (14) is atheroprotective in mice. In transgenic mice, a single-chain variable fragment of E06 counteracts with OxPLs in vivo, attenuating inflammation and progression of atherosclerosis (15). Accordingly, the available evidence suggests that OxPLs that contain PC are a risk factor for atherosclerosis-related diseases and that a therapeutic antibody against PC may represent an...
approach to improve coronary vascular function and attenuate atherosclerotic inflammation.

Positron emission tomography imaging with 18F-fluorodeoxyglucose (18F-FDG) is a noninvasive tool to measure inflammation in atherosclerotic lesions because 18F-FDG accumulates in inflammatory cells (16,17). Coronary flow reserve (CFR) in response to vasodilator stress is an integrated measure of blood flow through both the epicardial coronary arteries and microvasculature (18). Impaired CFR is a strong predictor of cardiovascular mortality in patients with suspected coronary artery disease (19). Both 18F-FDG uptake and CFR can serve as translational tools to assess the effects of anti-atherosclerotic therapy.

In the present study, we investigated whether a novel exogenous monoclonal IgG1 antibody against PC (designated X19-mu, with similar properties to the endogenous IgM E06 anti-PC) improves vascular function and reduces atherosclerotic inflammation in hypercholesterolemic low-density lipoprotein receptor deficient mice, expressing only apolipoprotein B100 (LDLR−/−ApoB100/100). Vascular function was studied by measuring CFR in response to adenosine with Doppler ultrasound and endothelium-mediated vasodilatation in response to methacholine. Inflammation in aortic atherosclerotic lesions was determined by the uptake of 18F-FDG and histological stainings of inflammatory markers. The effect of human PC antibody (PC-mAb) on human aortic endothelial cells (HAECs) stimulated with Lp(a) was studied in vitro.

METHODS

X19-mu AND HUMAN PC-mAb. The antigen-binding sequences of the X19-mu antibody were identified from a phage display library on the basis of their binding ability to PC and were converted to full IgG1 antibodies as part of the work that identified PC-mAb, a fully human monoclonal antibody against PC. This therapeutic, exogenous PC-mAb (Clone X19-A05, Athera Biotechnologies AB, Stockholm, Sweden) showed similar properties to the IgM E06 antibody in inhibiting OxLDL uptake in macrophages, binding to apoptotic cells, blocking OxLDL-induced release of monocyte chemoattractant protein 1 from monocytes, binding to inflammatory cells in human aortic atherosclerotic lesions, and preventing inflammation-driven vascular remodeling in mice (20,21). X19-mu (Athera Biotechnologies AB) differs from the fully human PC-mAb in that it has a murine Fc fraction to lower the risk of an immune reaction to the treatment, but it has identical antigen-binding sequences. The binding affinity to PC is similar between the fully human PC-mAb and the X19-mu antibody (Supplemental Figure 1).

ANIMALS AND INTERVENTIONS. The national Animal Experiment Board in Finland and the Regional State Administrative Agency for Southern Finland approved the studies (license ESAVI/2163/04.10.07/2015). They were carried out in compliance with European Union laws related to the conduct of animal experimentation. The animals were housed under standard conditions with a 12-h light–dark cycle with ad libitum access to water and food.

LDLR−/−ApoB100/100 mice (n = 45; strain #003000, The Jackson Laboratory, Bar Harbor, Maine) were fed a high-fat diet (0.2% total cholesterol, TD 88137, Harlan Teklad, Harlan Laboratories, Madison, Wisconsin) for 12 weeks, starting at the age of 8 weeks, to induce atherosclerosis. After 12 weeks on the high-fat diet, 34 mice were continued on a regular chow diet; after sex and sibling matching, they were randomized to receive intraperitoneal injections containing either 0.9% saline solution as a vehicle (n = 17), once a week, for 6 weeks. A separate group of mice (n = 11) was studied at week 0, after the 12-week high-fat diet.

CFR was assessed repeatedly before and after 6 week treatments in a randomly selected, pre-specified subgroup of mice (n = 10/treatment) and also in a separate group of healthy C57BL/6 mice (n = 11; age: 6.5 months). Aortic histology and 18F-FDG uptake were assessed at the end of the 6-week treatments, and also at week 0, in a separate group of mice. Endothelium-mediated vasodilatory response to methacholine was studied in a separate group of atherosclerotic mice after treatment with vehicle (n = 8) or X19-mu antibody (n = 7). For euthanasia in aortic histology and 18F-FDG studies, mice were anesthetized with isoflurane (2% to 3% inhalation), and blood was collected by cardiac puncture followed by cervical dislocation.

BLOOD SAMPLES. For details of the measurement of blood glucose, lipids and X19-mu antibody levels, see the Supplemental Appendix.

HISTOLOGY AND IMMUNOFLUORESCENCE. The aortic root was fixed with 10% formalin, embedded in paraffin, and cut into serial 5 μm cross sections at the level of the coronary ostium. Sections were stained with Movat’s pentachrome for measurement of the atherosclerotic lesion area and with Masson’s trichrome (Sigma-Aldrich, St. Louis, Missouri) for quantification of lesion collagen content. Macrophages were detected by double immunofluorescence using Mac-3 antibody and either CCR2 antibody that...
detected M1 polarized macrophages or CD206 antibody that detected M2 polarized macrophages. Macrophage apoptosis was studied by double immunofluorescence using Mac-3 and cleaved caspase-3 antibodies. Furthermore, vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, interleukin (IL)-1β, and monocyte chemoattractant protein 1 were detected by immunofluorescence. The presence of PC epitope in atherosclerotic lesions was detected by immunofluorescence using the fully human PC-mAb directly labeled with Cy5 and co-stained with the Mac-3 antibody and CD31 antibody that detected endothelial cells (Supplemental Table 1). For details, see the Supplemental Appendix. Control stainings with nonimmune IgGs are presented in Supplemental Figure 2.

**CFR and Endothelium-Mediated Vasodilatation.** A dedicated small animal Doppler ultrasound device (Vevo 2100, VisualSonics Inc., Toronto, Ontario, Canada) with a linear 22- to 55-MHz (MS550D) transducer was used to assess CFR as previously described (22,23). Mice were anesthetized with an intraperitoneal injection of midazolam (8 mg/kg; Hameln Pharmaceuticals GmbH, Hameln, Germany) and ketamine (60 mg/kg; Intervet International BV, Boxmeer, the Netherlands). A tail vein was cannulated, and body temperature was maintained with a heating pad. Blood flow in the middle left coronary artery was localized under color Doppler mapping using modified long-axis views, and the flow velocity spectrum was recorded by pulsed-wave Doppler, both at rest and during infusion (maximum of 2 min) of adenosine (140 μg/kg/min; Life Medical Sweden AB, Stocksund, Sweden). Anesthesia was reversed with a subcutaneous injection of flumazenil (0.5 mg/kg; Hameln Pharmaceuticals GmbH). CFR was calculated as the ratio of the mean diastolic flow velocity during maximal adenosine-induced hyperemia to that during rest. The mice were allowed to recover for a minimum of 72 h between ultrasound and 18F-FDG studies.

Endothelium-mediated vasodilatation was studied by measuring arterial blood pressure response to intravenous injection of methacholine in apolipoprotein E deficient (ApoE−/−) mice (Supplemental Appendix).

**Stimulation of HAECs with Donor-Derived LP(a).** To study the effect of PC-mAb on endothelial nitric oxide (NO) production and inflammatory responses, HAECs were stimulated with 1 mg/ml of LP(a) for 24 h in the presence of fully human PC-mAb or a non-specific IgG. Thereafter, cells were processed for gene expression measurements of VCAM1, ICAM1, IL6, and IL8, as well as protein measurements of IL-6 and IL-8, or lysed for measurement of intracellular nitrate reflecting NO production (Supplemental Appendix).

**18F-FDG Uptake.** The mice were fasted for 4 h, anesthetized with isoflurane, and then intravenously injected with 18F-FDG (11 ± 0.38 MBq) via the tail vein. At 90 min post-injection, the thoracic aorta from the sinotubular junction to the level of the diaphragm was excised and rinsed with saline. The aorta was frozen in cooled isopentane and cut into sequential longitudinal cryosections of 20 and 8 μm, which provided sections throughout the region on a single slide (n = 6 to 8 intervals per aorta). Digital autoradiography was performed using the previously described method (24,25). Cryosections were apposed to an imaging plate (Fuji Imaging Plate BAS-TR2025, Fuji Photo Film Co., Ltd., Tokyo, Japan) for 4 h and then scanned with a Fuji Analyser BAS-5000 (internal resolution of 25 μm, Fuji). Then, sections were stained with hematoxylin and eosin (20 μm) or immunohistochemically with Mac-3 antibody (8 μm), scanned with a slide scanner, and co-registered with autoradiographs. 18F-FDG accumulation was measured as photo-stimulated luminescence per square millimeter in regions of interest placed on the atherosclerotic lesions (n = 22/mouse) and vessel walls without lesions (n = 16/mouse), using Tina 2.1 software (Raytest Isotopenmessgeräte, GmbH, Straubenhardt, Germany). To assess the treatment effects, the average 18F-FDG uptake within all atherosclerotic lesions in 20-μm sections was calculated and divided by the average uptake in lesion-free vessel walls (expressed as lesion-to-wall ratio) in each mouse. To assess the effects of treatment on 18F-FDG uptake in atherosclerotic lesions with different macrophage densities, percentage of Mac-3-positive staining within the lesions was measured with Image J software and compared with the 18F-FDG uptake (lesion-to-wall ratio) in the same lesions in 8 μm sections (n = 7 randomly chosen mice/treatment; 8 lesions/mouse) as previously described (25). Measurement of 18F-FDG uptake in other organs is described in the Supplemental Appendix.

**Statistical Analysis.** Results are presented as individual data points with mean ± SD, unless otherwise indicated. Data were analyzed using SPSS Statistics software 22 (IBM, Armonk, New York). Normality was examined by a Shapiro-Wilk test, and equality of variances was tested with Levene’s test. Multiple comparisons were made by 1-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test for the week 0 group. Student’s t-test for unpaired or paired data was used for comparisons between intervention groups. Fisher’s exact test was used to evaluate...
histological scores. Mann-Whitney U test was applied for in vitro data analysis. Analysis of covariance was used for repeated measurements, which adjusted each subject’s follow-up measurement according to their baseline measurement, and was used to compare CFR values. There were no differences between female and male mice, except in the 18F-FDG study, in which the level of 18F-FDG uptake was higher in females. Sex by group interaction was examined and although it was not statistically significant, group and sex were included as fixed factors in the model. Two-way ANOVA was used to compare 18F-FDG uptake in lesions divided into tertiles according to macrophage density. Macrophage density and treatment were included as fixed factors in the model (no interaction between macrophage density and treatment was observed). A p value of <0.05 was considered statistically significant. Assuming an average CFR of 2.5 ± 0.3 (23) and 18F-FDG uptake of 1.8 ± 0.25 (25), sample sizes of 10 and 14 were calculated to be sufficient to detect a difference of 15% with 80% power and a type I error of 0.05, respectively.

**RESULTS**

Treatment with X19-mu was well tolerated, with the antibody remaining present in the blood until the end of the intervention; the average plasma X19-mu concentration 5 to 7 days after the last injection was 14 ± 9.8 μg/ml. One mouse was excluded due to a failure in the dosing of X19-mu (no detectable levels of X19-mu in plasma), and 1 mouse due to failure in dosing of 18F-FDG. Thus, the final study group consisted of 16 mice in the vehicle group and 16 mice in the X19-mu group.

As shown in Table 1, the body weights and fasting blood glucose levels were comparable between the vehicle- and X19-mu–treated groups. Compared with the mice studied at week 0, plasma levels of cholesterol, LDL, and high-density lipoprotein were lower (p ≤ 0.001) after 6 weeks on normal mouse chow in both the vehicle and X19-mu groups, with the X19-mu treatment not showing any incremental effect on lipids in comparison with vehicle.

**LESION HISTOLOGY.** All LDLR−/− ApoB100/100 mice showed prominent macrophage-rich atherosclerotic lesions in the aortic root (Figures 1 and 2). Compared with mice studied at week 0, the absolute lesion area in the aortic root was larger after the 6-week treatment in both the vehicle (0.32 ± 0.23 mm² vs. 0.68 ± 0.28 mm²; p = 0.004) and X19-mu–treated (0.32 ± 0.23 mm² vs. 0.62 ± 0.28 mm²; p = 0.017) mice. However, the lesion areas were similar after treatment with either vehicle or X19-mu (p = 0.57).

At week 0, the intimal area positive for Mac-3 staining was 25 ± 8.2%. In comparison with this initial value, the intimal area positive for Mac-3 did not differ after the 6-week treatment with vehicle (19 ± 8.7%; p = 0.19) or X19-mu (19 ± 10%; p = 0.21). X19-mu treatment had no effect in comparison with vehicle (p = 0.93) (Figure 1G). The expression of the M1 macrophage marker CCR2 after vehicle treatment was comparable to that in the X19-mu–treated group (44 ± 13% vs. 43 ± 13% of the Mac-3 positive area; p = 0.87) (Figure 1H), as was the expression of the M2 macrophage marker CD206 (48 ± 9.2% vs. 50 ± 13%; p = 0.55). At week 0, the percentages of M1 and M2 macrophages were 41 ± 9.2% and 50 ± 13%, respectively. These values were similar after 6-week treatment with either vehicle (p = 0.84 and p = 0.96, respectively) or X19-mu (p = 0.91 and p = 0.60, respectively).

The extent of lesion endothelium positive for VCAM-1 or ICAM-1 did not show significant differences after treatment with vehicle or X19-mu (median [25% and 75% percentiles] score: 2.5 [2.0 and 3.0] vs. 2.0 [2.0 and 3.0], p = 0.65 and 2.5 [2.0 and 3.0] vs. 2.0 [1.0 and 2.0]; p = 0.32) (Figures 2A and 2B, respectively). Compared with vehicle, treatment with X19-mu reduced the intimal area positive for IL-1β (24 ± 1.0% vs. 21 ± 0.83%; p = 0.044) (Figure 2C) but not for monocyte chemoattractant protein 1 (10 ± 1.0% vs. 10 ± 0.60%; p = 0.82) (Figure 2D). The number of macrophages containing cleaved caspase-3 was similar after treatment with vehicle or X19-mu (220 ± 65 cells/mm² vs. 230 ± 72 cells/mm²; p = 0.61) (Figure 2E). The lesion collagen content was similar after treatment with vehicle or X19-mu (39 ± 5.6% vs. 40 ± 7.9%; p = 0.68) (Figure 2F).

### Table 1: Characteristics of LDLR−/− ApoB100/100 Mice at the Time of Randomization (Week 0) and After 6-Week Treatment With Vehicle or X19-mu Antibody

|                          | Week 0           | Vehicle | X19-mu |
|--------------------------|------------------|---------|--------|
| Animals (f/m)            | 11 (5/6)         | 16 (8/8) | 16 (8/8) |
| Body weight (g)          | 28 ± 5.1         | 30 ± 6.0 | 30 ± 7.7 |
| Blood glucose (mmol/l)   | 11 ± 2.7         | 10 ± 2.6 | 10 ± 2.3 |
| Total cholesterol (mmol/l) | 29 ± 6.7     | 8.8 ± 1.6* | 9.0 ± 1.8* |
| LDL                      | 26 ± 6.3         | 6.8 ± 1.3* | 7.0 ± 1.6* |
| HDL                      | 5.9 ± 2.2        | 2.6 ± 0.67* | 2.8 ± 0.58* |
| Triglycerides (mmol/l)   | 1.7 ± 0.52       | 1.5 ± 0.45 | 1.5 ± 0.53 |

Values are mean ± SD. One-way analysis of variance with Dunnett post-hoc test. *p < 0.01 vs. week 0.

ApoB = apolipoprotein-B; F = female; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LDLR = low-density lipoprotein receptor; M = male.
FIGURE 1  Macrophage Proportion or Phenotype in Atherosclerotic Lesions Is Not Affected by X19-mu Treatment

An atherosclerotic lesion from serial sections of the aortic root stained with (A) Movat’s pentachrome, (B) antibodies against Mac-3 in macrophages, (C) CCR2 in M1 polarized macrophages, and (D) CD206 in M2 polarized macrophages, as well as double immunofluorescence stainings with (E) Mac-3 and CCR2, or (F) Mac-3 and CD206. The percentage of intimal area (G) stained with Mac-3 and the (H) proportions of CCR2- and CD206-positive macrophages within the Mac-3 area shown as individual data points with mean ± SD. Student’s t-test for unpaired and paired (CCR2 vs. CD206) measurements; n = 10 to 16/staining/group. Scale bar = 100 μm (10 μm in inserts in E and F).
FIGURE 2 The Effect of X19-mu Treatment on Lesion Histology in Aortic Root Sections

(A) Atherosclerotic lesions in the aortic root stained with vascular cell adhesion molecule (VCAM)-1 (green), (B) intracellular cell adhesion molecule (ICAM)-1 (green), (C) interleukin (IL)-1β (red), (D) monocyte chemoattractant protein (MCP)-1 (red), as well as (E) Mac-3 and cleaved caspase-3 (yellow). All sections are counterstained with 4',6-diamino-2-phenylindole (blue, nuclei). (F) Lesion collagen content is quantified from Masson’s trichrome stainings (blue). Quantitative results are shown as individual data points in addition to mean ± SD in histograms; Fisher’s exact test for histological scores (A and B), and Student’s t-test for unpaired measurements (C to F); n = 10 to 16/staining/group. Scale bar = 100 μm (10 μm in inserts in E).
Triple immunofluorescence staining demonstrated PC-positive staining that co-localized with Mac-3-positive macrophages within atherosclerotic lesions and CD31-positive endothelial cells covering the lesions (Figure 3).

**FIGURE 3** PC Epitope Is Present in Endothelial Cells and Macrophages in Atherosclerotic Lesions

Representative section of the (A) aortic root showing immunofluorescence staining with phosphorylcholine (PC) antibody (PC-mAb), (B) Mac-3 antibody (macrophages), (C) CD31 antibody (endothelial cells), and (D) 4’,6-diamino-2-phenylindole (DAPI, nuclei). White arrows indicate endothelial cells and yellow arrows macrophage-rich area. (E) PC-positive staining co-localizes with endothelial cells covering the lesion and macrophages (white color in merge). Scale bar = 75 μm. Abbreviation as in Figure 2.

X19-mu TREATMENT PRESERVED CFR AND ENDOTHELium-MEDIATED VASODILATATION. The CFR was measured as the ratio of coronary flow velocity in the left coronary artery during adenosine stress and rest by Doppler ultrasound (Figure 3A).
Analysis of covariance for repeated measurements showed that the treatment with X19-mu was associated with a 33% improvement in CFR ($p = 0.047$) compared with vehicle during the 6-week study period (Figure 4B). Compared with week 0, CFR was 24 ± 20% lower after the 6-week treatment with vehicle (1.9 ± 0.29 vs. 1.4 ± 0.23; $p = 0.006$), whereas there was a trend toward a higher (9.0 ± 23%) CFR after treatment with X19-mu (1.6 ± 0.24 vs. 1.7 ± 0.24; $p = 0.32$) (Figure 4B). In healthy age-matched C57BL/6 mice, CFR was higher (2.1 ± 0.39) than in atherosclerotic mice after either vehicle or X19-mu treatment ($p < 0.001$ and $p = 0.003$, respectively).

The absolute flow velocities are shown in the Supplemental Table 2. In a separate group of atherosclerotic ApoE$^{-/-}$ mice, methacholine injection induced a transient reduction in arterial blood pressure (vasodilatory response) in X19-mu–treated mice, but not in the vehicle group (Supplemental Appendix, Supplemental Figure 3).

**PC-mAb Treatment Preserved No Production in HAECs.** To investigate the mechanistic effects of human PC-mAb treatment on endothelium, HAECs were stimulated with isolated Lp(a), the main carrier of PC/OxPLs (11). In HAECs, intracellular nitrate concentration was decreased after 24-h stimulation with Lp(a) in the presence of nonspecific IgG (median [25% and 75% percentiles]: 144 [143 and 150] pmol/×10^6 cells vs. 118 [117 and 123] pmol/×10^6 cells; $p = 0.049$) but was preserved in the presence of PC-mAb (135 [133 and 172] pmol/×10^6 cells, $p = 0.049$ vs. nonspecific IgG) (Figure 5A). VCAM1, ICAM1, and IL8 gene-expression tended to be lower in the presence of PC-mAb than nonspecific IgG but were not statistically significant. IL6 gene-expression as well as IL-6 and IL-8 protein levels were similar in the presence of PC-mAb and nonspecific IgG antibodies (Figure 5B).

**X19-mu Treatment Reduced 18F-FDG Uptake in Atherosclerotic Lesions.** Autoradiography showed focal uptake of 18F-FDG in macrophage-rich atherosclerotic lesions within the aorta (Figures 6A to 6C). Compared with vehicle-treated mice and adjusted by sex, the average uptake of 18F-FDG in atherosclerotic lesions normalized to activity in the lesion-free vessel wall (lesion-to-wall ratio) was significantly lower after the 6-week treatment with X19-mu (1.7 ± 0.24 vs. 1.5 ± 0.17, $p = 0.002$).
If analyzed separately, $^{18}$F-FDG uptake was reduced after X19-mu treatment in both females ($p = 0.023$) and males ($p = 0.034$) compared with vehicle (Supplemental Figure 4). In comparison to the $^{18}$F-FDG uptake at week 0 in the mice fed a high-fat diet (2.3 ± 0.24), the lesion-to-wall ratios were lower after the 6-week treatment on normal mouse chow in both the vehicle- ($p < 0.001$) and X19-mu–treated ($p < 0.001$) groups.

The uptake of $^{18}$F-FDG was further compared in lesions with low (average 22%), intermediate (29%), or high (35%) density of macrophages. The $^{18}$F-FDG uptake was gradually increased in these lesions and was highest in lesions with high density of macrophages ($p = 0.039$). However, X19-mu treatment reduced $^{18}$F-FDG uptake in lesions with low, intermediate, and high macrophage density compared with vehicle ($p < 0.001$) (Figure 6E). The $^{18}$F-FDG uptake in other tissues is presented in Supplemental Table 3.

**DISCUSSION**

Our results revealed that 6-week treatment with an exogenous antibody targeting PC epitope on OxPLs preserved CFR and reduced $^{18}$F-FDG uptake in atherosclerotic lesions in mice. These results provided evidence that the therapeutic antibody against PC had beneficial effects on coronary vascular function and inflammatory activity in the arterial wall in atherosclerosis. Furthermore, our results indicated that CFR and $^{18}$F-FDG PET could be used as possible surrogate markers for the efficacy of PC antibody therapy in future clinical studies.

Endothelial cell injury at early stages of atherosclerosis may lead to exposure of antigens, including OxPLs, that are normally hidden, eliciting an immune response and secretion of various disease-modifying antibodies (4,26). Previous studies indicated that OxPLs cause endothelial dysfunction with impairment of NO-mediated vasodilatation in arterial preparations (3,27). In patients with stable coronary artery disease, levels of the PC epitope in LDL particles were shown to be significantly related to the severity of endothelial dysfunction after lipid-lowering therapy (2). Furthermore, a negative correlation between OxLDL levels and CFR, an integrated measure of coronary reactivity (18), was found in young individuals with hypercholesterolemia (28). Our results extended the previous findings by

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**FIGURE 5** PC-mAb Treatment Preserves NO Production in Human Aortic Endothelial Cells

(A) Intracellular nitrate reflecting nitric oxide (NO) production was significantly decreased after 24-h stimulation with lipoprotein(a) (Lp(a)) in the presence of a nonspecific immunoglobulin-G (IgG) but preserved in the presence of PC-mAb. (B) The pro-inflammatory mediators, VCAM1, ICAM1, and IL8 gene-expression tended to be lower in the presence of PC-mAb than the nonspecific IgG antibody, but it was not statistically significant (B). Results are expressed as median with 25th and 75th percentiles for 3 independent experiments; Mann-Whitney U test; *p < 0.05. Ctrl = control; other abbreviations as in Figures 2 and 3.
providing evidence that a therapeutic antibody against PC preserved CFR in vivo in response to adenosine in hypercholesterolemic mice that had impaired CFR, despite the absence of obstructive coronary artery disease (22).

In HAECs stimulated with Lp(a), the main carrier of PC/OxPLs in the human plasma (11), the PC-mAb preserved NO production compared with that of a nonspecific IgG. In vivo, the methacholine provocation test further demonstrated enhanced vasodilatory response after X19-mu treatment. We previously showed that blood pressure response to methacholine could be blocked by pre-treatment with 50 mg/kg of the NO synthase inhibitor, L-Nitro-Arginine-Methyl Ester, which indicated NO- and endothelium-mediated mechanisms (29). These results indicated that the beneficial effects of X19-mu on CFR were at least partly mediated via direct effects on endothelial cell NO metabolism. In line with that, our immunofluorescence stainings demonstrated PC-positive staining in the aortic root sections co-localizing with endothelial cells and macrophages. Alternatively,
improved vasodilatory response might be a result of an anti-inflammatory effect of X19-mu, because inflammation is well known to hamper endothelium-mediated vascular control (4). Although it showed a modest effect, PC-mAb treatment did not lead to a statistically significant difference in the expression of pro-inflammatory mediators.

Uptake of $^{18}$F-FDG in atherosclerotic lesions correlates with the quantity of inflammatory macrophages with high glucose consumption (16). Regardless of reflecting macrophage polarization (39), it has been shown that OxLDL stimulates macrophage $^{18}$F-FDG uptake (31), and that $^{18}$F-FDG uptake is particularly high in the early phase of foam cell formation (32). Recently, increased $^{18}$F-FDG uptake in the arterial wall was found in patients with elevated Lp(a). Ex vivo experiments showed that the arterial inflammation was due to the OxPLs bound to Lp(a), because the E06 antibody prevented the pro-inflammatory effects of Lp(a) (11). The present study demonstrated that administration of a therapeutic antibody against PC reduced $^{18}$F-FDG uptake in atherosclerotic lesions with different macrophage densities in vivo, which indicated reduced metabolic activity and possibly reduced anti-inflammatory effects in atherosclerosis. In line with this and a previous study (15), IL-1β was reduced in the lesions after X19-mu treatment. However, no changes in macrophage apoptosis were observed. The absolute amount of reduction in $^{18}$F-FDG uptake (13%) was in line with the degree of reduction in arterial $^{18}$F-FDG uptake seen in clinical studies that used cholesterol lowering intervention with atorvastatin (5% to 15%) (16,17). Previously, treatment with human recombinant IgG1 antibody against a malondialdehyde-modified ApoB100 peptide, another immunogenic epitope on OxLDL, did not reduce arterial $^{18}$F-FDG uptake in hypercholesterolemic minipigs (33) or patients with stable inflammatory vascular lesions (34). These differences can be explained by differences in PC (phospholipid) and malondialdehyde (protein) epitopes, with the former being specifically associated with OxPLs (11) that are more prevalent in advanced, inflamed lesions (35).

Despite reduced $^{18}$F-FDG uptake, we did not find a reduction in overall lesion macrophage quantity, the proportions of M1/M2 polarized macrophages, the lesion collagen content, or the atherosclerosis burden. Based on our previous validation study (24), mice that showed extensive pre-existing atherosclerosis at the beginning of therapy were studied, and therefore, it was unlikely that major plaque regression or changes in plaque cellular composition would have occurred within short-term treatment. Previous studies indicated that a metabolic marker such as $^{18}$F-FDG uptake was sensitive to changes caused by short-term interventions, despite changes in plaque burden, and aortic $^{18}$F-FDG signal provided an independent predictor of future cardiovascular events (16,17). There are also regional differences in the driving forces of atherogenesis in mice. Although histology was analyzed in the aortic root, $^{18}$F-FDG uptake was measured throughout the thoracic aorta in different regions (36). Aortic root is the most common and validated region for atherosclerosis quantification, but also contains the most advanced lesions (36). Finally, macrophages in the lesions were mainly of the reparative M2 type before therapy, and there was a high variation in the proportions of M1/M2 polarized macrophages between mice; therefore, we could not exclude small effects of PC antibody treatment on macrophage phenotype.

**STUDY LIMITATIONS.** We did not assess $^{18}$F-FDG uptake repeatedly in the same mice because high radiation exposure related to the high-resolution angiography might have influenced health of study animals, and the partial volume effects could have impaired the accuracy of quantification of the signal in the small atherosclerotic lesions in vivo (37). Although the LDLR$^{-/-}$ApoB$^{100/100}$ mouse is a widely used model of atherosclerosis, with a lipid profile that closely resembles human familial hypercholesterolemia, the findings could not be directly extrapolated to humans. Because of the high variation in CFR values observed in mice in general (from 1.2 to >2.2) (23,38) and in individual mice in our study, larger studies are needed to confirm the magnitude of the treatment effect on coronary vascular function. The high-fat diet was discontinued at the time of intervention, to prevent the toxic effects of high cholesterol and to mimic a clinical situation, where any therapy would be prescribed on top of cholesterol-lowering intervention. Chimeric mouse-human antibody was used in this study, because of the possible risk of formation of neutralizing antibodies with a fully human antibody in mice. Saline was used as a control treatment in the in vivo study because of challenges in the production of a corresponding mouse-human chimeric IgG1 not binding to PC, as well as to reduce the risk of interference of nonspecific IgG with the naturally occurring antibody responses (26).

**CONCLUSIONS**

Six weeks of treatment with X19-mu, a chimeric IgG1 antibody against PC on OxPLs, preserved coronary...
artery function and attenuated uptake of 18F-FDG in atherosclerotic lesions in mice. The present findings provide evidence that X19-mu exerts therapeutic actions on endothelial cell function and inflammatory processes in the vessel wall, despite changes in lesion burden or cholesterol levels. Our results suggest that noninvasive imaging techniques, CFR and 18F-FDG PET measures, represent translational tools to assess the effects of PC-targeted therapy on vascular function and atherosclerosis in clinical studies.

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KEY WORDS atherosclerosis, coronary flow reserve, inflammation, 18F-fluorodeoxyglucose positron emission tomography, phosphorylcholine

APPENDIX For expanded Methods and Results sections and supplemental tables and figures, please see the online version of this paper.
Targeting Phosphorylcholine in Established Atherosclerosis?

Dimitrios Tsiantoulas, PhD

Atherosclerosis is a lipid-driven chronic inflammatory disease that results in the formation of atherosclerotic plaques, which on rupture or erosion lead to myocardial infarction and stroke. The recent clinical trial CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) has demonstrated the therapeutic value of immunomodulation in atherosclerotic cardiovascular disease (CVD) in humans and has paved the way toward the development of additional therapeutic strategies against the maladaptive immune response underlying atherosclerotic plaque formation (1).

A crucial step in the initiation and progression of atherosclerosis is the oxidation of low-density lipoprotein (OxLDL) in the subendothelial space of arteries. OxLDL displays oxidation-specific epitopes (adducts generated on oxidative modification) that are immunogenic and are recognized by different cell types including endothelial cells, T cells, monocytes, and macrophages, resulting in the triggering of proinflammatory responses (2). Oxidation-specific epitopes include phosphorylcholine (PC)-containing oxidized phospholipids that are also present on OxLDL and apoptotic cells and exhibit a strong proinflammatory and proatherogenic effect in vivo (3). Natural immunoglobulin M (IgM) antibodies (pre-existing germline-encoded products) are an important arm of humoral immunity and have the capacity to recognize oxidation-specific epitopes in both mice and humans. Mice lacking soluble IgM antibodies display dramatically accelerated atherosclerosis (4). Moreover, whereas the association of OxLDL-specific IgG levels in plasma with cardiovascular disease risk in humans requires further investigation, several epidemiological studies have demonstrated that anti-PC IgM levels in plasma are inversely associated with CVD (4). Thus, immunomodulatory strategies targeting PC may be beneficial in mitigating atherosclerosis.

In this issue of JACC: Basic to Translational Science, Ståhle et al. (5) investigated the effect of a monoclonal IgG1 anti-PC antibody (X19-mu) on inflammation and vascular function in established atherosclerosis in Ldlr−/− ApoB100/100 mice that were first fed an atherogenic diet for 12 weeks and then switched to regular chow diet for 6 weeks while they were treated weekly with the X19-mu antibody or saline. Treatment with the X19-mu antibody did not alter LDL, high-density lipoprotein, and blood glucose levels in plasma. Furthermore, the lesion size in mice treated with the X19-mu antibody was similar to lesions in the mice that received saline. In addition, X19-mu treatment did not alter the collagen deposition and total macrophage area as well as the proportions of M1- and M2-type macrophages in lesions. Despite similar macrophage and phagocytic cell content, mice that were treated with the X19-mu antibody displayed a modest reduction in interleukin (IL)-1β content in the lesions. Notably, compared with cells that were collected from control mice, peritoneal macrophages isolated from transgenic mice overexpressing a single chain variant of the anti-PC E06 antibody that had been injected with the oxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC), displayed strongly reduced IL-1β expression (5). Thus, it is conceivable that X19-mu treatment may contribute to reducing specific proinflammatory...
responses in macrophages and/or phagocytic cells in atherosclerotic lesions. Along this line, Ståhle et al. (5) showed reduced $^{18}$F-fluorodeoxyglucose uptake in lesions of X19-mu-treated mice compared with in lesions from control mice.

It is important to point out a significant limitation of the study by Ståhle et al. (5): their study lacks of a group of mice treated with an isotype control antibody. This is particularly important because only the Fc portion of the X19-mu is of murine origin, and thus an immune reaction against X19-mu clone during the 6-week treatment cannot be excluded, which could also potentially limit the effect of the antibody. Furthermore, the different effector functions of the Fc portion of IgG antibodies that display different affinity to Fcγ receptors may also determine the effect of such a therapeutic approach. Fcγ receptors are divided into 2 main categories—the activating and inhibitory receptors—which confer both proatherogenic and atheroprotective effects (6). In addition, the capacity to activate the complement system differs among the different IgG classes and thus these properties could be of importance with respect to the efficacy of an anti-PC IgG-based therapeutic strategy (6).

Previous studies have shown the atheroprotective effect of PC-based immunization or anti-PC IgM passive infusion strategies in the aortic root and vein-graft atherosclerosis (4). Ståhle et al. (5) showed that in contrast to saline-treated mice, treatment with the X19-mu antibody preserved the coronary flow reserve (on adenosine stress) in the left coronary artery before and after 6 weeks of treatment in mice with established atherosclerosis. These data show that anti-PC targeting has the capacity to improve coronary vascular function, which is highly relevant for human disease. To explore the underlying mechanism of the effect of X19-mu antibody intervention on coronary vascular function, Ståhle et al. (5) treated human aortic endothelial cells with lipoprotein(a). Treatment with lipoprotein(a) led to a decreased intracellular nitrate concentration in endothelial cells treated with the isotype control antibody, whereas cells treated with the fully human PC-specific monoclonal antibody X19-A05, which has comparable binding affinity to PC as the X19-mu antibody, preserved intracellular nitrate concentration. These data are consistent with the protective effect of the PC-specific IgM E06 antibody in inhibiting IL-8 production by endothelial cells stimulated with apoptotic cells (6).

In summary, the study by Ståhle et al. (5) provides new insights into the therapeutic effect of anti-PC immunotherapy in atherosclerosis, particularly with respect to the homeostasis of the coronary vascular function.

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KEY WORDS atherosclerosis, coronary flow reserve, inflammation, $^{18}$F-fluorodeoxyglucose positron emission tomography, phosphorylcholine
Systems Analysis Implicates WAVE2 Complex in the Pathogenesis of Developmental Left-Sided Obstructive Heart Defects

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VISUAL ABSTRACT

Edwards, J.J. et al. J Am Coll Cardiol Basic Trans Science. 2020;5(4):376–86.
HIGHLIGHTS

- Combining CHD phenotype-driven gene set enrichment and CRISPR knockdown screening in zebrafish is an effective approach to identifying novel CHD genes.
- Mutations affecting genes coding for the WAVE2 protein complex and small GTPase-mediated signaling are associated with LVOTO lesions.
- WAVE2 complex genes brk1, nckap1, and wasf2 and regulators of small GTPase signaling cul3a and racgap1 are critical to zebrafish heart development.

Genetic variants are the primary driver of congenital heart disease (CHD) pathogenesis. However, our ability to identify causative variants is limited. To identify causal CHD genes that are associated with specific molecular functions, the study used prior knowledge to filter de novo variants from 2,881 probands with sporadic severe CHD. This approach enabled the authors to identify an association between left ventricular outflow tract obstruction lesions and genes associated with the WAVE2 complex and regulation of small GTPase-mediated signal transduction. Using CRISPR zebrafish knockdowns, the study confirmed that WAVE2 complex proteins brk1, nckap1, and wasf2 and the regulators of small GTPase signaling cul3a and racgap1 are critical to cardiac development. (J Am Coll Cardiol Basic Trans Sci 2020;5:376-86) © 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/).

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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.
Congenital heart disease (CHD) is the most common clinically devastating birth defect (1). Among the multiple factors that drive CHD pathogenesis, genetic variants appear to be a primary driver (2,3). Despite greater understanding of the molecular mechanisms of heart development, our ability to definitively identify specific genetic causes for most patients has progressed more slowly.

A barrier to unraveling genotype-phenotype associations is the high degree of genetic heterogeneity, such that analysis of even a moderate-sized cohort results in a relatively low number of genes observed to be mutated recurrently (4,5). Hence, identifying novel CHD genes is well suited for network analysis, wherein discrete mutations and affected genes can be connected based on prior knowledge of gene-gene functional networks such as associations with developmental pathways and known protein-protein interactions. Together, these functional data can provide a platform to identify molecular pathways that link variants to prioritize identification and lead to the discovery of novel candidate causal CHD genes. Validation of selected genes in model systems will ultimately expand our ability to detect genetic etiologies for patients.

Initial whole exome sequencing from the Pediatric Cardiac Genomics Consortium (PCGC) identified a burden of de novo exonic mutations in genes with higher fetal heart expression (HHE) in sporadic, severe CHD accounting for 10% of cases (4). Gene Ontology enrichment analyses identified altered epigenetic regulators, particularly histone modifiers, as important for CHD pathogenesis. Subsequent PCGC whole exome sequencing studies have identified a significant overlap between HHE and high fetal brain expression for mutated genes identified in CHD probands—thus providing a potential mechanism for the frequent co-occurrence of extracardiac anomalies or neurodevelopmental delays (6). More recently, whole exome sequencing of this growing cohort of sporadic, severe CHD characterized the contribution of recessive variants in genes well established to cause CHD in human and mouse studies (7). To expand on these, we performed unbiased global analyses of phenotype-specific CHD-associated variants to prioritize candidate causal genes and identify pathways relevant for CHD pathogenesis.

**METHODS**

**EXOME SEQUENCING AND VARIANT FILTERING.** Whole exome sequencing results from 2,881 sporadic, severe CHD trios enrolled in the PCGC or Pediatric Heart Network were compared with whole exome sequencing from 900 control trios in Simon’s Foundation Autism Research Initiative Simplex Collection (6). The research protocols were approved by the Institutional Review Boards at each participating center—Boston’s Children’s Hospital, Brigham and Women’s Hospital, Great Ormond Street Hospital, Children’s Hospital of Los Angeles, Children’s Hospital of Philadelphia, Columbia University Irving Medical Center, Icahn School of Medicine at Mount Sinai, Rochester School of Medicine and Dentistry, Steven and Alexandra Cohen Children’s Medical Center of New York, and Yale School of Medicine. Briefly, sequencing for case and control trios was performed at Yale Center for Genome Analysis using NimbleGen v2.0 exome capture reagent (Roche, Basel, Switzerland) and Illumina HiSeq 2000, 75-bp paired-end reads (Illumina, San Diego, California). Three independent analysis pipelines were used to process reads and were mapped to hg19 using Novoalign (Novocraft Technologies, Selangor, Malaysia) and Genome Analysis Toolkit 3.0 (Broad Institute, Cambridge, Massachusetts) best practices at Harvard Medical School and BWA-mem at Yale School of Medicine and Columbia University Medical Center (8,9). Variant calls were made using GATK HaplotypeCaller (8). De novo variants not meeting the following criteria after pooling from the 3 pipelines were filtered out: depth (minimum 10 reads total and 5 alternate allele reads), alternate allele balance (minimum 20% if alternate read depth ≥10 or minimum 28% if alternate read depth <10), and parental read characteristics (minimum depth of 10 reference reads and alternate allele balance <3.5%).

Variant pathogenicity was assessed using in silico prediction from PredictSNP2 (Loschmidt Laboratories, Brno, Czech Republic), which employs an ensemble approach by integrating data from multiple in silico tools, for all variants other than frameshift mutations (10). As PredictSNP2 does not score frameshift mutations, we used Combined Annotation Dependent Depletion v1.4 for these variants (11). Combined Annotation Dependent Depletion, 1 of the tools which contributes to PredictSNP2, yields PHRED-scaled C scores, such that a score of ≥30 correlates to the top 0.1% of all possible variants and in prior studies has been used to discriminate pathogenic from tolerated frameshift variants (11). Variants were also filtered for those affecting HHE genes (4). The HHE gene set was previously identified using RNA sequencing of isolated strain 129/SvEV mouse hearts including atria, ventricles, and all 4 valves at embryonic day 14.5 to dichotomize 16,676 genes with identified human-mouse orthologues with a minimum of 40 reads per million mapped reads into
the top quartile of expression (4). The HHE gene list consists of 4,169 genes, and the low heart expression list consists of 12,507 genes.

We considered that the low heart expression genes may contain some genes critical to cardiogenesis, so in an orthogonal variant filtering, we excluded genes unlikely to have a role in cardiovascular development based on knockout mouse phenotypes using the Mouse Genome Informatics (MGI) knockout phenotype gene library (12). Specifically, a gene was excluded if its knockout had been phenotyped, and the knockout was not found to cause any cardiovascular related phenotype, embryogenesis phenotype, or embryonic or postnatal lethality.

Cardiac diagnoses were obtained from the PCGC Data Hub (13). Left ventricular outflow tract obstruction (LVOTO) (n = 802) included hypoplastic left heart syndrome, aortic coartation, and aortic stenosis. Conotruncal defects (CTDs) (n = 1120) included D-transposition of the great arteries, tetralogy of Fallot, double outlet right ventricle, truncus arteriosus, ventricular septal defects, and abnormalities of the aortic arch patterning. Heterotaxy (HTX) (n = 274) included patients with left-right isomerism as the primary defect. The remaining patients not included in 1 of the 3 phenotype categories consisted of a heterogeneous group of defects including atrial septal defects and anomalous pulmonary venous connections, pulmonary valve lesions, atrioventricular canal defects, double inlet left ventricle, and tricuspid atresia.

**Gene Set Enrichment Analysis.** We performed gene set enrichment analysis for Gene Ontology biological processes (GOBP) terms and Comprehensive Resource of Mammalian Protein Complexes (CORUM) using HHE in silico and MGI library in silico-filtered variants and the hypergeometric test. Enrichment analyses were performed with Enrichr (Icahn School of Medicine at Mount Sinai, New York, New York), which, in addition to implementing the hypergeometric test, also employs a ranking method that combines the adjusted p value with a deviation from the expected rank based on enrichment analysis applied to random gene sets (z-score) (14). In addition, we repeated the enrichment analysis with the loading of 2 different background reference lists; 1 made of the 4,169 HHE genes and the other made of the entire human genome excluding the MGI library phenotype negative genes using WebGestalt (Vanderbilt University Medical Center, Nashville, Tennessee) with the default settings (15). The reference and background gene lists are available in supplemental information. A Benjamini-Hochberg-adjusted p < 0.05 using both tools, Enrichr and WebGestalt, was used for significance with significant terms ranked by Enrichr’s combined score. Gene Ontology enrichment analysis was performed for controls, all cases, and LVOTO, CTD, and HTX gene lists.

Given that among the CHD phenotype-specific groups, significant enrichment was only observed for the LVOTO group, we performed a protein-protein interaction (PPI) network analysis only for this group to prioritize candidate genes. We generated an LVOTO-specific PPI network, using GeNets (Broad Institute, Cambridge, Massachusetts), by inputting all LVOTO genes associated with all GOBP and CORUM terms identified as enriched including terms which were observed as enriched when using only Enrichr or WebGestalt enrichment analyses. Then we ranked genes associated with the consistently enriched terms by their number of connections identified in the LVOTO-specific PPI network.

A flow diagram of the variant filtering and enrichment analyses is provided in Supplemental Figure 1.

**Modeling Loss of Candidate Genes in Zebrafish Embryos.** Clustered regularly interspaced short palindromic repeats (CRISPR)-mediated knockdown experiments were performed for candidate genes in zebrafish F0 embryos to assess morphological phenotypes, as described previously (16). Briefly, wild-type AB strain zebrafish embryos were injected at the 1- to 2-cell stage with zebrafish-optimized Cas9 protein and CRISPR RNAs targeting abbi, brki, cyfp1, cul3a, nckap1, racgap1, or wasf2. CRISPR design and construction were performed by the University of Utah Mutation Generation and Detection Core using standard best practice procedures. Single guide RNAs were designed for high efficiency and low off-target effects, and concentrations for single guide RNA and cas9 protein were also titrated for optimal impact. The mutagenic efficiency of each CRISPR and validation of double-stand breaks was assessed using high-resolution melting analysis performed on genomic DNA from individual injected embryos (17). CRISPR target sequences and high-resolution melting analysis primer sequences are listed in Supplemental Table 1. Zebrafish embryos were phenotyped at 2 days past fertilization. To evaluate cardiac morphology and function, wild-type and mutant lines of zebrafish were evaluated on a cmlc2-GFP background and visualized with a fluorescent microscope.

**Statistics.** The burden of de novo mutations in controls was compared with that found in all CHD cases and then separately for the LVOTO, CTD, and HTX phenotype groups. A 2-tailed chi-square analysis was performed to generate the odds ratio with 95%
confident interval comparing the number of filter-selected variants (in silico, HHE in silico, or MGI library in silico) to filtered-out variants for each group compared with controls. As described previously, gene set enrichment was assessed using a hypergeometric test. All reported p values were adjusted using the Benjamini-Hochberg method. A p value <0.05 was considered statistically significant and R software version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

**DATA AVAILABILITY.** Whole exome sequencing data have been previously deposited in the database of Genotypes and Phenotypes (dbGaP) under accession number phs000571.v1.p1, phs000571.v2.p1, and phs000571.v3.p2.

**RESULTS.**

**EXOME SEQUENCING AND VARIANT FILTERING.** Consistent with the initial PCGC cohort and as published for this expanded PCGC cohort, the rate for all de novo variants in patients with CHD was not significantly different from healthy controls (1.04 vs. 1.05 variants/individual) (4,6,7). This was also true for the LVOTO (n = 802), CTD (n = 1120), and HTX (n = 274) groups (1.08, 1.03, and 0.90 de novo variants per individual, respectively). Variant pathogenicity was assessed using in silico prediction from PredictSNP2 and Combined Annotation Dependent Depletion (10,11). With the exception of HTX, which did not demonstrate significant burden with any combination of filters, all cases, LVOTOs, and CTDs exhibited increasing burden with each layer of filtering (Table 1). The HHE in silico filter demonstrated the greatest burden, with odds ratios of 1.7, 2.0, and 1.7 (p < 0.001, for all) for all cases, LVOTOs, and CTDs, respectively, with comparable burden identified using the MGI library in silico.

**GENE SET ENRICHMENT ANALYSES.** We performed GOBP and CORUM gene set enrichment analyses using the hypergeometric test with the MGI library in silico- and HHE in silico-filtered gene lists derived from all cases, CHD phenotypes, and control groups. Hypergeometric tests were performed using Enrichr and WebGestalt as described in the Methods (14,15). Using the MGI library in silico-filtered gene set from all cases, we identified 181 GOBP and 4 CORUM enriched terms. Similarly, using WebGestalt, we identified 140 GOBP and 3 CORUM enriched terms, of which 17 common GOBP and 2 common CORUM terms identified as significant with both tools (Supplemental Table 2). The relatively low overlap in enriched terms between Enrichr and WebGestalt is likely due to different methods to convert the Gene Ontology tree into a gene set library and different versions of the Gene Ontology tree. Notable enriched terms included the following developmental pathways and processes: heart morphogenesis, vasculogenesis, VEGF receptor signaling, beta-catenin-TCF complex, and regulation of actin filament-based processes. Using the same approach for the LVOTO phenotype genes, we identified enrichment for 23 GOBP and 3 CORUM enriched terms using Enrichr and 39 GOBP and 2 CORUM enriched terms using WebGestalt, with 2 from each library consistently enriched using both tools: regulation of actin filament-based processes, VEGF receptor signaling, WAVE2 complex, and ITGA6-ITGB4-Laminin 10/12 complex (Table 2).

Similar themes emerged from enrichment results using HHE in silico-filtered gene sets for all cases and LVOTO groups but with overall narrower overlapping results. From all cases, 4 GOBP and 1 CORUM term were consistently identified as enriched: heart morphogenesis, ephrin receptor signaling, regulation of small GTPase-mediated signal transduction, actomyosin structure organization, and BAF complex (Supplemental Table 3). For LVOTO, the highest-ranked CORUM protein complex identified by Enrichr was WAVE2. One GOBP term was consistently enriched using both tools: regulation of small GTPase-mediated signal transduction (Table 2).
Much less enrichment was observed for the CTD, HTX, and control gene lists, with no terms reaching statistical significance for the control or the HTX MGI library in silico or HHE in silico gene lists using either tool. For CTDs, only 1 GOBP term—pericardium—was consistently enriched using the MGI library in silico-filtered genes. No term was consistently enriched using HHE in silico-filtered CTD genes. Complete results of patient phenotypes and variants and complete enrichment analyses results are included in the Supplemental Appendix.

Across all enrichment analyses, LVOTO-driven WAVE2 complex enrichment demonstrated the highest Enrichr combined score and WebGestalt fold enrichment. Variants affecting 3 of the 5 genes encoding proteins within the WAVE2 complex and the direct regulator of WAVE2 (RAC1) were identified in LVOTO probands with none identified in other phenotypes: nonsense variants in ABI1 (p. R106X) and NCKAP1 (p. E1057X) and damaging missense variants in CYFIP1 (p. S35L) and RAC1 (p. R68C). PredictSNP2 scores ranged from 0.69 to 1.0 (maximum) and C scores were >32 (0.06% most damaging variants) for these 4 variants. The WAVE2 complex functions downstream of the small GTPase RAC1 to regulate branched actin synthesis and influence actin cytoskeleton organization in multiple cellular processes, including cell migration through formation of lamellipodia (18,19). In this context, it is notable that in addition to consistent enrichment observed for actin filament-based processes and regulation of small GTPase signaling, terms related to lamellipodium and actin cytoskeleton development were identified as enriched in at least 1 context.

We considered that visualizing protein network interactions between genes associated with the LVOTO enriched terms would allow us to better prioritize candidate genes for downstream validation. To that end, we used Metanetwork v1.0 (Broad Institute) predicted-protein-interactions available from GeNets to interrogate for interactions between the 35 genes associated with the 5 consistently enriched GOBP and CORUM terms and all genes associated with all LVOTO enriched terms (Figure 1) (20). These 35 genes had a median of 3 connections in this LVOTO-specific protein network with CUL3, CDC42, CYFIP1, NCKAP1, NF1, NOTCH1, RAC1, RACGAP1, and RAF1 being in the top quartile with 7 to 14 connections each. Unsurprisingly, most of these genes are already strongly implicated in cardiac development and CHD (21–25). Notably, both CUL3+/- and RACGAP1+/- result in embryonic lethality, suggesting a possible role in heart development, which is further supported by a more recent cardiomyocyte-specific CUL3+/- demonstrating a developmental cardiomyopathy and postnatal lethality (26–28).

The role of the WAVE2 complex, which consists of the 3 previously mentioned genes as well as BRICK1 and WASF2, in cardiogenesis is mostly unknown. Mouse knockout studies of Abi1, Brck1, and Cyfip1 resulted in embryonic lethality, with the only reported cardiac phenotype being hemorrhagic pericardial edema or discontinuous cardiac tissue layers from loss of Abi1 (29–32). Additionally, loss of Wasf2 resulted in reversed cardiac looping in 1 of 2 mouse models and loss of Nckap1 resulted in cardiaca biffida in mice (33–35). Therefore, we selected the 5 WAVE2 complex genes, CUL3a, and RACGAP1 for further validation in zebrafish embryos.

| Gene List         | Library | Term                                      | Combined* | Fold†  | Adjusted p Value† | Genes                                      |
|-------------------|---------|-------------------------------------------|-----------|--------|-------------------|--------------------------------------------|
| MGI library in silico | CORUM   | Wave-2 complex                            | 1709.0    | 32.6   | 0.011             | ABI1;CYFIP1;NCKAP1                         |
| MGI library in silico | CORUM   | ITGAV-ITGB4-Laminin10/12 complex           | 692.9     | 32.6   | 0.011             | LAMAS5;LAMB1;LAMC1                         |
| MGI library in silico | GOBP    | Regulation of actin filament-based process | 77.1      | 3.5    | 0.003             | ABL2;DLC1;TEM1;ASAP3;SPTA1;S5H2;ANK2;TSC1;SMAD4;CYFIP1;NCKAP1;LRP1;RAC1;CDC42;RYR2;MTR;ROCK1 |
| HHE in silico     | GOBP    | Regulation of small GTPase-mediated signal transduction | 73.6      | 3.8    | 0.002             | FOXM1;NF1;NOTCH2;NOTCH1;ARAP3;DLC1;CUL3;SIPA1L1;ITSN2;KALRN;TNFAIP3;JHOT2;RACGAP1;RAF1;RAC1;CDC42 |
| MGI library in silico | GOBP    | Vascular endothelial growth factor receptor signaling pathway | 63.5      | 5.3    | 0.017             | MYOF;ABI1;CYFIP1;NCKAP1;RAC1;CDC42;ROCK1 |

Gene set enrichment was performed using HHE and MGI library in silico-filtered genes with the hypergeometric overrepresentation test implemented using Enrichr and WebGestalt. Terms were filtered for statistical significance if Benjamin-Hochberg-adjusted p < 0.05 using both tools. *Combined score derived from Enrichr, which is a unique ranking system that combines the adjusted p value with a deviation from expected ranking for each term based on inputting random gene sets. †Fold enrichment and adjusted p values presented from WebGestalt using background gene list correction. CORUM = Comprehensive Resource of Mammalian Protein Complexes; GOBP = Gene Ontology biological processes; other abbreviations as in Table 1.
**MODELING LOSS OF CANDIDATE GENES IN ZEBRAFISH EMBRYOS.** Zebrafish was selected for gene knockdown as its ability to survive via oxygen diffusion in the absence of a functional cardiovascular system permits the study of lethal cardiac defects later in development (36). Using CRISPR-guided knockdown, we observed cardiac phenotypes in F0 embryos with mosaic loss of brk1, cul3a, nckap1, racgap1, and wasf2 (Table 3). Reversed cardiac looping was observed in brk1, cul3a, nckap1, and wasf2 knockdown embryos, while racgap1 knockdown resulted in zebrafish embryos with a small, poorly contractile ventricle often
with associated atrial dilation (Figure 2). Notably, all 7 of these genes are both highly expressed in the developing heart and brain of the mouse, but a brain phenotype was only observed in racgap1 knockdown embryos (6).

**DISCUSSION**

**HUMAN GENETICS.** In this study, we used whole exome sequencing of 2,881 well-phenotyped, sporadic CHD trios and compared these with 900 control trios to identify de novo predicted damaging mutations using in silico, developmental heart expression, and previous implications in development or cardiac disease in knockout mouse phenotypes. As gene set enrichment analyses are sensitive to both the length of the gene list associated with each term and the background gene list, we performed enrichment analysis with different methods and considered not only statistical significance, but also consistency across tools and algorithms.

Through phenotype-driven elaborate gene set enrichment analyses, we identified a novel association between the LVOTO phenotype and genes associated with the WAVE2 complex, actin-filament based processes, and small GTPase signal transduction. The WAVE2 complex comprises 5 proteins (gene symbols in parentheses when different)—WAVE2 (WASF2), HSPC300 (BRK1), CYFIP1, NCKAP1, and ABI1.

**TABLE 3**

| Target Gene | Reversed Heart Looping | Atrial and Ventricular Size | Circulation | Head/Brain/Eye Structures |
|-------------|------------------------|-----------------------------|-------------|---------------------------|
| cul3a       | 8/64 (13)              | Normal                      | Normal      | Normal                    |
| nckap1      | 6/105 (6)              | Dilated atria and small ventricle 42/105 (42) | Normal | Hypoplastic 99/105 (95) |
| brkt        | 23/122 (19)            | Normal                      | Normal      | Normal                    |
| nckap1      | 16/83 (19)             | Normal                      | Normal      | Normal                    |
| cyfpl1      | 1/75 (1.3)             | Normal                      | Normal      | Normal                    |
| abtl        | 1/72 (1.4)             | Normal                      | Normal      | Normal                    |

Values are n/N (%). Most (5 of 7) candidate genes demonstrate abnormal cardiac development in CRISPR-mediated knockdown, including reversed cardiac looping in 3 WAVE2 complex genes (brkt, nckap1, and nckap1 and cul3a). Loss of racgap1 also demonstrated extracardiac anomalies in addition to a small ventricle and atrial dilation.

CRISPR = clustered regularly interspaced short palindromic repeats.

**FIGURE 2**

Crispr-Mediated Candidate Gene Knockdown in Zebrafish

(A, C) Wild-type (WT), (C) cul3a, and (D) racgap1 knockout (KO) zebrafish at 2 days past fertilization. Reversed cardiac looping illustrated in cul3a KO as compared with WT, both on a cmic2-GFP background. The racgap1 KO zebrafish demonstrate atrial dilation and pericardial edema. At = atria; CRISPR = clustered regularly interspaced short palindromic repeats; Ve = ventricle.
and ABI1—and is regulated by the small GTPase RAC1 to mediate branched actin synthesis, a key contributor to actin cytoskeleton organization, via Arp2/3-mediated actin polymerization (18). Enrichment analyses also identified CUL3 and RACGAP1 as mediators of small GTPase signaling. Placing the protein products of these genes within a PPI network we further highlight their strong connections within an LVOTO-specific network.

In contrast to LVOTO, the other phenotype groups demonstrated significantly less term enrichment. Although CTDs had similar variant burden to LVOTO, we hypothesize that genetic and phenotypic heterogeneity within this group may have limited our ability to identify consistent term enrichment with our approach. For HTX, we failed to identify a burden of mutations or identify any significant term enrichment using any combination of enrichment tools or gene filter. The smaller size of the HTX cohort compared with the other phenotypes may have limited our power for enrichment analysis, but limiting to de novo variants may also have missed significant genetic contributors. Recent results from the PCGC highlight variants that recessively inherited variants contribute substantially to pathogenesis of patients with CHD and laterality defects, suggesting that this model of inheritance needs to be incorporated into all future gene pathway enrichment studies for patients with HTX (7).

**Candidate Gene Validation in Zebrafish.** Given consistency of LVOTO gene set enrichment, we selected candidate genes from this group for validation. We identified reversed cardiac looping with loss of brk1, cul3a, nckap1, and wasf2 and a small ventricle with atrial dilation and pericardial edema with loss of racgapt1 in F0 zebrafish embryos. It is difficult to directly compare reversed cardiac looping in zebrafish with LVOTO defects in humans. Being comprised of only 2 chambers renders the developing zebrafish heart vulnerable to looping defects from mechanisms other than perturbed sidedness including altered myocardial cell polarity, cell number, or blood flow (37). Thus, while these results in zebrafish cannot definitively implicate these genes in LVOTO, we can conclude their critical nature to cardiac development and establish an association with mutations in these genes and LVOTO in humans.

**Proposed Molecular Mechanisms.** Recently, the Lo lab has developed a mouse forward genetics screen coupled with fetal imaging and whole exome sequencing of founder mice exhibiting a cardiovascular malformation to identify recessively inherited variants in novel CHD genes (38). This model has illustrated a role for complex genetic inheritance in multiple CHD phenotypes including the severest form of LVOTO, hypoplastic left heart syndrome, in the Ohia mouse line (Sap130<sup>m/m</sup>/Pcdha9<sup>m/m</sup>) (39). These genes were further validated in LVOTO pathogenesis after identifying mutations affecting SAP130 and a related gene, PCDHA13, in patients with hypoplastic left heart syndrome. Kyoto Encyclopedia of Genes and Genomes network analysis of differentially expressed genes in the Ohia right and left ventricular tissue by RNA sequencing and chromatin immunoprecipitation sequencing implicated multiple developmental pathways including Notch, Wnt, Tgfβ, and hedgehog signaling, as well as biological processes including extracellular matrix receptors, regulation of actin cytoskeleton, axon guidance, and metabolism. WAVE2 is a highly conserved regulator of actin cytoskeleton and cell morphology during development, a process that is critical to regulating cell polarity, cell migration, cytokinesis, and tissue architecture (35,40,41). Interestingly, the noncanonical Wnt planar cell polarity pathway, which regulates cell polarity during development via small RhoGTPase regulation including RAC1, was identified as enriched using Enrichr with both MGI library in silico and HHE in silico-filtered LVOTO gene lists and could provide a mechanistic link between altered WAVE2 complex activity and CHD (42).

Knockout studies in mouse and zebrafish demonstrate that planar cell polarity genes influence heart development by regulating directional migration of progenitor cells, septation of the primitive heart tube, and patterning of cardiac structures (43). In loss of Wnt5a and mutants of Vangl2 and Dvl2 (which connect planar cell polarity to RhoGTPase signaling), abnormal outflow tract development, reduced cardiomyocyte polarity, and actin polymerization defects in cardiac progenitor cells are observed (44). The direct link between planar cell polarity and WAVE2 complex signaling is the RhoGTPase RAC1. Early lethality has prohibited studying global loss of Rac1, but second heart field-specific knockdown of Rac1 resulted in abnormal right ventricular cardiomyocyte polarity with inhibited second heart field progenitor cell migration and concomitant decreased expression of WAVE2 complex genes and Arp2/3 in embryonic right ventricular tissue (22). Taken together, these studies illustrate how altered signaling via planar cell polarity/Rac1/WAVE2 can disrupt normal mammalian heart development.

In addition to contributing to small GTPase-mediated signaling, loss of CUL3 may also contribute to CHD through its role in ubiquitination (45). In HEK293 cells, SAP130, a regulator of
ubiquitination via cullin-RING ligases and 1 of 2 proteins implicated in the mouse *Ohia* line, binds CUL3 with higher affinity than other CUL proteins (46). Additionally, *Lztr1* knockout in the mouse and loss of function *LZTR1* mutations in humans have been implicated in Noonan syndrome through decreased CUL3-mediated RAS ubiquitination and ultimately increased RAS/MAPK signaling (47,48). Further, in the LVOTO-specific protein network generated in this study, the canonical ubiquitination regulator *UBC* demonstrated the highest number of connections including with *CUL3*. Thus, the mechanism of CUL3 loss leading to developmental cardiac defects might also be related to dysregulated ubiquitination.

Finally, loss of *cypf1* and *abi1a*, unlike the other WAVE2 complex genes, was not found to impact zebrafish heart development. Zebrafish, unlike mouse or human, have 2 orthologs for *ABI1* (*abi1a* and *abi1b*) with *abi1b* likely compensating to maintain normal cardiac development. Similarly, loss of *cypf1* may be compensated by *irsp53*, which in mice has been shown to also facilitate binding of activated Rac to WAVE2 (49).

**CONCLUSIONS**

Despite rigorous efforts to unravel the genetic mechanisms for severe forms of LVOTO pathogenesis, the etiology for most patients has largely remained elusive with only recent evidence confirming a role for *SAP130* and *PCDHA13* (39). Here, we exploited the strength of gene pathway enrichment analyses from whole exome sequencing results of sporadic, complex CHD trios to identify an association with LVOTO in humans and WAVE2 complex, actin-filament regulation, and small GTPase signaling genes. Furthermore, we confirmed a role for *brk1*, *cul3a*, *nckap1*, *racc1*, and *wasf2* in cardiac development using CRISPR mediated knockdown in zebrafish. Ultimately, we illustrate that combining phenotype-driven gene set enrichment analyses with validation in zebrafish is as an effective approach for identifying novel CHD genes. Given evidence linking planar cell polarity pathway to WAVE2 complex activity via small GTPase signaling, we propose this as a promising framework for future mechanistic investigation into LVOTO.

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**PERSPECTIVES**

**COMPETENCY IN MEDICAL KNOWLEDGE:** Genetic variants including single gene mutations or larger genomic changes, such as copy number variations or aneuploidy, contribute substantially to CHD pathogenesis. With the decreasing cost of next-generation sequencing, there is more widespread genetic testing, including single gene mutations or larger genomic changes, such as copy number variations or aneuploidy, contribute substantially to CHD pathogenesis. With the decreasing cost of next-generation sequencing, there is more widespread genetic testing, yet an unequivocal genetic cause is not identified in up to 80% of CHD patients. Closing this gap in our fund of knowledge has significant implications on genetic counseling for our patients and their families.

**TRANSLATIONAL OUTLOOK:** Predicted damaging mutations affecting genes in the WAVE2 complex and related genes are probably causative of CHD and, more specifically, LVOTO.

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APPENDIX For supplemental databases, tables, and a figure, please see the online version of this paper.
Limitations of Animal Studies for Predicting Toxicity in Clinical Trials

Part 2: Potential Alternatives to the Use of Animals in Preclinical Trials

Gail A. Van Norman, MD

SUMMARY

Dramatically rising costs in drug development are in large part because of the high failure rates in clinical phase trials. The poor correlation of animal studies to human toxicity and efficacy have led many developers to question the value of requiring animal studies in determining which drugs should enter in-human trials. Part 1 of this 2-part series examined some of the data regarding the lack of concordance between animal toxicity studies and human trials, as well as some of the potential reasons behind it. This second part of the series focuses on some alternatives to animal trials (hereafter referred to as animal research) as well as current regulatory discussions and developments regarding such alternatives.

In increasingly, toxicologic testing in animals and preclinical animal studies in drug development have been questioned because of poor correlation with in-human results (1). In addition, public opinion plays a key role in determining how animal research is funded and regulated, and public opinion polls both in the United States and Europe demonstrate a steadily growing unease among the public sector for use of animals in industrial and pharmaceutical toxicity testing. Trends in public opinion regarding animal research in Great Britain, for example, demonstrated relatively steady approval (75%) of animal research between 1999 and 2010, so long as there was no alternative. However, public opinion began to shift significantly around 2012, with approval of animal research in the face of no alternative dropping to 66%, and less than one-half of the public agreeing that scientists could be trusted to be truthful about the experiments they were running (2). Annual Gallup polls in the United States demonstrated a steady decline in public opinion about the “moral acceptability” of animal research, from 65% approval in 2001 to 51% in 2017 (3). Opposition to animal research grew during the same period from 25% to 44%. Less than one-half of Americans 18 to 34 years of age felt that medical testing on animals was acceptable (4).

In 2003, the seventh amendment to the European Union’s Cosmetics Directive (5) stipulated an end to animal testing in the European Union for cosmetic purposes by 2009, regardless of the availability of alternative models by that time. In 2013, the National Institutes of Health (NIH) announced a program to phase out all research involving chimpanzees, and in
To end all funding for invasive research involving chimpanzees (6, 7). In 2016, Congress called on the NIH to review its policies related to research involving all nonhuman primates, and in 2017, a spending bill was passed by Congress and signed into law that required the Department of Veterans Affairs to suspend all dog research not specifically approved by the agency’s secretary (8). In other parts of the globe, similar trends are demonstrated across Europe (9) and in New Zealand, Australia, and Japan (2). Animal research will not be entirely replaced by other methods anytime soon, but the potential for faster and ultimately less expensive commercialization of therapeutics, together with the reduction of animal use in the early phases of drug development, has helped to increase research funding for alternatives.

Overall, the use of alternative methods for product testing and preclinical testing of medical drugs and devices has increased dramatically over the last quarter of a century. Between 1990 and 2015, the number of published papers using “alternative animals” (e.g., insects, fish, worms and shrimp) and in silico analysis increased over 900%. In 2015, more than 88,000 studies were published using in silico modeling, versus just 7,405 studies in 1990. Over the same period, testing and research use of guinea pigs and rabbits, previous favorites in the cosmetic industry, fell by 68% and 40%, respectively, although the use of rats and dogs remained stable, and the use of mice increased (10).

**Predicting negative side effects of putative therapeutic agents is an important goal in drug development:** prohibitive side effects or toxicity are a major reason why drugs that reach clinical trials fail to progress to market (lack of efficacy and lack of commercial interest are others). Animal toxicity testing fails to predict toxicity in almost 50% of drugs in the pipeline between Phase I trials and early post-market withdrawals (1), well after significant time and resources have been spent on what will prove to be a “failed” therapeutic. Testing that predicts probable late and expensive “drop outs” during drug development—termed a “fail early” strategy—would not only reduce costs, but also allow development resources to be redirected toward agents more likely to pass clinical trials.

Alternative methods of drug testing that provide more consistent, rapid, and translatable results will also increase human safety.

**In silico modeling.** Early prediction of human toxicity is critical in decreasing the costs of drug development, and in silico testing has recently been promoted as an important, human-based tool for preclinical evaluation (11). In silico, or computational, modeling refers to experiments or elements of experiments that are performed on computer computational models or via computer simulation. An in silico method is not simply a statistical analysis of known experimental data, but rather is a modeling approach using known characteristics of a chemical or chemical similar to it, information about the underlying chemical or biological system in which it will be used, and, when available, known preclinical and clinical data to predict untested system-level behaviors of a given chemical. Mayourian et al. (12) provide a detailed review of various in silico techniques particularly relevant to cardiovascular (CV) research.

One of the most important advantages of in silico testing involves scale—in the number of chemicals that can be tested quickly (throughput), the types of endpoints and biological pathways covered, and the range of conditions that can be rapidly simulated. Computational research does not yet provide complete replacement of animal experimentation in drug development but can significantly increase the scale and speed of preclinical drug development, thus reducing expense in animal testing phases, leveraging information from fewer experimental animals to increase drug development, decreasing the timeline for new drugs to enter the market by reducing the time for preclinical testing, reducing overall costs of drug development, and improving access to new and novel therapies for patients in need. Today, practically all toxicological research already includes in silico elements (13).

Another potentially important advantage of in silico testing is its potential to identify areas for rescue or repurposing of existing drugs (14). Rescue refers to redirecting drugs that have already failed efficacy trials for one application, but then can be successfully reintroduced into clinical trials for another indication. Repurposing of a drug (also called “repositioning” or “repurposing”) involves reapplying drugs that are either in clinical use already, or might have been retired from further development or marketing—because of production of a more efficacious drug, risks of adverse effects, or commercialization considerations—to new indications. In both cases, preclinical phases and animal testing can often be bypassed altogether, and if human clinical phase
trials are needed, they may be abbreviated to late-phase studies for the new indication, saving years of clinical testing (15). Costs of drug approval for repurposing or rescue are estimated by some authors to be around $40 to $80 million, compared with $1 to $2 billion for the development of an entirely new entity (16). Repurposing of approved drugs may even be accomplished without going through reapproval at all, as once the U.S. Food and Drug Administration (FDA) has issued a marketing approval for any purpose, off-label use for other purposes is at the discretion of the physician, so long as the physician feels it is “medically appropriate for their patient” (17). A contemporary example of repurposing an established drug is the proposed use of chloroquine—an approved treatment for malaria, long used to treat some autoimmune diseases such as lupus—for treatment of coronavirus infection. Chloroquine has the advantage of already being approved (which means that animal toxicity studies do not need to be repeated) and it has a human side effect profile that is largely understood, at least in its current usages. It is an FDA-approved drug that could therefore be used off label without FDA-approved human clinical trials, and is immediately available. However, it is critical to point out that, as of this writing, it is not known to be efficacious against coronaviruses, and it has significant potential human toxicity. It is vital that reasonable human data be generated before it can be determined whether or not it should be recommended for coronavirus patients—to avoid unknown toxicities and to avoid creating a shortage of drug that would not only be of minimal benefit to pandemic victims, but also prevent patients who are known to benefit from it from being treated.

While computer models alone would seem to be an unlikely predictor of drug behavior within complex physiologic systems or human beings, such modeling is nevertheless proving to be both effective and clinically relevant. In 2012, University of California, San Francisco researchers were able to successfully “predict” side effects (i.e., not merely the adverse side effects) of 656 drugs on the market, based on a computer model that compared the similarity of the drugs’ chemical structures to other molecules known to cause the same side effects (18), showing that such models could potentially be used early in drug development to prioritize which agents to pursue or repurpose. Of note, 26% of the side-effect targets identified by the computer model were entirely unrelated to the previously known targets of the drugs (39 of 151), suggesting that previously unrecognized therapeutic effects might be found for existing drugs, allowing them to be repurposed for new diseases and conditions. Despite these exciting findings, however, the model suffered from similar problems of poor specificity to that of animal studies: almost one-half (46%) of its predictions of adverse drug activity proved to be false. Luechtedefeld et al. (19) demonstrated that a computer comparison algorithm predicted toxicity for thousands of chemicals across 9 types of tests—from inhalational injury to hazards to aquatic environments—with accuracy similar to that of animal models, and with better reproducibility.

CV toxicity, specifically related to QT prolongation and arrhythmogenicity, is a major cause of drug relabeling or market withdrawal, second only to hepatotoxicity; from 1990 to 2006, it constituted 26% of drug withdrawals from the market (20). As a result, guidelines for new drug development include clinical testing for electrophysiologic changes that might predict QT changes specifically (21). In vitro assays relevant to this change are relatively easy to perform but demonstrate marginal likelihood ratios for predicting toxicity (22). When combined with computational simulations, Lancaster and Sobie (23) were able to use solely in vitro preclinical data to correctly classify 86 drugs as torsadogenic or non-torsadogenic approximately 90% of the time. In 2017, Passini et al. (24) found that a computer simulation of human heart cells was better able to predict risk of arrhythmogenicity of 62 drugs—including analgesics, antihistamines, and antibiotics—than animal testing (accuracy of 89% to 96% vs. 75% to 85%, respectively). In 2019, Moreno et al. (25) used computational modeling to design a novel in silico mexiletine “booster” that may improve the efficacy of mexiletine in suppressing arrhythmias.

There are a number of problems yet to solve regarding computational methods. As with animal research, lack of specificity could prompt unnecessary testing during drug development, or alternatively stop a safe and efficacious compound from progressing to further drug development because of false positive toxicity findings (26). Currently there is lack of widespread understanding of computational model construction, creating a “black box effect” that limits trust and acceptance of in silico data in preference to the familiarity of animal research, which may be less reliable. Additionally, a major challenge to computer modeling is achieving the computational power necessary to sufficiently simulate complex mechanical and physiological systems.

The CompBiomed Project, initially funded by the European Union in 2016 and re-funded in 2020 for another 4-year period, has the goal of creating an
entire in silico human organism for use in drug testing, disease modeling, and even personalized approaches to individual patient therapy based on patient-specific modeling and simulations (27).

While the FDA has expressed interest in in silico testing for drug research (28), as of 2020, there are no guidelines that allow in silico tests to replace preclinical animal testing in drug development, although in silico data can certainly be submitted in support of animal findings, and may reduce the number of animal experiments that are required.

The FDA supports modeling and simulation research to help predict clinical outcomes, inform clinical trial design, support evidence of efficacy, identify the most relevant patients to study, and predict product safety (29). The FDA has published guidances on the formats for submitting in silico data in support of regulatory advancement of devices on its website (30). However, development of drug and biologicals and toxicological evaluation of metabolites of these entities is still vested in animal studies (31), which can be particularly problematic for testing metabolites that occur in human subjects but cannot be replicated in an animal model.

**ANIMAL-FREE RECOMBINANT ANTIBODIES.** Not only are antibodies used in a variety of research, diagnostic, and regulatory applications, but also antibodies and other biologicals are increasingly becoming mainstream therapeutic agents themselves. Traditionally, the development of antibodies has required the expensive process of immunizing animals, and then sometimes fusing antibody-producing B cells with “immortalized” cell cultures to increase supply. More than 300 companies now supply antibodies for such research and development, with an estimated market value in 2011 of $1.6 billion (32). However, antibodies produced from immunized animals exhibit variability in DNA sequencing and variability in target binding. The lack of reproducibility in research using animal-based antibodies has been deemed a “crisis,” with some scientists claiming that over one-half of all commercially available antibodies are unreliable in binding their intended targets (32). One proposed way to tackle the problems of variability in animal-produced antibody structure and targeting is the use of antibodies manufactured in engineered “recombinant” cells, and the use of antibodies from “human antibody libraries” (33). Advantages include faster production, more consistency in quality, and less biological variation with better potential for research and therapeutic reproducibility. Overall, costs would be eventually be reduced, but only after initial increased expenses of changing to recombinant antibody production from current methods.

**TISSUE ENGINEERING**

Perhaps one of the most “futuristic” approaches to drug and device development is represented by the emerging field of tissue engineering (TE). Unlike cell suspensions and “tissue culture” cellular monolayers, TE constructs have 3-dimensional (3D) structure. TE can more closely mimic the considerable influence that 3D cell-to-cell and cell-to-matrix interactions have over cell behavior in actual tissue and organ systems; something that cell and tissue cultures cannot. In addition, although a considerable body of TE research utilizes animal cells and tissues, TE can allow the creation of 3D tissue structures utilizing human cells—the actual therapeutic target—and likely increase the probability that activity in the engineered human tissue will more accurately reflect or predict the outcomes in human patients. Research regarding TE is burgeoning: the average annual number of published papers more than doubled between 1991 and 2010 (34). The largest body of publications presented various 3D constructions of human skin, made from human karyocytes—derived from neonatal foreskin and mammary tissue discarded after plastic surgery—that vary in complexity from more simple epidermal layers on collagen substrates, to tissue that included epidermis, dermis, and immune cells, and even vascularized skin equivalents with a vascular network (35). TE models have been created that mimic human corneal epithelium and stroma, urothelium, and human oral and vaginal mucosa. Emerging TE studies include engineered human liver tissue, and human neurospheres, as well as models for corneal innervation and the interaction between metastatic tumor cells and bone (34).

**TE AND THE HUMAN HEART.** CV medicine, and the heart in particular, affords many challenges for reducing animal research, as it involves not merely the pharmacologic effects of drugs on the CV cells and systems, but also the effects of topographical properties, motion, and forces in cardiac and vascular shape and mechanical function. As a result, some of the most complex and dynamic alternative research is evolving in this field. The combination of TE with the methodology of 3D printing has resulted in some startling innovations with promise to revolutionize CV bioprosthesis interventions as well as pharmacologic testing and drug development.

For more than 2 decades, biologists have been able to turn embryonic stem cells into beating heart muscle cells in a dish (36). Cardiac cells have also long
been known to have intrinsic capacity to self-assemble into spontaneously beating spheroids, or syncytia (37)—making them particularly interesting with regard to 3D TE. Furthermore, cardiac constructs from primary cardiac myocytes develop a primitive vascular network with or without addition of endothelial cells (38).

Engineered 3D constructs of heart muscle permit measurement of virtually all parameters of heart function, including twitch force, kinetics, beating rate, rhythm, diastolic tension, and intracellular calcium movement (39–43). Engineered cardiac tissue has the potential for use as a research tool, as a replacement for animal toxicity testing of therapeutic agents, and as a therapy unto itself. The models are easy manipulated genetically, and they behave quantitatively much like native muscle, making them reasonable platforms for disease modeling and toxicity testing. In 2018, for example, Truitt et al. (44), used an engineered 3D cardiac microtissue model to characterize mechanisms of human toxicity of sunitibnib, a drug with considerable cardiotoxicity that is used widely to treat renal cell, gastrointestinal, and neuroendocrine tumors.

However, the pharmaceutical industry has been slow to adopt such models as drug development tools, and regulatory agencies have not yet put forth guidelines for how these technologies can or should be integrated into preclinical and clinical safety trials, nor whether they can begin to replace animal models in either capacity. The process of defining how engineered tissue might be approved and introduced as a therapy, in contrast, has quickly developed (45–48), but even the expedited processes available at the FDA for such therapeutics generally still follow the pathway of preclinical animal studies followed by human trials.

**HUMAN TISSUE-ON-CHIP ENGINEERING.** Cell-based toxicity assays (via traditional “tissue culture” approach) are problematic in predicting drug toxicity in preclinical testing because cultivated cells often do not retain their original organ functions and morphologies when taken out of the context of intraorgan connection and interactions. In tissue culture, for example, it is difficult to maintain cellular functions for sustained periods of time. Tissue culture cells receive nutrients, oxygen and other substances almost solely by diffusion. However, in vivo, cells obtain oxygen, nutrients, and other substances that regulate their function via blood flow, and they experience and respond to physical stimulation such as stretching and sheer forces within their complex environments. Such differences may account for rapid deactivation, senescence, and cellular loss in many in vitro cultures. In addition, interactions between organs cannot be directly tested in tissue culture.

Microfabrication techniques and microfluidic technology combined with computer technology has led to a new type of in vitro organ model: the so-called...
organ-on-chip (OC), which has been further combined into multiorgan chip interactions to mimic whole-body responses, or “body-on-chip” (BC). Creating a hybrid of human tissue on a computer chip that can replicate the structure and function of human organs may seem like the realm of science fiction; however, this new technology not only is a current reality, but also is quickly entering the regulatory framework for assessing new therapeutic compounds.

In OCs, transparent chips about the size of an AA battery contain microchannels that are lined with cultured human cells and have microsensor capabilities (e.g., photolithography). These channels allow microfluid (or air) flow that mimic breathing motions, muscle contractions, and other physiologic stressors (Figure 1). The chips are then placed into a research system similar to a computer, in which toxins, chemicals, and medicines can be introduced to test the OC’s response and behavior. In recent developments, at the Wyss Institute at Harvard University, OCs have been connected together to mimic multiorgan interactions within a body (49). Software within the research system allows the investigator to manipulate cell architecture, tissue-to-tissue interfaces, mechanical forces in the environment, and biochemical changes within the environment of the OCs or BCs.

Such OCs and BCs have undergone rapid advancement in the last 5 years. They include the “lung-on-chip” or “breathing lung” chip developed at Harvard University (48), in which the 2-layer channel structure of the microchip is separated by a microporous, stretchable silicone membrane on which alveolar cells and vascular endothelial cells are cultured. The chip mimics the physiological expansion and contraction of alveolar movement by altering pressure on both sides of the membrane via vacuum chambers (Figure 2). Researchers were able to reproduce inflammatory reactions, and even to allow neutrophils to enter via a side channel to respond to bacterial invasion. Other organs mimicked by OCs include the liver, kidney, and gut (50).

In 2017, the FDA announced a multiyear research and development agreement with Emulate, Inc. (Cambridge, Massachusetts), a biotech spinout from the Wyss Institute specializing in OCs (51). Earlier this year, researchers from Wake Forest School of Medicine demonstrated that multiorganoid BC systems were both stable and capable of detecting hepatic and cardiotoxicity at human-relevant doses in almost all members of a panel of drugs they tested that had to be recalled by the FDA for hepatic or cardiotoxicity (52). Bear in mind that for all of these drugs,
preclinical animal studies and all phases of human clinical trials had failed to detect significant toxicity.

**HEART-ON-A-CHIP.** Despite being the most frequent cause of death in the United States and a cause of almost 18 million deaths annually worldwide (53), the number of novel drug approvals for CV disease has undergone progressive decline (54). In 2019, just 2 drugs were approved under the classification of CV disease, both of which were for treatment of a rare form of cardiomyopathy—transhyretin-mediated amyloidosis (55). There were no new drug therapies approved for coronary artery disease and hypertensive cardiac disease, which is responsible for 60% of CV deaths (56). CV toxicity is a major cause of drug withdrawals from the market (16%), second only to hepatic toxicity (57).

The ability to mimic physiologic and mechanical effects in the microchip environment has vastly extended the CV research applications of OC technology in the last 10 years (38,58,59). In 2011, Grosberg et al. (60) developed a “heart-on-chip” platform with anisotropically organized cardiomyocytes, which could be stimulated to mimic in vivo production of electrical impulses by pacemaker cells—demonstrating that the heart-on-chip could be used to study and measure contractile behavior, cellular alignment, and functionality of cardiac cells, and suggesting that chip technology could be used to study and evaluate pharmacologic interventions on cardiac contractile function. A similar approach demonstrated that in vitro “chip” testing of pharmacologic effects of isoproterenol was comparable to results of in vivo studies in rats (61). McCain et al. (62) have successfully created a “failing heart-on-chip” platform, further suggesting that chip technology can be used to create disease models, and not merely as a high-throughput method of testing drugs. Ren et al. (63) fabricated a heart-on-chip to mimic hypoxic myocardial injury. Researchers are exploring chip technology as a means of evaluating the use of stem cells to assess therapies for myocardial repair, as well as to generate in vitro models of cardiac disease. In one case, an in vitro disease model of Barth syndrome was developed, and then used to test potential pharmacological and genetic therapeutic options (64).

**“ACCURACY” OF OCs IN PREDICTING HUMAN DRUG TOXICITY AND MODELING DISEASE.** Before the FDA and other regulatory bodies will agree to allow OC technology to replace any phases of animal testing, the technology will have to be proven at least as accurate, if not more so than current testing. Early data are scarce but promising.

In a pulmonary edema model created in an OC, the response to a low-molecular-weight pulmonary edema therapeutic was shown to be similar in the OC to results obtained in animal models (65). InSpheroAG (Schlieren, Switzerland), a commercial developer of a liver chip assay, claims that the sensitivity of the test (in predicting human toxicity) over animal testing is increased by a factor of 2 (66). During development of a vascularized human bone marrow (BM) OC, researchers demonstrated that it reproduced aspects of BM injury, including myeloidrethoid toxicity after clinically relevant exposures to chemotherapeutic drugs and ionizing radiation, and myeloid recovery after drug-induced myelosuppression, suggesting that a BM OC system may be useful for predicting human toxicity and designing in-human trials. When the BM OC was constructed using cells from patients with a rare genetic disorder, it not only reproduced key hemopoietic defects of the disease, but also led to

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**TABLE 1 Correlation of Liver Chip Results for Drugs Halted in Clinical Trials (i.e., Drugs That Had Passed Animal Toxicity Studies)**

| Drug                                      | Clinical Trial Results                                                                 | Possible Mechanism of Liver Injury                                                                 | Liver Chip Results                                                                 |
|-------------------------------------------|----------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| Fialuridine (antiviral)                    | Discontinued in phase II; liver failure and deaths in 5 of 15 patients due to microvesicular steatosis. Animal toxicity studies did not predict severe hepatic injury in humans. | Drug-induced mitochondrial injury                                                                  | Significant hepatocyte lipid accumulation, increased liver injury markers          |
| TAK-875 (G-protein-coupled receptor 40 agonist) | Discontinued in phase III clinical trials due to treatment-related elevations in transaminases and several instances of drug-induced liver injury. In vivo and in vitro studies had detected formation of active metabolites. | Formation of reactive metabolites, suppression of mitochondrial respiration, inhibition of hepatic transporters | Reactive metabolites, hepatic transporter inhibition, mitochondrial dysfunction, lipid accumulation, markers of oxidative stress, release of inflammatory cytokines |
| Janssen proprietary compound JNJ-1 (colony-stimulating factor receptor kinase inhibitor). | Discontinued in phase I clinical trial due to very high elevations in liver transaminases in 2 subjects. Although minimal elevations were seen in rats and dogs, no microscopic liver changes were found in the animal models. | Kupffer cell depletion                                                                               | Kupffer cell depletion, decreased IL-6 and MCP-1 in clinically significant concentrations |

For reference, please see Jang et al. (68).

IL = interleukin; MCP-1 = monocyte chemotaxtractant protein-1.
the discovery of a previously undescribed neutrophil maturation abnormality (67). Thus, the BM OC may be useful in discovering new, human-relevant therapeutic targets.

In late 2019, Jang et al. (68) developed a multi-species liver OC that was able to differentiate species-specific toxicities of test compounds in human, canine, and rat species. In one case, a proprietary test compound from Janssen Pharmaceuticals (Raritan, New Jersey), which had shown liver inflammation in rats and was subsequently discontinued from further development, showed replication of the rat findings but demonstrated a lack of toxicity in human cells. With a second proprietary compound, the canine portion of the canine chip was able to replicate the liver inflammation that had been found in dogs, and also suggested changes in human cells, suggesting a high likelihood of human toxicity. In addition, the authors tested their chip against drugs that had survived preclinical animal tests, only to be discontinued during human trials due to liver toxicity, and found that the chip accurately predicted human toxicity (Table 1). This is particularly intriguing news, as liver injury is the most frequent reason for post market withdrawal of drugs (69). Cross-species chips may be able to hone animal testing to only those species whose toxicity has been shown by chip analysis to be relevant to humans, sparing animals, time, and expense while preserving human safety.

Despite academic enthusiasm for OC technology, industry has been cautious in adopting it for preclinical drug screening. In 2018, only about 20% of the top 50 pharmaceutical companies routinely used OC screening (66). OC technology is currently most widely applied in screening research for therapeutic targets, in preclinical testing in which no regulatory submissions are in question, than for toxicity testing, which at this time carries considerable regulatory uncertainty. A significant impediment is lack of standards for validation parameters for compounds, endpoints, exposure times, and thresholds for sensitivity and specificity, which contribute to the hesitation about acceptance/rejection criteria from the FDA and other regulators, including whether OC technology can at least partially replace animal testing in preclinical phases.

In order to overcome some of these obstacles, the NIH National Center for Advancing Translational Sciences has collaborated with the FDA in the Tissue Chip for Drug Screening program to develop human tissue chips that accurately model the structure and function of human organs and predict drug safety in humans more rapidly and effectively. Updated information about funding opportunities for chip research can be found on their website (70).

### ADDITIONAL REGULATORY STEPS

In December of 2017, the Toxicology Working Group at the U.S. FDA released its Predictive Technology Roadmap, which was formulated with a mission to strengthen the FDA’s commitment to promoting the development and use of new technologies to better predict human, animal and environmental responses to a wide range of substances relevant to FDA’s regulatory mission (71). In a September 2018, public hearing for stakeholder feedback on the roadmap, Commissioner Scott Gottlieb expressed a desire to apply OC technology across the life cycle of regulated product development, observing that “results in animals are not always predictive of results in humans” (66). Although the FDA has emphasized support for alternative research methods, to date most therapeutics require animal testing in the preclinical phases, and do not allow substitution of alternative methods. Under the 21st Century Cures Act (72), certain therapeutics wholly or partially including human cells, tissues, and “therapeutic tissue engineering products” may achieve status as “regenerative medicine advanced therapies” and be eligible for certain expedited pathways at the FDA for approval, including focused and expedited FDA review, acceptance of retrospective data in lieu of some clinical study requirements, and other measures (48). However, these programs do not yet generally allow substitution of alternative research for preclinical animal studies.

In 2006, the European Union passed REACH (Registration, Evaluation, Authorization, and restriction of Chemicals) legislation that set a goal of reducing animal testing, and REACH does embrace some in silico methods (48,73). The European Medicines Agency has also put forth guidelines to eliminate or significantly restrict animal testing for certain human medicine products in order to reduce animal use in drug regulatory approvals (74–76).

### SUMMARY

Goals of reducing the time and cost of drug development, together with reduced public support for animal research, are driving attempts to find alternatives to animal testing, which does not sufficiently identify human safety and toxicity for therapeutics. Reducing pursuit of drugs that prove in late phase of development to have intolerable human toxicity is an
important step in meeting those goals. In addition, accurately identifying agents that are human safe but currently fail animal testing will likely increase potential effective therapeutics in human disease. Although alternatives to animal research, such as cell and tissue platforms, computational in silico modeling, 3D tissue platforms and QC research have shown great promise in facilitating drug development while decreasing time and expense, they have yet to make significant inroads as replacements for preclinical animal testing. Although both the United States and European Union have recognized the value of pursuing alternative methods of research, such methods still await wider regulatory acceptance to replace animal testing in drug approval processes.

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KEY WORDS animal research, drug development, toxicity, translational research
Targeting Vascular Calcification in Chronic Kidney Disease

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HIGHLIGHTS

- Vascular calcification is a highly regulated, cell-mediated process that is strongly associated with CKD and confers increased risk for incident CV events.
- Multiple pathways link vascular calcification with CKD; however, they remain incompletely understood, and the development of targeted therapies has been underwhelming.
- Illumination of the causal steps and natural history that link vascular calcification to CV events will affirm its role as a CV risk factor and accelerate drug discovery and therapeutic translation.

SUMMARY

Cardiovascular (CV) disease remains an important cause of morbidity and mortality for patients with chronic kidney disease (CKD). Although clustering of traditional risk factors with CKD is well recognized, kidney-specific mechanisms are believed to drive the disproportionate burden of CV disease. One perturbation that is frequently observed at high rates in patients with CKD is vascular calcification, which may be a central mediator for an array of CV sequelae. This review summarizes the pathophysiological bases of intimal and medial vascular calcification in CKD, current strategies for diagnosis and management, and posits vascular calcification as a risk marker and therapeutic target. (J Am Coll Cardiol Basic Trans Science 2020;5:398–412) © 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/).

Chronic kidney disease (CKD) is a major global public health problem. Defined by the sustained presence of either kidney damage (albuminuria) or reduced kidney function (estimated glomerular filtration rate [eGFR] <60 ml/min/1.73 m²) (1), CKD is believed to affect 10% to 15% of the population and is estimated to contribute to 5 to 10 million deaths annually (2,3). Despite improvements in the care of patients with CKD, life expectancy remains significantly reduced across all stages of kidney disease (4), and the global burden of kidney disease continues to rise (5). In this context, cardiovascular disease (CVD) is a significant contributor to the high morbidity experienced by patients with CKD and CV death is the most common cause of death in this population (Figure 1).

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Large contemporary datasets have repeatedly shown linear relations between CV mortality and reduced eGFR and proteinuria or albuminuria (6). The effect is most pronounced for those with end-stage kidney disease (ESKD) in whom 85% of adults commencing dialysis at older than 45 years of age will have some form of CVD and experience a relative risk of CV death that exceeds 20 times that of the general population (7). In adults younger than 30 years of age, the relative risk is even more dramatic; incident ESKD confers a ~150-fold risk of CV death compared with an age-matched population (8). Expressed another way, persons with mild to moderate (stages 1 to 3) CKD are at higher risk of CV events and CV mortality than they are for progression to ESKD (9,10).

CKD imparts increased risk for a wide array of CVDs (Figure 2). Although atherosclerotic CVD is the most frequent manifestation, leading some to consider CKD a coronary-artery risk equivalent (11), rates of heart failure (12), stroke (13), valvular heart disease (14), arrhythmia (15), and sudden cardiac death (16) are all significantly increased across the spectrum of CKD. Clustering of traditional risk factors such as type 2 diabetes mellitus and hypertension were originally believed to drive propensity for CVD in these patients; however, meta-analyses have consistently identified significant residual, CKD-specific risk (17,18). Once CVD is established, CKD confers a significantly worse prognosis with a more aggressive disease phenotype, higher risk of complications, and premature cause-specific death (18). Reduced efficacy of proven secondary preventative therapies (e.g., statins) likely also contribute to the divergent outcomes experienced by patients with CKD (19–21).

Multiple systemic perturbations observed in CKD can lead to cardiac and vascular damage, and likely underscore the increased risk of CV events observed in this population. Vascular calcification, the pathological deposition of calcium salts in the arterial wall, has been observed among patients with CKD at between 2- and 5-fold the rate of age-matched non-CKD patients. A large body of evidence has subsequently supported biologically plausible, temporal (22), and dose–response (23) relations between vascular calcification and CV risk in patients with CKD. However, whether the regression or halting of vascular calcification is possible and subsequently results in improved CV outcomes remains to be determined.

**TYPES OF CALCIFICATION**

There are several distinct phenotypes of CV calcification: intimal vascular, medial vascular, and valvular. Although valvular calcification is increasingly recognized as an important contributor to morbidity and mortality in patients with CKD, particularly those on dialysis (24), for the purpose of this review we focused on the vascular entities of intimal and medial calcification. Intimal and medial vascular calcification are defined by their location within the arterial wall, which is a distinction that helps separate predisposing etiology, regional distribution, and clinical sequelae (Figure 3). Although this has intrinsic appeal, noninvasive imaging is unable to discern intimal from medial calcification; thus, our insights into the natural history and clinical relevance of each type of vascular calcification are limited.

**INTIMAL CALCIFICATION.** Intimal calcification occurs almost exclusively in the context of atherosclerosis, which has a predilection for medium to large arteries and areas of abnormal flow, such as arterial vessel bifurcations. Although generally increased in patients with CKD, intimal calcification is not specific to CKD and instead is associated with traditional atherogenic risk factors such as dyslipidemia, diabetes, hypertension, and cigarette smoking (25). Of note, adolescents on dialysis who do not have traditional risk factors develop medial calcification almost exclusively (26,27), which is similar to animal models of CKD that are not exposed to an additional metabolic stimulus (e.g., through low-density lipoprotein (LDL) receptor knockout or a high cholesterol diet) (28). CKD may promote, accelerate, or catalyze an already established, synchronous atherosclerotic calcification process rather than incite it (29). Regardless, the complications of intimal calcification are believed to be local and mediated through the development of luminal stenosis, obstruction, and distal ischemia or infarction (i.e., myocardial infarction, stroke, or limb events). In general, intimal calcification appears to be dynamic and may be considered a barometer for the atherosclerotic cycle; microscopic flecks are visible during the earliest stages of intimal thickening and diffuse, confluent regions are observed in the most advanced fibrocalkis lesions (30). Although spotty calcification (approximately 1 mm in size) observed on both computed tomography (CT) and intravascular imaging has been associated with plaque instability and propensity for atherothrombotic events, confluent calcific lesions appear to be associated with a more stable phenotype (30) and may instead represent a teleological response to injury (25). These confluent
and often dense lesions on intravascular imaging are more common in patients with CKD (31). Whether the accelerated atherosclerotic or intimal calcification observed in CKD is causally linked to adverse CV outcomes or may be acting as a synchronous biomarker for medial calcification remains undetermined.

**MEDIAL CALCIFICATION.** Unlike the often patchy distribution of intimal calcification, medial calcification tends to be more diffuse, forming sheets in topographic areas typically devoid of lipid or atherosclerotic change (26) (Central Illustration). Although medial calcification is seen in vessels of all calibers, it is conspicuous for location in territories usually spared from atherosclerosis, such as the internal mammary, radial, and digital arteries. Medial calcification is more specific to CKD because it appears to be associated with calcium-phosphate exposure, abnormal bone and mineral metabolism, severity of CKD, and dialysis vintage. In addition to CKD, medial calcification is also observed with diabetes mellitus and advanced age; these 3 entities potentially share linking pathophysiology of chronic inflammation and cellular senescence (32). Compared with intimal calcification, medial calcification is rarely associated with local luminal compromise, and instead is linked to the systemic manifestations of increased arterial stiffness. Following seminal observations linking the presence of (mostly medial) vascular calcification with increased arterial stiffness (33,34), the latter has been considered a functional surrogate for the former. A reduction in vascular compliance augments systolic blood pressure, which increases cardiac work and causes left ventricular hypertrophy (35) (Figure 3). This is a finding observed in up to 75% of patients with higher than or equal to stage 3 CKD (36), providing a mechanistic basis to the increased rates of heart failure (37) and atrial fibrillation observed in CKD (38).

**DETECTION AND PROGNOSIS**

Most vascular calcification is detected incidentally through imaging obtained for other indications. In the research setting, several methods have been developed to quantitate vascular calcification and stratify risk. The most studied is the Agatston score (39) (often synonymous with “calcium score”) for coronary artery calcification on CT scans. However, the Kauppila index (40) for abdominal aortic calcification and the Adragao score (41) for both lower abdominal aorta and peripheral arteries have also been shown to have a prognostic value. As alluded to earlier, noninvasive imaging is unable to discern intimal from medial calcification; a limitation that is amplified in CKD in which there is a greater degree of coexistence of both processes, particularly in the coronary arteries, peripheral arteries, and the aorta. For this reason, there has been enthusiasm for evaluating arterial beds that are generally devoid of atherosclerosis, thereby providing more specific measures of medial calcification. Another approach has been to assess the functional consequences of calcification by evaluating indexes of arterial stiffness, reviewed elsewhere in detail (42).
CT CORONARY ARTERY CALCIFICATION SCORE.
Over 30 years of data in the general population has shown that CT coronary artery calcification (CAC) scoring with the Agatston score closely correlates with the atherosclerotic plaque burden, is linearly and independently associated with CV outcomes, and may outperform other established biomarkers for CV risk prognostication, including the ankle–brachial index, high-sensitivity C-reactive protein, and carotid intima media thickness (43). Seminal observations in the 1990s reported that patients with ESKD who received dialysis had an average CAC score that was an order of magnitude greater than what was generally considered a high-risk threshold (4,290 vs. >400 Agatston units) (44). Findings some years later affirmed the presence of a graded relationship between severity of CKD and CAC score (45,46). Until recently, data on the prognostic role of CAC in CKD was conflicting because of single-center studies, small sample sizes, or recruitment of narrow CKD severity grades (47). In the largest study of patients with CKD published in 2017, investigators evaluated the CAC scores of 1,541 participants with an eGFR so low that 0 to 70 ml/min/1.73 m² without established CVD as part of the CRIC Chronic Renal Insufficiency Cohort trial (48). Over a median follow-up of 6 years, a total of 60 myocardial infarctions, 120 index heart failure events, 27 strokes, and 137 all-cause deaths occurred. There was a stepwise association between CAC severity (CAC score 0, 0 to 100, >100) and composite CV outcome. In multivariable modeling, the relative hazard ratios (HRs) associated with a 1 SD of log CAC were 1.40 (95% confidence interval [CI]: 1.16 to 1.69; p < 0.001) for the composite CVD outcome (myocardial infarction, heart failure, and stroke), 1.44 (95% CI: 1.02 to 2.02; p = 0.04) for myocardial infarction, and 1.39 (95% CI: 1.10 to 1.76; p = 0.006) for heart failure. CAC was not associated with all-cause mortality. This study from CRIC, as did another study (49), found CAC offered incremental prognostic value for CV events beyond traditional risk factors. The similar magnitude hazard ratios for atherosclerotic CVD and for HF suggest CAC scoring in the CKD population may be less specific for atherosclerotic burden and instead be an integrated marker of both medial and intimal calcification. This is consistent with autopsy findings of patients with established coronary artery disease in which high rates of coexistent medial calcification was observed only in those with CKD (50).

Reduction in radiation dose, application of semi-automated scoring mechanisms, ease of access, and validated age-standardization have resulted in the CT CAC score becoming the gold standard endpoint for trials that evaluated change in vascular calcification.

PLANE RADIOGRAPHY. The Kaupila score is a semiquantitative scoring method that attributes an ordinal value to calcification (0 to 3) at 8 sites along the abdominal aorta (total maximal score 24) as...
viewed on a lateral lumbar spine plane radiograph (40). Although the score has reasonable interobserver reproducibility, the progressive reduction in vertebral height with age (thus, length of quantified aorta) and superimposition of the vertebra on the aorta are known shortcomings. Studies in patients with ESKD have shown stepwise increases in abdominal aortic calcification (AAC) that correlate with CAC score (51) and that associate with worse CV outcomes (52). However, there are few data to support its accuracy at discerning temporal trends, particularly in non-ESKD cohorts in which calcification may be more modest, nor has there been formal validation of the Kauppila score against a quantitative method. Another x-ray-based scoring system that was described by Adragao involves semiquantitative scoring of linear calcification on pelvic and hand radiographs (41). The deliberate focus on pattern of calcification and inclusion of hand vessels increases the specificity for medial calcification, which is a theory supported by its correlation with arterial stiffness. In a study of 742 participants with ESKD, an elevation in either the Adragao (≥3) or Kauppila (≥6) score was associated with all-cause and CV mortality; however, only the Adragao score remained significant after adjustment for covariates (HR: 3.46; 95% CI: 1.27 to 9.45; \( p = 0.02 \)) (53).

**BREAST IMAGING.** Breast arterial calcification is an attractive method for measuring exclusively medial artery calcification because atherosclerosis has not been shown to occur in these vessels. Studies in patients with advanced CKD confirm mammography is not only capable of sensitive, temporal measurement of breast arterial calcification compared with CT (54), but also demonstrates that breast arterial calcification is strongly associated with both measures of peripheral arterial calcification (55) and CV outcomes (56). Although such a modality may inform mechanistic insights around the natural history of medial calcification, this is a modality limited to the assessment of women, and at present, has only been retrospectively analyzed.

**SCREENING.** Proponents of routine screening for vascular calcification among patients with CKD suggest there is significant incremental value in identifying a high-risk CV cohort. Conversely, others
have argued that all patients with CKD should be considered at the highest CV risk and, in the absence of specific vascular calcification therapies, screening for vascular calcification does not influence management. Routine vascular calcification screening using either lateral pelvic x-ray or CT imaging received a weak (level 2C) recommendation in the 2017 Kidney Disease Improving Global Outcomes guidelines, reflecting the paucity of outcomes-driven data (57,58). The wording used in the 2017 updated guidelines was nearly identical to that used in the 2009 Guidelines, highlighting the lack of progress in the search for specific therapies proven to reverse, arrest, or attenuate vascular calcification (59).

**MECHANISMS**

Our understanding of vascular calcification has evolved from a benign, passive deposition of minerals
to what is now regarded as a pathological, tightly regulated, and cell-mediated process that resembles bone formation and turnover. Despite this growth in understanding, the temporal sequence of events and the exact cellular steps remain incompletely understood and the focus of ongoing research. Moreover, the observed events are unlikely to occur in a stepwise manner and instead are more likely to represent synchronous and interrelated processes (Table 1).

**PHOSPHATE AND MINERAL METABOLISM.** Phosphate excretion is regulated at the proximal tubule of the kidney; however, the balance of bone formation and resorption is an important determinant of serum levels. Hormonal regulation of phosphate at the renal and gastrointestinal levels via parathyroid hormone (PTH), fibroblast growth factor (FGF)-23, klotho and 1,25-dihydroxyvitamin D (calcitriol) maintains this mineral in a narrow serum range (2.5 to 4.5 mg/dl). As impaired kidney function progresses to advanced stages, with reduction in functional nephron mass, phosphate excretion is impaired, bone remodeling slows, and together with persistent dietary intake, serum phosphate concentrations rise. Multiple studies have suggested that there is a link between hyperphosphatemia and the propensity for accelerated vascular calcification (46,60), particularly in patients on hemodialysis (61,62). Because phosphate is a key building block of hydroxyapatite crystals, it is conceivable that increased serum levels alone in the context of CKD could contribute directly to precipitation of hydroxyapatite in tissue (63). Although this may occur to some degree, not all patients with sustained hyperphosphatemia develop vascular calcification; seminal in vitro observations suggest a more active process wherein exposure to elevated phosphate induces a dose- and time-dependent phenotypic change in vascular smooth muscle cells (VSMCs) (64). The presence of a pro-calcific CKD milieu permits (and potentially promotes) the upregulation of phosphate channels (Pit-1 and Pit-2) which, in the context of hyperphosphatemia, mediates transdifferentiation of VSMCs to an osteochodrogenic cell phenotype (65); an event that can be, in part, inhibited by competitive antagonism of Pit-1 (64).

Beyond phosphate, other metabolic features of the mineral-bone axis have been implicated in vascular calcification, including calcium and PTH, although their effects may be more complex. Several lines of evidence support a direct role of PTH: elevated PTH is associated with higher rates of vascular calcification (66); and animal models of synthetic PTH infusion develop extensive calcification regardless of subtotal nephrectomy/sham and independent of presence or absence of hypercalcemia (67). In subtotally nephrectomized rats, submaximal suppression of PTH by treatment with a calcimimetic or parathyroidectomy slows rates of aortic calcification independent of serum calcium and phosphate concentrations (68). That stated, vascular calcification may also be potentiated in the setting of overzealous treatment of hyperparathyroidism, especially with excessive use of calcium-containing phosphate binders, which results in low bone turnover and reduced mineralization (69).

Although complex and incompletely understood, states of low bone turnover and reduced mineralization are likely to attenuate the skeletal capacity for effective calcium and phosphate homeostasis. A reduction in skeletal buffering capacity may expose the vasculature to greater fluctuations in extracellular calcium and phosphate, thereby increasing the propensity for vascular calcification (70).

**VSMCs.** As alluded to, the transdifferentiation of VSMCs into an osteoblast-like secretory phenotype is central to the underlying pathophysiology of vascular calcification. This process is marked by downregulation of smooth muscle genes and subsequent expression of bone markers Runx2, osteopontin, osteocalcin, and alkaline phosphatase. The biology governing VSMC phenotypic switching remains to be fully appreciated; however, a number of triggers have been identified, including oxidative stress, imbalance of pro-calcific and/or anticalcific mediators (see the following), pro-calcific microRNAs, advanced glycation end products, cellular senescence [potentially through Prelamin A (71)], and hyperphosphatemia (as previously described). Once established, these osteoblast and/or chondrocyte-like cells behave in a manner consistent with other bone-forming cells by producing a collagen matrix through the secretion of calcium and phosphorous-laden vesicles (see the following).

**FAILURE OF INHIBITORY MECHANISMS.** Mineralization occurs throughout the body at physiological calcium and phosphate levels; thus, a dynamic balance of promoters and inhibitors are required to ensure mineralization is supported at desired sites (bone) and is prevented from occurring elsewhere (ectopic, metastatic). Two prominent inhibitors are fetuin-A and matrix Gla protein (MGP). Fetuin-A is a glycoprotein synthesized in the liver that is recycled by VSMCs where it acts as a mineral chaperone, binding serum calcium in calciprotein complexes and preventing it from crystallization (72). Mice deficient in fetuin-A develop widespread calcification (73), whereas the addition of fetuin-A to bovine VSMC lines provides dose-dependent inhibition of
calcification (74). Supporting data in patients with CKD suggest levels of fetuin-A are inversely associated with vascular calcification (75), and low levels have been linked to inflammation and all-cause mortality in the dialysis population (76).

MGP is a widely expressed protein and has been shown to accumulate in calcified tissue. Its precise role remains unconfirmed; however, the two prevailing, potentially synergistic, theories are that MGP may bind calcium ions and calcium crystals, thereby inhibiting crystal growth (77), or that MGP may interfere with bone morphogenic protein(s) signaling and prevent unwanted cell-induced mineralization (78). Animal MGP-knockout models developed rapid and extensive aortic medial calcification, which is associated with rupture and early hemorrhagic death (79). In vitro studies showed that MGP was down-regulated in states of vitamin K deficiency and had less affinity for calcium in the setting of hyperphosphatemia, which are 2 common abnormalities observed in CKD (80,81). MGP requires vitamin K–dependent carboxylation to assume its inhibitory qualities, thus a reduction in MGP activity may not necessarily relate to absolute levels but to functional decreases through persistence of a decarboxylated state. Supportive translational data demonstrated the administration of vitamin K antagonists (e.g., warfarin) was associated with increased rates of vascular calcification in cell lines, animal models, and patients (82,83). These findings subsequently spawned interest in the potential therapeutic capacity of vitamin K supplementation (see the following).

Other known inhibitors of vascular calcification include pyrophosphate and osteopontin. Pyrophosphate is produced by arterial smooth muscle and directly inhibits hydroxyapatite formation (84). Osteopontin is a phosphoprotein that regulates mineralization through multiple functions, including mediating angiogenesis and responses to inflammation and mechanical stress. The relationship between osteopontin and vascular calcification appears complex. Although there is evidence of upregulation in calcified vessels (85), serum levels do not readily correlate with calcific burden (86).

**MATRIX VESICLES AND APOPTOSIS.** Matrix vesicles, a form of extracellular vesicle observed in the vascular wall, have been more broadly implicated in both transdifferentiation of VSMCs and the calcification environment (87). Extracellular vesicles are phospholipid membrane-bound particles, have molecular cargo (protein, RNA, or lipid), and are released by many cells and cell types in response to cell activation or apoptosis. Under pro-calcifying conditions the vesicles released by VSMCs are altered and resemble those released by osteoblasts (88). These vesicles act as nucleating points that have the capacity for calcium binding and extracellular matrix production. There is emerging evidence that these vesicles may permit cross-talk among VSMCs but potentially also between endothelial cells and other vascular cells (89), lending biological support for bidirectional positive feedback between intimal and medial calcification (90). In vesicles that contain genotypic and/or phenotypic information such as microRNA, the content of the specific sequences released by VMSCs may either induce or inhibit nearby pro-calcific phenotypic change (91). An additional paracrine-type effect may be mediated through osteoblastic- and/or osteochondroblastic-like cell activity on surrounding tissue. Subsequent changes in relative collagen composition and the degradation of elastin (92) have been shown to promote hydroxyapatite formation and provide scaffolding for further mineralization (93).

**FGF-23 AND KLOTHO.** Most FGF-23 is produced by bone (94) and although the mediators of its release remain incompletely understood (95), associations with PTH (96), iron deficiency (97), calcium (98), and vitamin D (99) have been consistently observed. The primary function of FGF-23 is to orchestrate phosphate and calcium homeostasis by stimulating urinary phosphate excretion and suppressing circulating concentrations of calcitriol (100). The activity of FGF-23 to regulate phosphate homeostasis in the kidney requires the presence of klotho, a co-receptor that facilitates binding of FGF-23 to the FGF-receptor (101). FGF-23 excess is associated with poor outcomes in CKD (102), although its links to vascular calcification are less clear, and multiple studies have conveyed conflicting results. Human and animal VSMCs exposed to FGF-23 in the presence or absence of klotho, and in the presence of normal or high phosphate, showed increased, decreased, or no effect at all on vascular calcification (103–107). These conflicting results posit a more complex role of FGF-23 in vascular calcification; however, it is also possible that FGF-23 is not causal factor at all. Unlike FGF-23, in vitro and in vivo evidence support a protective role of klotho in vascular calcification. Addition of klotho to a rat VSMC line directly suppressed Pit-1 and Pit-2 activity and subsequently prevented phosphate-induced osteogenic transdifferentiation. In addition, klotho knockout models demonstrated increased expression of Pit-1 and Pit-2 receptors, which suggested that the progressive klotho deficiency observed in advancing CKD might result in
upregulation of these receptors, which would subsequently promote phosphate uptake and drive VSMC transdifferentiation (108).

**THERAPEUTIC APPROACHES**

A large investment into targeting intermediates of bone and mineral metabolism over several years has yielded little more than possible slowing of vascular calcification. Critically, neither evidence of halting nor evidence of regression has been observed. Further data questioning the validity of a passive (or restorative) therapeutic approach comes from elegant animal work in which calcified aortas from uremic mice were orthotopically transplanted into non-CKD mice and observed for 34 weeks (109). Although there was some superficial loss of calcium, there was no significant active resorption or regression of established calcified material.

Despite the failures to date, novel therapeutic approaches are emerging that actively target calcified material and may offer fresh insights into drug discovery in this area of unmet need.

**CALCIMIMETICS.** The calcium-sensing receptor (CaR) is expressed in multiple tissues but particularly in the parathyroid glands, which makes it an effective target for treating secondary hyperparathyroidism and disordered mineral metabolism in patients with ESKD. Calcimimetics bind to the CaR and allosterically increase the parathyroid cell sensitivity to extracellular calcium, thereby suppressing the release of PTH and resulting in a reduction of serum calcium. In patients with ESKD, calcimimetics also decrease serum phosphate, presumably through a reduction in PTH-mediated bone resorption, which otherwise releases bone phosphate into the circulation. Beyond modulating PTH release, the CaR is also found on VSMCs (110) and its stimulation is associated with a reduction in phosphate- and calcium-driven vascular mineralization both in vitro and in animal models (111,112).

These findings prompted the design and execution of 2 key clinical studies that evaluated cinacalcet: ADVANCE (A Randomised Study to Evaluate the Effects of Cinacalcet Plus Low Dose Vitamin D on Vascular Calcification in Subjects with Chronic Kidney Disease Receiving Hemodialysis) and EVOLVE (Evaluation of Cinacalcet Hydrochloride Therapy to Lower Cardiovascular Events). ADVANCE enrolled 360 patients who received hemodialysis across 3 continents with secondary hyperparathyroidism and CAC (113). Participants were randomized to open-label cinacalcet and low-dose vitamin D analogs or flexible vitamin D therapy and were followed for 12 months. At follow-up, there was a smaller numerical increase (i.e., attenuation of progression) in the CAC score of the cinacalcet-treated patients compared with those treated with flexible vitamin D therapy; however, this did not reach statistical significance (progression of CAC: 24%; 95% CI: –22% to 119% vs. 31% (95% CI: –9% to 179%; p = 0.073). A secondary analysis by CAC volume score in the coronary and the aortic valve suggested a potential beneficial effect of cinacalcet, although this was only of nominal significance. It remained unclear if excess vitamin D use might have mitigated the benefits of cinacalcet, or whether a 12-month follow-up might have been too short to observe a significant change. In comparison, the EVOLVE trial randomized 3,883 patients with secondary hyperparathyroidism on maintenance hemodialysis to cinacalcet or placebo in the setting of conventional therapy (e.g., phosphate binders and calcitriol or active vitamin D analogs) (114). After a median follow-up of 17 (placebo) to 21 (cinacalcet) months, there was an observed trend for relative reduction in the HR for the primary composite CV outcome (i.e., time until death, myocardial infarction, hospitalization for unstable angina, heart failure, or a peripheral vascular event) (HR: 0.93; 95% CI: 0.85 to 1.02; p = 0.11). Post hoc adjustment for age yielded lower relative HRs with nominally significant results in favor of cinacalcet, as did the analyses that accounted for differential withdrawal of study drug and commercial use of cinacalcet in the placebo arm.

Etelcalcetide, an intravenous calcimimetic that acts at a different site on the CaR, was highly efficacious at lowering PTH and FGF-23 in placebo-controlled trials (115,116), and in a head-to-head study, was more effective than cinacalcet on biochemical endpoints (117). The effects of etelcalcetide on vascular calcification or clinical events have not been evaluated.

**VITAMIN K.** As previously described, vitamin K is required to decarboxylate MGP to obtain its calcification inhibitory capacity. Consistent with this physiology, both vitamin K antagonism and vitamin K deficiency have been linked to vascular calcification (118,119). In a biochemical proof-of-concept study conducted in 53 patients who underwent hemodialysis, daily supplementation of vitamin K was safe and resulted in a significant increase in MGP activation (120). Whether increased MGP activation translates into slowing of calcification is being tested in the VitaVasK study. This trial enrolled 348 patients who underwent hemodialysis with a CAC score of at least
100 and randomized them to either oral vitamin K1 orally thrice weekly supplementation or placebo (121). The primary endpoint is progression of thoracic aortic calcification and CAC at 18 months. Major adverse CV events will be assessed at 3 to 5 years after treatment initiation; results are expected towards the end of 2020. The other way of testing this hypothesis is to evaluate for differential effects on vascular calcification in patients who receive direct acting oral anticoagulants compared with vitamin K antagonists (e.g., warfarin). The largest trial evaluating this hypothesis is the IRIVASC-Trial (Rivaroxaban Compared to Vitamin K Antagonist Treatment Upon Development of Cardiovascular Calcification; NCT02066662), which has enrolled participants with an eGFR >15 ml/min/1.73 m² and who have either atrial fibrillation or pulmonary embolism that requires anticoagulation.

**PHOSPHATE BINDERS.** Patients with advanced CKD who have developed hyperphosphatemia frequently require treatment with phosphate binders when dietary restriction is inadequate. Because phosphate and the attendant increase in FGF-23 and PTH have been linked to vascular calcification (46,64) (as previously described), lowering or even maintaining stable phosphate levels near normal could be associated with favorable overall vascular outcomes. Serum phosphate can be reduced with either calcium-containing binders (acetate, carbonate) or calcium-free binders (sevelamer, lanthanum, iron compounds, magnesium). In the dialysis population, the totality of data suggest exposure to exogenous calcium through calcium-containing binders is associated with higher rates of vascular calcification (62,122,123). Although subsequent meta-analyses have shown a mortality benefit for calcium-free binders (124), it remains unclear whether this is due to increased risk in the comparator arms due to excess calcium loading or to an absolute benefit in the calcium free arms. Moreover, because the degree of phosphate lowering has been similar across most patients in the trials, it is unclear whether any benefits at all are due to the management of phosphatemia (or potentially its effect on intermediates, such as FGF-23) or are instead due to agent-specific effects, such as improvements in lipid profile and C-reactive protein observed with sevelamer. Importantly, no clinical trial has demonstrated a long-term benefit of phosphate binders on clinical outcomes in ESKD nor has any trial established the optimal serum phosphate target for ESKD.

In the nondialysis CKD population, data are even less convincing. A recent trial that compared phosphate control with either calcium-containing or calcium-free binders against placebo in patients with stage 3 to 4 CKD showed only a modest reduction in phosphate and a paradoxical increase in CAC (albeit largest in those receiving calcium-containing binders) (125). A further trial is currently underway, IMPROVE-CKD (Impact of Phosphate Reduction on Vascular Endpoints in Chronic Kidney Disease), which is comparing lanthanum against placebo in patients with stage 3 to 4 CKD. This trial will evaluate arterial stiffness as the primary endpoint in 488 participants at 96 weeks with secondary endpoints that include aortic calcification, left ventricular mass using magnetic resonance imaging, and bone and/or phosphate markers among others (126). Whether IMPROVE-CKD will fill the evidence gap between control (or reduction) of serum phosphate with slowing or halting of vascular calcification, or potentially further highlight the limitations of serum phosphate as an overall measure of the total phosphate pool (and thus, propensity to calcification) remains to be seen. Adding complexity is the recently completed COMBINE (CKD Optimal Management With Binders and Nicotinamide study) trial that evaluated a similar cohort of patients with stage 3 to 4 CKD; not only was lanthanum poorly tolerated (despite the addition of nicotinamide), there was minimal effect on phosphate or FGF-23 over 12 months (127).

**MAGNESIUM.** Recent studies have highlighted a potential role for magnesium to prevent vascular calcification. In vitro (128–130) and animal studies (131) showed that magnesium modulates the development of phosphate-induced calcification in a dose-dependent manner. Small human clinical studies have shown that oral administration of magnesium to patients with moderate CKD through to ESKD, either as a phosphate binder or as a supplement, directly slowed CAC progression or indirectly reduced the propensity for calcification (132–134). Based on this favorable signal, an open-label randomized controlled trial evaluated the effect of magnesium oxide on CAC progression in patients with CKD stages 3 to 4 (135). The trial was stopped early when the control arm demonstrated a median CAC change of 39.5% (interquartile range: 19.0% to 81.3%), whereas the magnesium arm showed a progression of only 11.3% (interquartile range: 0% to 30.8%; p < 0.001). Because this was an open label trial that was halted early and included <100 participants, larger studies are required to confirm and progress these findings.

**SNF472: MYO-INOSITOL HEXAPHOSPHATE.** A compound with promising results in early phase studies is SNF472, which is a hexasodium salt of the active
ingredient, myo-inositol hexaphosphate (IP6), or phytate. SNF472 inhibits the development and progression of calcification by binding to growth sites of hydroxyapatite crystal; this mechanism appears to be agnostic to the underlying cause of calcification and may represent an opportunity to inhibit the final common pathway of vascular calcification.

Pre-clinical studies demonstrated that the intravenous administration of SNF472 to vitamin D–supplemented rodent models reduced the development of CV calcification (136) and prevented progression of established calcification (137). Recently, the results of the CalLIPSO (Cal for calcium and ipso meaning the item itself) trial were reported; in this Phase IIb study, 274 patients with ESKD who underwent dialysis were randomized to SNF472 administered at 2 different doses (300 mg or 600 mg) or placebo (1:1:1) for 52 weeks (138). For the primary endpoint at 12 months, using data from the combined dose group, administration of SNF472 resulted in significant slowing of progression of CAC (11%; 95% CI: 7% to 15% vs. 20%; 95% CI 14% to 26%; p = 0.016). Secondary endpoints also showed slowing of progression of aortic valve calcification (14%; 95% CI: 5% to 24% vs. 98%; 95% CI: 77% to 123%; p < 0.001) and a directionally consistent but nonsignificant difference in progression of thoracic aorta calcification (23%; 95% CI: 16% to 30% vs. 28%; 95% CI: 19% to 38%; p = 0.40). Notwithstanding these promising data, larger studies are required to delineate the clinical efficacy and safety of this compound.

**CONCLUSIONS**

Biologically plausible, temporal, and dose-dependent relationships exist between vascular calcification and CV outcomes in patients with CKD. Despite a large investment in potential therapeutic strategies, little more than slowing of calcification has been documented in only a handful of previous studies.

The reasons for a lack of progress are multiple and varied. At a pre-clinical level, there are a lack of animal models that faithfully recapitulate a chronic, progressive CKD calcification process. The extension of pre-clinical findings derived from the current acute injury models may be misleading and translate to early clinical phase failure. The modification of an adenine murine model that does not require transgenic manipulation or surgical intervention appears promising (139). From an imaging perspective, a metric that reliably and quantitatively measures medial calcification with fidelity over time would be a welcome addition to trial endpoints of novel therapies. That said, currently enrolling studies have included imaging of multiple vascular beds or included functional assessments of arterial stiffness. A combination of these predominantly intimal versus predominantly medial modalities will provide unique insights into natural history, relative modifiability and potential differential drug efficacy.

Furthermore, conventional thinking about vascular calcification has been relatively uni-dimensional, aiming to restore or reverse components of the uremic milieu in individuals in whom calcification is established, and inhibitory mechanisms exhausted. The underwhelming results may be due to late intervention at a point of inexorable vascular damage, in which case trials such as IMPROVE-CKD may demonstrate the potential benefits of earlier, more preventative intervention. Alternatively, because of the multiple and synergistic processes involved in the development of vascular calcification in CKD, addressing individual upstream factors in isolation, even if dominant, may not be sufficient. Instead, actively targeting the final common pathway of calcification, such as with an anticalcifying agent agnostic to the upstream processes, may provide critical insight on this type of therapeutic approach.

After decades of translational research fueling heated debate and polarized opinion, the next wave of evidence may finally provide the answer to whether arterial calcification, particularly in the

### TABLE 1 Key Mediators of Medial Vascular Calcification

| Category                                      | Key Mediators                                      |
|-----------------------------------------------|---------------------------------------------------|
| Biochemical and Pathological Changes          | Phosphate, bone, and mineral metabolism           |
|                                               | Failure of/reduction in calcification inhibitors   |
|                                               | Matrix Gla protein                                 |
|                                               | Fetuin-A                                          |
|                                               | Klotho (FGF-23)                                   |
|                                               | Pyrophosphate                                     |
|                                               | Osteopontin                                       |
|                                               | Increase in calcification promoters                |
|                                               | Osteocalcin                                       |
|                                               | Alkaline phosphatase                              |
|                                               | Inflammatory cytokines                            |
|                                               | Runx2                                             |
| Phenotypic transdifferentiation of VSMCs      | Pro-calciﬁc matrix vesicles                       |
| (osteochondroblastic change)                  | Uremic milieu                                     |
|                                               | Cellular apoptosis                                 |
|                                               | Pro-calciﬁc miRNAs                                |

FGF = ﬁbroblast growth factor; miRNA = micro RNA; VSMC = vascular smooth muscle cell.
media, sits on the causal pathway of CVD in CKD. Perhaps the more critical question in managing the growing number of patients with CKD is whether targeting vascular calcification will lead to meaningful improvements in CV outcomes.

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On March 28, 2020, during the JACC Journals’ reception, JACC: Basic to Translational Science was proud to recognize and congratulate the following recipient of the JACC: Basic to Translational Science 2019 Young Author Award.

Kathryn C. Chatfield, MD, PhD

*Paper: Elamipretide Improves Mitochondrial Function in the Failing Human Heart*

Dr. Kathryn Chatfield is an assistant professor of pediatrics and cardiology at the University of Colorado School of Medicine, and and Director of the Cardiac Genetics Clinic. Dr. Chatfield’s clinical interests include pediatric heart failure, pediatric cardiomyopathy, and heart transplantation. In addition to the clinical work that is the foundation of her research program related to energy defects in pediatric dilated cardiomyopathy, she directs the Cardiac Genetics Clinic at the Children’s Hospital of Colorado. This clinic serves children and adolescents with genetic forms of cardiovascular disease, including familial and metabolic forms or cardiomyopathy, inherited forms of congenital heart disease, Noonan spectrum disorders, and connective tissue disorders.

Mentor: Brian Stauffer, MD

Dr. Brian Stauffer is a professor of medicine and integrated physiology at the University of Colorado Anschutz Medical Campus and the School of Medicine. He is the chief of the Division of Cardiology at Denver Health Medical Center. Dr. Stauffer has an adjunct appointment in integrative physiology at the University of Colorado Boulder and is the medical director of the CU Boulder clinical translational research unit of the Colorado Clinical Translational Sciences Institute. His research has focused on basic and translational approaches to understand mechanisms underlying heart failure and vascular endothelial (dys)function.
On March 28, 2020, during the JACC Journals’ reception, JACC: Basic to Translational Science was proud to recognize and congratulate the following recipient of the JACC: Basic to Translational Science 2019 Young Author Award.

Paola C. Rosas, MD, PhD, BSPharm

*Paper: Cardiac Myosin Binding Protein-C Phosphorylation Mitigates Age-Related Cardiac Dysfunction: Hope for Better Aging?*

Dr. Paola Rosas currently is a research assistant professor in the Department of Physiology and Biophysics, College of Medicine, University of Illinois at Chicago. Dr. Rosas was previously a postdoctoral research associate at the Texas A&M University College of Medicine. Dr. Rosas’ research focuses on investigating the causes of gender-related differences in heart failure due to different stressors such as aging, obesity, and diabetes, with the ultimate goal of finding a common denominator with the potential of being translated into therapy.

Mentor: Carl W. Tong, MD, PhD

Dr. Carl Tong cares for patients as an advanced heart failure/transplant cardiologist and conducts basic translation research. He is an associate professor in the Department of Medical Physiology, Texas A&M University College of Medicine. He also serves as the Medical Director at CHI St. Joseph Heart Failure Clinic. Previously, Dr. Tong served as the Interim Medical Director of the Heart Transplant and Mechanical Circulatory Support Program at Baylor Scott & White Health Central Texas and as an active duty Captain in the U.S. Air Force as an electrical engineer. His research effort focuses on elucidating myofilament-based mechanisms that can improve diastolic and systolic functions of the heart. After elucidation, Dr. Tong aims to translate these discoveries to new treatments for heart failure.
Chinese Health Care Workers and COVID-19
For Whom the Bell Tolls

Douglas L. Mann, MD, Editor-in-Chief, JACC: Basic to Translational Science

“No man is an island, entire of itself; every man is a piece of the continent, a part of the main. If a clod be washed away by the sea, Europe is the less, as well as if a promontory were, as well as if a manor of thy friend’s or of thine own were: any man’s death diminishes me, because I am involved in mankind, and therefore never send to know for whom the bells tolls; it tolls for thee.”
—John Donne, Devotions Upon Emergent Occasions (1)

In mid-December 2019, Chinese physicians working in Wuhan, a port city of 11 million people in the central province of Hubei, reported several cases of an undisclosed pneumonia. Using a surveillance mechanism that was established following the 2003 severe acute respiratory syndrome (SARS) outbreak, the initial 4 reported cases of “pneumonia of unknown etiology” were linked to the Huanan Seafood Wholesale Market, which consequently was shut down on January 1, 2020. Based on the results of deep sequencing and investigations by 5 independent laboratories, Chinese officials reported on January 7, 2020, that the causative agent of the mystery pneumonia was a novel coronavirus (nCoV). Sequence analysis revealed that the 2019-nCoV possesses a typical genome structure of coronavirus belonging to the cluster of betacoronaviruses that includes Bat-SARS-like (SL)-ZC45, Bat-SL ZXC21, SARS-CoV, and Middle East respiratory syndrome-CoV. Based on the genomic content of coronavirus disease viruses, 2019-nCoV appears to be more closely related to bat-SL-CoV ZC45 and bat-SL-CoV ZXC2, and more distantly related to SARS-CoV (2). On January 11, 2020, China reported the first death from nCoV in a 61-year-old man who purchased goods from the Huanan Seafood Wholesale Market. In the weeks that followed, the coronavirus outbreak spread across China, slithering into neighboring countries and continents. By January 13, the World Health Organization reported a case of 2019-nCoV in a woman in Thailand, who had arrived from Wuhan. Thus began what we now recognize as the coronavirus disease 2019 (COVID-19) pandemic.

How the 2019-nCoV spread from a seafood market to become a worldwide pandemic that collapsed economies worldwide within a matter of months will continue to be the grist for the medical, economic, and political mills, now and for the foreseeable future. Certainly, there is plenty of blame to go around, beginning with the early handling of the outbreak by Chinese officials, as well as the agonizingly slow roll out of nCoV testing in the United States. Notwithstanding the reflexive finger-pointing and blame-gaming, there is an inspiring backstory of human sacrifice, heroism, and courage on the part of the health care workers in China, who were grappling with treating patients with SARS flu-like symptoms, but without any specific knowledge about the virulence or pathogenicity of the disease they were treating. Moreover, the frontline Chinese health care workers were providing care without proper personal protective equipment to shield them from harm. In a sobering article in the Los Angeles Times titled “Doctors and Nurses Fighting Coronavirus in China Die of Both Infection and Fatigue” (3), Alice Su reports that there were “18 reported deaths of medical workers involved in the COVID-19 response as of Monday [February 24, 2020], including nurses and doctors who died not because of infection but because of cardiac arrest or other ailments due to overwork and fatigue. One victim was hit by a car while taking...
temperatures on a highway.” The article also mentions Dr. Peng Yinhua (age 29), who postponed his planned wedding on February 1, so that he could continue to provide care to COVID-19 patients. Dr. Yinhua promised his pregnant fiancée that they would have a ceremony after the outbreak had passed: he died of COVID-19 on February 21.

In his epic work Devotions Upon Emergent Occasions, John Donne argues that the death of any individual is something others can learn from, should they understand it properly (1,4). Fully understanding the COVID-19–attributable deaths of health care workers in China, and for that matter, the COVID-19–attributable deaths of health care workers all around the world will take some time to process, insofar as many of these deaths were preventable. As noted, there is plenty of blame to go around. The unbelievable courage of the men and women who provided care to COVID-19 patients, knowing that they might die from the same mysterious illness that was killing their patients, is an example of what is truly remarkable and best about humanity. Their lesson of sacrifice and selfless caring is one that can never be washed away, nor ever diminished. Not ever. To this end, we dedicate this issue of JACC: Basic to Translational Science to the Chinese health care workers who died from COVID-19, as well as all of the other health care workers around the world who are caring for COVID-19 patients. A partial list of the names of the frontline Chinese physicians and nurses who died of COVID-19 (online search date March 20, 2020) follows at the end of this Editor’s Page.

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IN MEMORIAM

Dr. Wenliang Li, 35 years old, ophthalmologist, Central Hospital of Wuhan.
Dr. Zhongming Mei, 57 years old, ophthalmologist, Central Hospital of Wuhan.
Dr. Heping Zhu, ophthalmologist, Central Hospital of Wuhan.
Dr. Xueqing Jiang, 56 years old, thyroid and breast surgeon, Central Hospital of Wuhan.
Dr. Zhengbin Lin, 62 years old, kidney transplant surgeon, Tongji Hospital of Huazhong University of Science and Technology.
Dr. Yinhua Peng, 29 years old, pulmonary physician, First People’s Hospital of Jiangxia District, Wuhan, Hubei province.
Dr. Sisi Xia, 29 years old, gastroenterologist, Xiehe Jiangbei Hospital, Wuhan, Hubei province.
Dr. Wenjun Huang, 42 years old, pulmonary physician, Xiaogan Central Hospital, Hubei province.
Dr. Zhiming Liu, 51 years old, Dean of Wuchang Hospital, Wuhan, Hubei province.
Dr. Hui Xu, 51 years old, Vice Dean of Nanjing Hospital of Chinese Medicine, Nanjing, Jiangsu province.
Dr. Junxiao, 50 years old, general surgeon, Red Cross Hospital of Wuhan, Wuhan, Hubei province.
Dr. Yanghong Mao, 52 years old, Vice Dean of Xianyang Central Health Center, Nanping, Fujian province.
Dr. Jijun Jiang, 52 years old, infectious physician, Taizhou People’s Hospital, Taizhou, Jiangsu province.
Dr. Jinbo Jiang, 58 years old, Disease Control Center of Dayu, Ganzhou, Jiangxi province.
Nurse Fan Yang, 55 years old, Wuchang Hospital Community Health Service Center, Wuhan, Hubei province.
Dr. Debu Wu, 69 years old, Ezhou Hospital of Traditional Chinese Medicine, Ezhou, Hubei province.
Dr. Yingjie Song, 28 years old, Maji Health Center, Donghu, Hunan province.
Dr. Tucheng Wang, 37 years old, Changge, Henan province.
Dr. Zhengrong Zhu, 48 years old, Nanyang Community Health Service Center, Nantong, Jiangsu province.
Dr. Xiansheng Du, 55 years old, Yangjiang Hospital of Li Autonomous County, Qiongzhong, Hainan province.
Dr. Wucheng Liang, 62 years old, Hubei Hospital of Integrated Traditional Chinese and Western Medicine.
Dr. Yunhua Song, 46 years old, Baoshan, Yunnan province.
Dr. Yangyang Yuan, 36 years old, Baofeng Hospital of Traditional Chinese Medicine, Henan province.
Dr. Jinxing Yuan, 32 years old, Jinlingfeng Town Health Center, Hezhou, Guangxi province.