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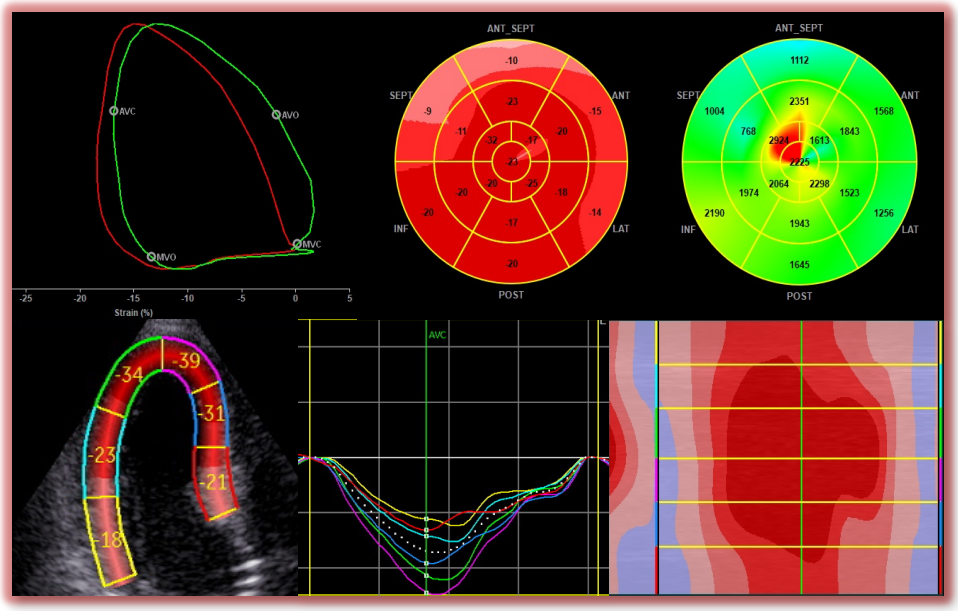

New echocardiographic and translational tools in valvular and ischemic heart disease

PhD Thesis

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"More important than the quest for certainty is the quest for clarity"

Francois Gautier

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CHAPTER 1

General introduction and outline of the thesis

Echocardiography is the most widely used cardiac imaging technology in clinical setting (1). Its non-invasiveness, broad availability and reproducibility represent the main advantages of this powerful investigative tool, whose utilization has considerably increased in recent years. Actually, the advent of new methodologies such as tissue Doppler imaging, 2D speckle tracking for myocardial deformation and 3D echocardiography (2) has provided an additional tool, capable of finer and earlier diagnosis of myocardial dysfunction.

Speckle Tracking Echocardiography (STE) is an advanced method that allows a fast and accurate assessment of global and regional myocardial function, independently of the angle of insonation and in-plane translational motion (3) It is based on the observation that the interaction between the ultrasound beam and the myocardium generates acoustic markers, defined *speckle*, which can be tracked in their displacement during the cardiac cycle, thanks to a dedicated software. Before STE, only the myocardial tissue tagging with cardiovascular magnetic resonance (CMR) was able to evaluate the cardiac deformation without an angle dependence (4). Although considered the reference standard in this area of study, the routine use of tagged CMR is limited by its high costs, poor availability, relative complexity of acquisitions, and time-consuming image analysis (5). About 15 years ago, the first paper regarding STE validated this technique against sonomicrometry and CMR tagging, showing accurate and angle-independent measurements of left ventricular (LV) dimensions and strains (6). Since then, STE has become a widely used method to evaluate myocardial contractile function. By tracking the displacement of speckles during the cardiac cycle, STE allows semiautomated elaboration of myocardial deformation in 3 spatial directions: longitudinal, radial, and circumferential. (7) The semiautomated nature of STE guarantees good intraobserver and interobserver reproducibility.(8) The longitudinal strain (LS) of the LV represents the myocardial

shortening along its longitudinal axis and it is identified by negative curves during systole and by positive ones in diastole. LS is evaluated in 2-, 3- and 4-chamber apical views: LV is divided into 6 segments in each view and global LS (GLS) calculated as a the mean value of the deformation peak at end systole.

Although STE was introduced for the exclusive analysis of the LV deformation, several studies have recently extended its applicability to other cardiac chambers, such as the left atrium (LA), right atrium and right ventricle (RV) (9). Currently, a dedicated software for the evaluation of other heart chambers is not available on the market. Nevertheless, considering the limits of using the same software used to assess LV strain, despite the different wall thickness of the different heart chambers, both RVLS and LA strain have shown good feasibility and reproducibility.(9-10) For the RV, the segments of interest are obtained in 4-chamber apical view and they are 6 (3 for the free wall and 3 for the interventricular septum). Also for the RV, the averaged value of strain curve of each segment represent the global RVLS. Moreover, if the region of interest of the strain analysis is focused only on basal, mid, and apical segments of RV free wall, it is possible to analyze singularly free wall RVLS, that has emerged as an even more accurate parameter in detecting RV dysfunction in some clinical settings (11).

Evaluation of LA deformation parameters is a promising approach for analyzing LA mechanics. Early detection of LA dysfunction can be anticipated with strain measurement; it can also provide new insights into pathophysiology and perhaps guide clinical management. Two longitudinal strain parameters of the LA are recognized: the peak atrial longitudinal strain (PALS), measured at the end of the atrial reservoir phase, and the peak atrial contraction strain (PACS), identified just before the start of the active atrial contraction.(12) The mean value is defined respectively global PALS and global PACS, taking into account 12 segments (6 in 4-chamber apical view and the other 6 in 2-chamber apical view). Although normal ranges of LA function have been reported in some previous studies, age related normal references have not been explored.

Technological progression of 2D speckle-tracking software has also enabled the estimation of layer-specific strain, thus allowing to differentiate endocardial and epicardial longitudinal strain. This differentiation has clinical implications as the longitudinal fibers of endocardial layer could be firstly involved in the progression of myocardial dysfunction in the majority of cardiac pathologies. Accordingly, the clinical usefulness of the multilayer strain software has already been successfully tested in some clinical settings, including coronary artery disease (CAD), myocardial infarction, arterial hypertension, and heart failure. (13-16) Furthermore, the study of layer-specific strain could provide useful insight in the pathogenesis of aortic stenosis (AS) related myocardial dysfunction. Previous studies, in fact, have suggested that endocardial layer could be affected first by the pathological changes (hypertrophy, increased wall stress, reduced arterial compliance) associated with AS. (17-18)

In the recent years, myocardial work (MW) has emerged as an alternative tool for myocardial function assessment. This new parameter derives from GLS, with the advantage to incorporate information on afterload, through interpretation of strain in relation to dynamic non-invasive LV pressure. Russel et al. demonstrated that non-invasively pressure-strain loops (PSLs) could estimate LV performance deriving LV pressure curves during a cardiac cycle from non-invasively acquired brachial artery cuff pressure (19-20). Myocardial work is approximatively calculated as the area of PSL. Experimental studies have shown a strong correlation of LV-PSL area with cardiac metabolism, assessed by fluorine 18 fluorodeoxyglucose-positron emission tomography (19). The clinical application of MW measurement has been shown in several pathological conditions, including LV dyssinchrony (21-22), ischemia (23-24) , hypertrophic (25), hypertensive and dilated cardiomyopathy (26). In this thesis we report the first multicenter study which established normal reference limits for MW indices in healthy adults and the correlation of MW indices with systolic and diastolic parameters.

In general, STE may allow an in-depth evaluation of myocardial systolic and diastolic dynamics across a broad range of physiologic and pathologic conditions beyond traditional

echocardiographic techniques. In sight of the increasingly wide availability of these new advanced techniques, which appear to be more sensible than traditional echocardiographic parameters (such as LVEF) in the detection of pathologic conditions, STE has become a pivotal target of several studies in patients with cardiomyopathies and valvular disease.

Therefore, in this thesis we explored the feasibility, efficacy and prognostic role of these new diagnostic tools mainly in two clinical setting: valvular and ischemic heart disease.

AS represents a high prevalent disease in developed country, especially among elderly population (27). Currently, in presence of severe AS, valve replacement is indicated when symptoms occur or when LVEF is reduced (<50%) (28). Conversely, the management of patients with asymptomatic severe AS, particularly the choice between early intervention vs watchful waiting, continues to be a matter of debate. Symptoms related to AS (limiting dyspnea, syncope, dizziness) sometimes are unrecognized, mainly in elderly patients who often reduce the level of their daily activity as a mechanism of protection to avoid symptoms. In this context, exercise testing has proven to be a useful tool to unmask exercise-limiting symptoms (29-30), but this test is not available in all centers and not all patients are able to perform it. Thus, the identification of new parameters able to predict prognosis both in asymptomatic severe AS patients and in those undergoing transcatheter aortic valve implantation (TAVI) could be helpful to guide the decision-making and the correct timing of intervention.

In the last years, strain parameters, mostly LV GLS, have gained growing importance in the diagnosis and prognosis of CAD. Studies in patients with acute coronary syndrome (ACS) have demonstrated that GLS is related to peak levels of cardiac troponin T (31) and LV infarct size (32) and, also in patients with preserved LVEF, values <-14% are predictive of hospitalization for acute heart failure and CV mortality (33). In chronic coronary syndrome (CCS), both LV GLS (34) and RV GLS (35) are important predictors of outcome during a long-term follow-up. Besides its prognostic value, strain parameters have also shown to improve diagnosis of significant CAD in acute (32,36-37) and chronic setting (38-39).

In patients with CCS and multivessels disease, stress echocardiography finds indication in detecting myocardial ischemia to guide revascularization (40). Actually, dobutamine stress echocardiography (DSE) have demonstrated good sensibility in revealing presence and location of significant coronary stenosis (41-42), despite is limited by a subjective interpretation, dependent on operator experience and image quality (43-44). In this context, the application of STE, which provides a more objective and quantitative analysis of myocardial contraction, would improve the diagnostic power of the stress test.(45) In this thesis, we report a study aimed at investigate the feasibility and accuracy of global and regional LS during DSE in detecting significant coronary stenosis.

In some circumstances, CAD is not associated with an a significant obstruction of an epicardial coronary artery, but is expression of coronary microvascular dysfunction (MVD) (46). MVD is commonly related to detrimental effects of CV risk factors on the arterial wall, hypersensitivity of the vascular smooth muscle cell layer, or both. Coronary flow reserve (CFR), which in absence of significant epicardial coronary stenosis is an accurate expression of coronary microvascular function, might be tested non-invasively by transthoracic doppler echocardiography (47). Pharmacological agents used to induce maximal endothelium-independent hyperemia mainly include adenosine and dipyridamole. Hyperemia may even be provoked by a completely endothelium-dependent stimulus such as cold pressure test (CPT), which is performed by hand immersion in ice water for few minute and acts through the production and release of several vasoactive mediators such as nitric oxide (NO) (48). It's known that traditional CV risk factors (such as dyslipidemia, hypertension and diabetes mellitus) can influence CRF, through reduction of NO's bioavailability, increase of reactive oxygen species (ROS) production and alteration of endothelial function (49), but also interfere with platelets function, allowing therefore, development of atherosclerotic lesions and acute CV events (50). Identification of molecules able to counteract negative effects of hypercholesterolemia, hyperglycemia, and oxidative stress has been object of our studies.

Outline of the thesis

The thesis is divided in five parts:

Part I. Feasibility and reference ranges for longitudinal strain and myocardial work indices in EACVI NORRE study population. In the first part of the thesis we report our research projects focused on the validation of feasibility, reproducibility and clinical value of the more recent, advanced echocardiographic parameters: LA strain and strain rate (chapter 2), LV multilayer strain (chapter 3), myocardial work (chapter 4) and its correlates (chapter 5). Normal values according to age, gender and body surface area of the parameters mentioned above were provided in a large cohort of healthy individuals enrolled in the Normal Reference Ranges for Echocardiography Study (NORRE Study).

Part II. Multiparametric approach in the diagnosis, prognosis and treatment of aortic stenosis. In this part of the thesis, we focused our research project on patients with moderate to severe AS, from the asymptomatic status until TAVI. We started with the investigation of the natural history and outcomes of patients with moderate and severe AS (chapter 6), followed by the description of the role of stress echocardiography in the assessment of severity and symptoms (chapter 7), then the identification of predictors of symptom occurrence (chapter 8 and chapter 9) and worse prognosis (chapter 11). Finally, we evaluated the role of STE in the detection of early myocardial recovery after TAVI (chapter 12), also in a specific subgroup of patients with radiation-associated AS (chapter 13).

Part III. **Ischemia-driven coronary revascularization: how stress echocardiography can make difference.** In this section, we explored recent evidence and current indication to perform a complete revascularization in acute and chronic coronary syndrome (chapter 14), and how STE applied to DSE could help in the detection of myocardial ischemia (chapter 15).

Part IV. Platelet and microvascular function. This part of the thesis is centered on the evaluation of endothelial and platelet function in specific clinical settings: dyslipidemia (chapter 16), diabetes mellitus (chapter 17), chronic kidney disease (chapter 18) and ACS (19).

Part V: Discussion and conclusions. The last section of the thesis is a broad discussion of the addressed topics with the conclusions.

Part I

Feasibility and reference ranges for longitudinal strain and myocardial work indices in EACVI NORRE study population

ESC European Society of Cardiology

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Echocardiographic reference ranges for normal left atrial function parameters: results from the EACVI NORRE study

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Aims To obtain the normal ranges for echocardiographic measurements of left atrial (LA) function from a large group of healthy volunteers accounting for age and gender.

Methods and results A total of 371 (median age 45 years) healthy subjects were enrolled at 22 collaborating institutions collaborating in the Normal Reference Ranges for Echocardiography (NORRE) study of the European Association of Cardiovascular Imaging (EACVI). Left atrial data sets were analysed with a vendor-independent software (VIS) package allowing homogeneous measurements irrespective of the echocardiographic equipment used to acquire data sets. The lowest expected values of LA function were 26.1%, 48.7%, and 41.4% for left atrial strain (LAS), 2D left

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atrial emptying fraction (LAEF), and 3D LAEF (reservoir function); 7.7%, 24.2%, and -0.53/s for LAS-active, LAEF-active, and LA strain rate during LA contraction (SRa) (pump function) and 12.0% and 21.6% for LAS-passive and LAEF-passive (conduit function). Left atrial reservoir and conduit function were decreased with age while pump function was increased. All indices of reservoir function and all LA strains had no difference in both gender and vendor. However, inter-vendor differences were observed in LA SRa despite the use of VIS.

Conclusion The NORRE study provides contemporary, applicable echocardiographic reference ranges for LA function. Our data highlight the importance of age-specific reference values for LA functions.

Keywords adult echocardiography • left atrial function • deformation imaging • reference values

Introduction

The left atrium is extremely sensitive to sustained volume and pressure overload secondary to increased left ventricular filling pressures,[1] and the stepwise backward effects of loss in left atrial (LA) functional properties are a reduction in lung vessel compliance and vascular remodelling that trigger right ventricular overload and dysfunction.[2] In contrast to left ventricular measures, there is a strong linear relationship between volumetric and longitudinal deformation indices of left atrium.[3] Early detection of subclinical LA dysfunction plays a crucial role in the evaluation of many cardiac diseases.[4–9] Although normal ranges of LA function have been reported in recent studies,[10–13] age related normal references remain unknown. The Normal Reference Ranges for Echocardiography (NORRE) study is the first European, large, prospective, multicentre study performed in 22 laboratories accredited by the European Association of Cardiovascular Imaging (EACVI) and in one American laboratory. The NORRE study has already provided reference values for all 2D echocardiographic measurements of the four cardiac chambers,[14] Doppler parameters,[15] aortic dimensions,[16] left ventricular strains,[17] and 3D echocardiographic measurements of LV volumes and strain.[18] This report aimed (i) to establish normal reference limits, using vendor-independent software (VIS), for 2D and 3D measurement of LA function in healthy adults and (ii) to examine the influence of age, gender, and vendor on the reference ranges.

Methods

Patient population

A total of 734 healthy European subjects constituted the final NORRE study population. The local ethics committees approved the study protocol. After the exclusion of patients that had incompatible image format and/or poor image quality, the final study population consisted of 371 normal subjects. Baseline clinical characteristics of patients included in and excluded from this study are shown in Supplementary data online, *Table S1*.

Echocardiographic examination

A comprehensive echocardiographic examination was performed using state-of-the-art echocardiographic ultrasound systems (GE Vivid E9; Vingmed Ultrasound, Horten, Norway, and/or iE33; Philips Medical Systems, Andover, MA, USA) following recommended protocols approved by the EACVI.[19,20] All echocardiographic images were recorded in a digital raw-data format (native DICOM format) and centralized for further analysis, after anonymization, at the EACVI Central Core Laboratory at the University of Liège, Belgium.

LA functions and stiffness analysis

Based on previous validated studies and guidelines of the American Society of Echocardiography/EACVI, quantification of LA 2D strain, 2D volume, and 3D volume was performed using commercially available VIS (2D Cardiac Performance Analysis and 4D Cardio-View, TomTec Imaging System, Munich, Germany) (*Figure 1*).[10,21] 2D analyses were performed in the apical four- and two-chamber views. The most suitable cardiac cycle was chosen for each view. The reference point was set at the beginning of the QRS complex. Left atrial end-systole was identified as the time point in which the LA cavity was the smallest. The endocardial border was traced in end-systole. The accuracy of tracking was visually confirmed throughout the cardiac cycle and confirmed from the morphology of the strain curves. If necessary, the region of interest was readjusted. In the measurement of LA 3D volumes, end-diastole was identified as the time point in which the LA cavity is the largest and end-systole as the time point at which the cavity was the smallest. The definition of LA reservoir, pump, and conduit function in this study is demonstrated in *Figure 2*. Left atrial reservoir function was assessed using the left atrial strain curve (LAS), left atrial emptying fraction (LAEF) in both 2D and 3D. As shown in *Figure 2*, LA pump function was assessed using LAS-active: LA strain at the onset time of the P wave, LAEF-active: (LA volume at the onset time of the P wave - LA minimum volume)/LA volume at the onset time of the P wave and LA strain rate during LA contraction (SRa).[10] Left atrial conduit function was assessed by using LAS-passive: LAS - LAS-active, and LAEF-passive: (LA maximum volume - LA volume at the onset time of the P wave)/LA maximum volume. The ratio of mitral inflow *E*/*e*' to LA reservoir function (LAS, LAEF) was used to estimate LA stiffness.[22]

Statistical analysis

Normality of the distribution of continuous variables was tested by the Shapiro–Wilk test. All data are presented as the mean ± standard deviation or median (interquartile range) as appropriate. Group differences were evaluated using the Student's *t*-test for normally distributed continuous variables and the Mann–Whitney *U* test for non-normally distributed continuous variables. Correlation between continuous variables was performed Pearson's or Spearman's correlation coefficient as appropriate. The lowest (2.5th percentile) and highest (97.5th percentile) expected values for left atrial parameters were estimated in 1000 bootstrap samples to generate sampling distributions. For each of these values, the mean and standard errors were estimated from the simulated sampling distribution. Multiple linear regression analyses were performed to examine the independent correlates between LA functions and baseline parameters including cardiovascular risk factors (age, gender, body mass index, systolic blood pressure, diastolic blood pressure, glycaemia, and cholesterol level) and vendor. Intra-observer (T.S.) variability was assessed in 20 randomly selected subjects using intraclass correlation coefficient (ICC). A *P*-value of <0.05 was considered as statistically significant. Data were analysed using open source statistical software, R version

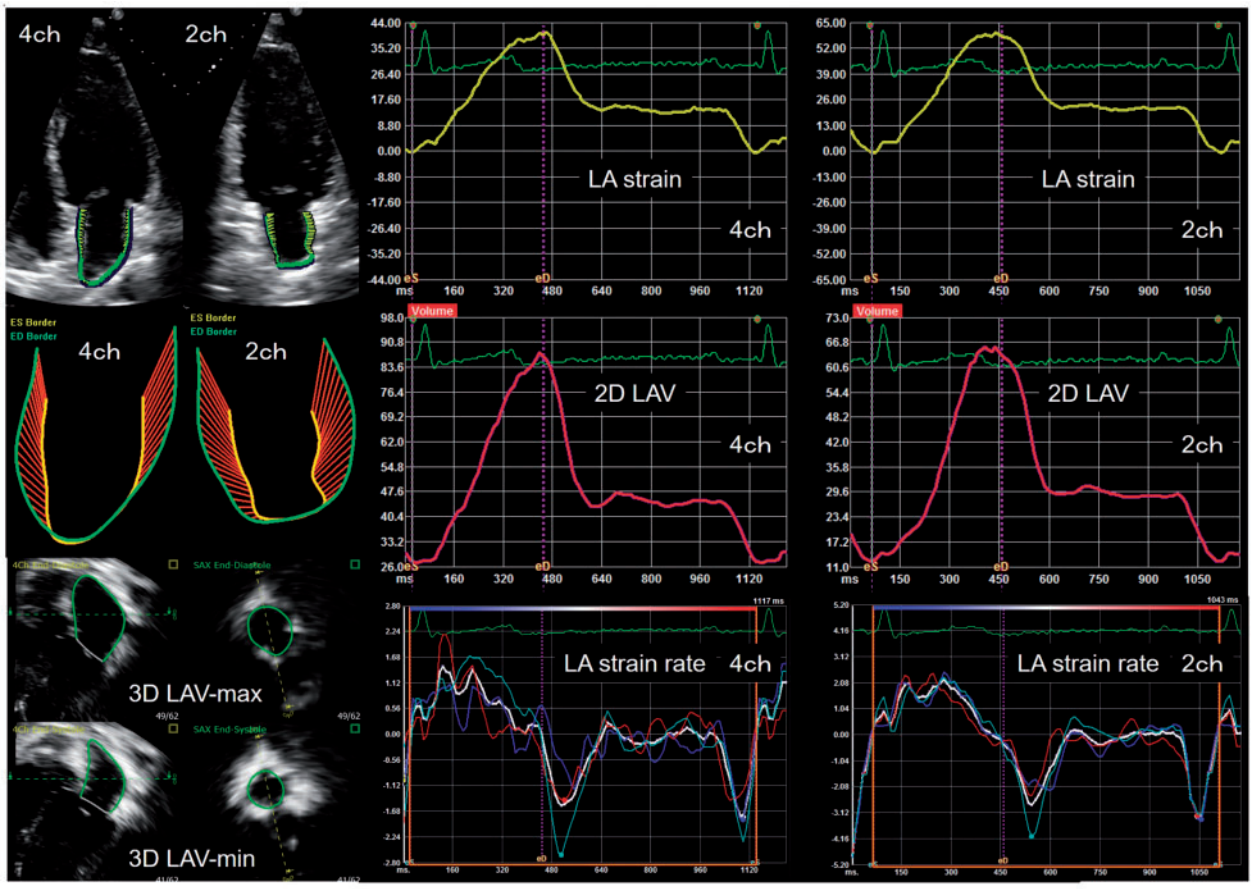

Figure 1 Measurements of LA strain and strain rate by 2D speckle-tracking echocardiography analysis and LA volume by 2D and 3D echocardiography analysis using VIS. VIS, vendor-independent software; LA, left atrial; LAV, left atrial volume.

3.3.2 (R Foundation for Statistical Computing, www.R-project.org) and SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

Results

Demographic data

Table 1 summarizes the demographic data of the cohort of the NORRE population analysed in this study. A total of 165 men and 206 women were included. There was no significant association between age and 3D LA volume index on univariable analysis ($R = 0.09$, $P = 0.07$) and no differences in 3D LA volume index for gender. Values for LA reservoir, pump, and conduit function obtained from analysis of LA volume and strain curves for the entire study population are summarized in *Table 2*. The lowest limits of normality were 26.1%, 48.7%, and 41.4% for LAS, 2D LAEF, and 3D LAEF (reservoir function), respectively; 7.7%, 24.2%, and -0.53/s for LAS-active, LAEF-active, and LA SRa (pump function), respectively; and 12.0% and 21.6% for LAS-passive and LAEF-passive (conduit function), respectively. All LA parameters except SRa were significantly associated with age. Gender differences were observed in LAEF-passive. Vendor differences were observed in 2D LAEF, LAEF-active, and LA SRa. Multivariable analysis for LA functions showed that LAS, 2D LAEF, 3D LAEF, LAS-passive, and LAEF-passive decreased whereas 3D LA volume index and LAS-active increased with age. After adjusting for variables including basic parameters and vendor, LAEF-passive was higher in women than in men and 3D LA volume index was higher and LA SRa was lower when acquired with GE platforms compared with Philips equipment, (*Table 3*). *Figure 3* shows two representative cases of LA functional assessments (middle vs. advanced age).

Age and LA functions relationship

Left atrial reservoir, pump, and conduit function and stiffness in the three subgroups according to age (20–40, 40–60, and ≥60 years) are displayed in *Table 4*. The lowest expected values for LAS were 31.1% in 20–40 years of age, 27.7% in 40–60 years, and 22.7% in ≥60 years. The highest expected values for LA stiffness calculated from LAS were 0.22 in 20–40 years of age, 0.42 in 40–60 years, and 0.55 in ≥60 years.

Vendor and LA functions relationship

Multivariable analysis showed that 3D LA volume index was higher [GE, 27.8 (24.2–32.2) mL/m^2, $n = 189$ vs. Philips, 25.5 (22.8–29.3) mL/m^2, $n = 184$, $P = 0.001$] and LA SRa was lower [GE, -0.98 (-1.16 to -0.73)/s vs. Philips, -1.99 (-2.42 to -1.58)/s, $P < 0.001$] with GE

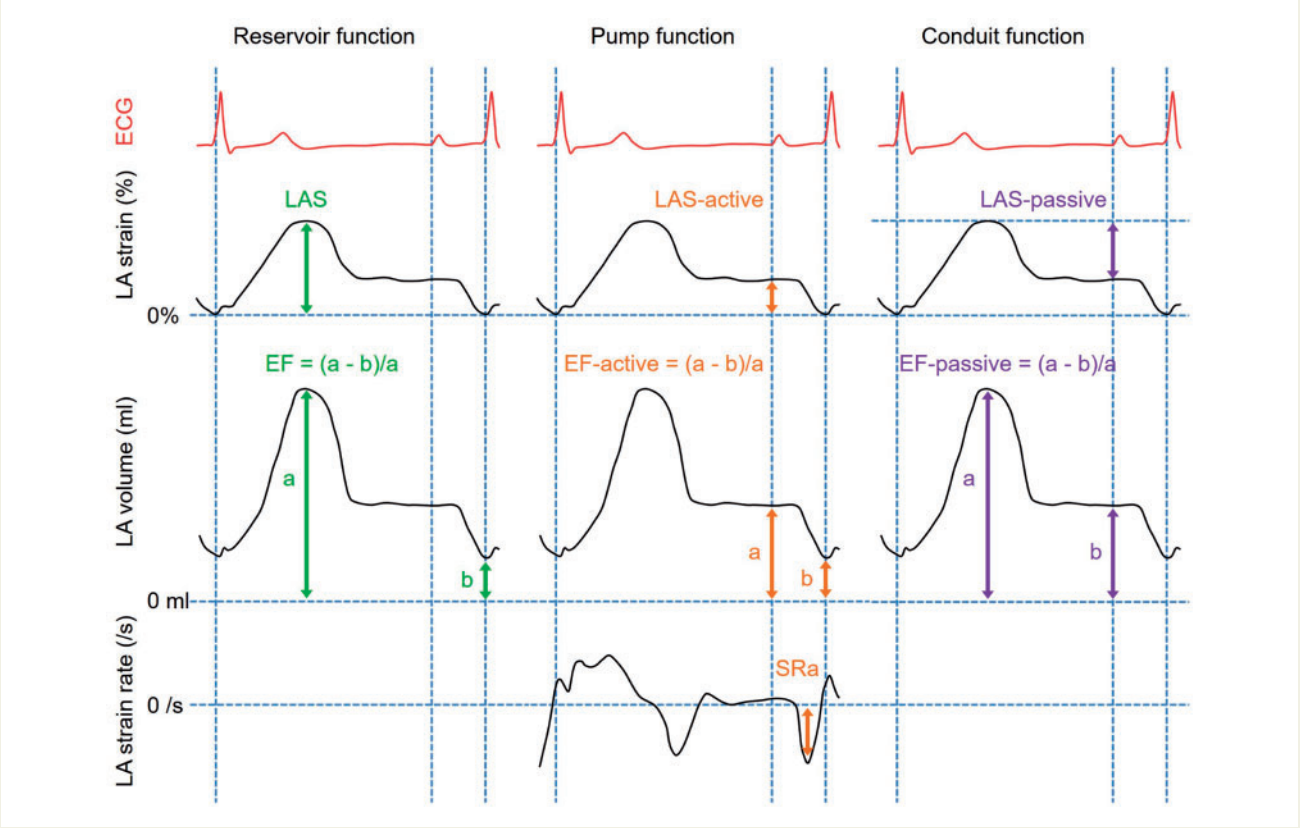

Figure 2 Assessments of LA reservoir, pump and conduit function using LA strain analysis and LA volume. EF, emptying fraction; LA, left atrial; LAS, left atrial strain; SRa, strain rate.

Table 1 Characteristics of the population

Parameters	Total ($n = 371$)	Male ($n = 165$, 44%)	Female ($n = 206$, 56%)	*P*-value
Age (years)	45 (34–55)	46 (33–57)	44 (34–54)	0.54
Height (cm)	170 ± 9	177 ± 7	165 ± 7	<0.001
Weight (kg)	69 (60–78)	77 (71–84)	63 (57–69)	<0.001
Body surface area (m^2)	1.79 (1.66–1.94)	1.94 (1.85–2.05)	1.68 (1.61–1.78)	<0.001
Body mass index (kg/m^2)	23.9 (22.0–26.0)	24.7 (23.2–26.4)	23.2 (21.1–25.4)	<0.001
Systolic blood pressure (mmHg)	120 (110–130)	121 (117–130)	116 (108–126)	<0.001
Diastolic blood pressure (mmHg)	75 (70–80)	77 (70–80)	73 (68–80)	0.002
Glycaemia (mg/dL)	90 (85–98)	91 (87–98)	90 (83–96)	0.017
Cholesterol level (mg/dL)	181 (163–197)	183 (160–197)	179 (164–196)	0.86

compared with Philips equipment for the total population. The same tendency was observed for the apical four-chambers [-0.84 (-1.12 to -0.65) vs. -1.81 (-2.28 to -1.32)/s, $P < 0.001$] and two-chambers [-1.11 (-1.31 to -0.82) vs. -2.25 (-2.65 to -1.70)/s, $P < 0.001$] LA SRa. The number of patients whose LA SRa could not be identified on LA strain analysis was significantly higher with GE than Philips (8% vs. 0% and 38% vs. 15% in apical four- and two-chambers view, $P < 0.001$, respectively). The lowest expected values for LA SRa were -0.47/s (GE) and -0.86/s (Philips). The highest expected values for LA volume index were 41.3 mL/m^2 (GE) and 39.6 mL/m^2 (Philips).

Repeatability

Intra-observer analysis showed excellent repeatability in LAS, LAS-active, LA SRa, and 3D LA volume (ICC = 0.85, 0.71, 0.79, and 0.90, $P < 0.01$, respectively).

Table 2 Left atrial reservoir, pump, and conduit function

	Total		Age		Differences between gender	Differences between vender
	Mean ± SD or medial (IQR)	Limits of normality ± SE[a,b]	*R*	*P*-value	*P*-value	*P*-value
3D LA volume (mL)	47.7 (40.8 to 57.1)	78.7 ± 2.2[a]	0.10	0.06	<0.001	0.001
3D LA volume index (mL/m^2)	26.3 (23.1 to 31.1)	40.6 ± 1.1[a]	0.09	0.07	0.07	0.001
Reservoir function						
LAS (%)	42.5 (36.1 to 48.0)	26.1 ± 0.7[b]	-0.47	<0.001	0.49	0.47
2D LAEF (%)	68.5 (63.2 to 73.2)	48.7 ± 1.9[b]	-0.31	<0.001	0.42	0.02
3D LAEF (%)	57.3 (52.4 to 61.9)	41.4 ± 1.1[b]	-0.17	<0.001	0.53	0.76
Pump function						
LAS-active (%)	16.3 (12.9 to 19.5)	7.7 ± 0.3[b]	0.15	0.003	0.34	0.35
LAEF-active (%)	43.1 ± 9.4	24.2 ± 1.4[b]	0.14	0.008	0.13	0.002
LA SRa (/s)	-1.31 (-1.99 to -0.95)	-0.53 ± 0.03[b]	-0.1	0.054	0.08	<0.001
Conduit function						
LAS-passive (%)	25.7 (20.4 to 31.8)	12.0 ± 0.5[b]	-0.61	<0.001	0.06	0.98
LAEF-passive (%)	43.0 ± 10.3	21.6 ± 0.9[b]	-0.55	<0.001	0.008	0.85

IQR, interquartile range; SD, standard deviation; SE, standard error; other abbreviations as in *Figure 2*.
[a]Highest expected values.
[b]Lowest expected values.

Table 3 Multivariable analysis for left atrial functions

Variables	Basic parameters[a]		Basic parameters[a] + vendor	
	β-coefficients ± SE	*P*-value	β-coefficients ± SE	*P*-value
3D LA volume index (mL/m^2)				
Age (years)			0.07 ± 0.03	0.029
Cholesterol level (mg/dL)	-0.03 ± 0.01	0.033	-0.03 ± 0.01	0.009
Male gender			1.62 ± 0.77	0.037
Vendor (GE as referent)			-3.94 ± 0.74	<0.001
LAS (%)				
Age (years)	-0.32 ± 0.04	<0.001	-0.33 ± 0.04	<0.001
Body mass index (kg/m^2)	-0.39 ± 0.18	0.03	-0.42 ± 0.18	0.02
Glycaemia (mg/dL)	-0.09 ± 0.04	0.049		
2D LAEF, (%)				
Age (years)	-0.20 ± 0.04	<0.001	-0.21 ± 0.04	<0.001
3D LAEF (%)				
Age (years)	-0.11 ± 0.04	0.008	-0.10 ± 0.04	0.013
LAS-active (%)				
Age (years)	0.06 ± 0.02	0.02	0.06 ± 0.03	0.02
Glycaemia (mg/dL)	-0.07 ± 0.02	0.005	-0.07 ± 0.02	0.006
LAEF-active (%)				
Body mass index (kg/m^2)	0.40 ± 0.20	0.047		
Glycaemia (mg/dL)	-0.10 ± 0.05	0.036	-0.10 ± 0.05	0.045
LA SRa (/s)				
Age (years)	-0.01 ± 0.004	0.01		
Vendor (GE as referent)			-1.08 ± 0.08	<0.001
LAS-passive (%)				
Age (years)	-0.37 ± 0.04	<0.001	-0.38 ± 0.04	<0.001
Body mass index (kg/m^2)	-0.53 ± 0.16	0.001	-0.56 ± 0.16	<0.001
LAEF-passive (%)				
Age (years)	-0.43 ± 0.05	<0.001	-0.43 ± 0.05	<0.001
Male gender			-2.42 ± 1.18	0.04
Body mass index (kg/m^2)	-0.52 ± 0.19	0.007	-0.55 ± 0.19	0.005

SE, standard error; other abbreviations as in *Figure 2*.
[a]Age, gender, body mass index, systolic blood pressure, diastolic blood pressure, glycaemia and cholesterol level.

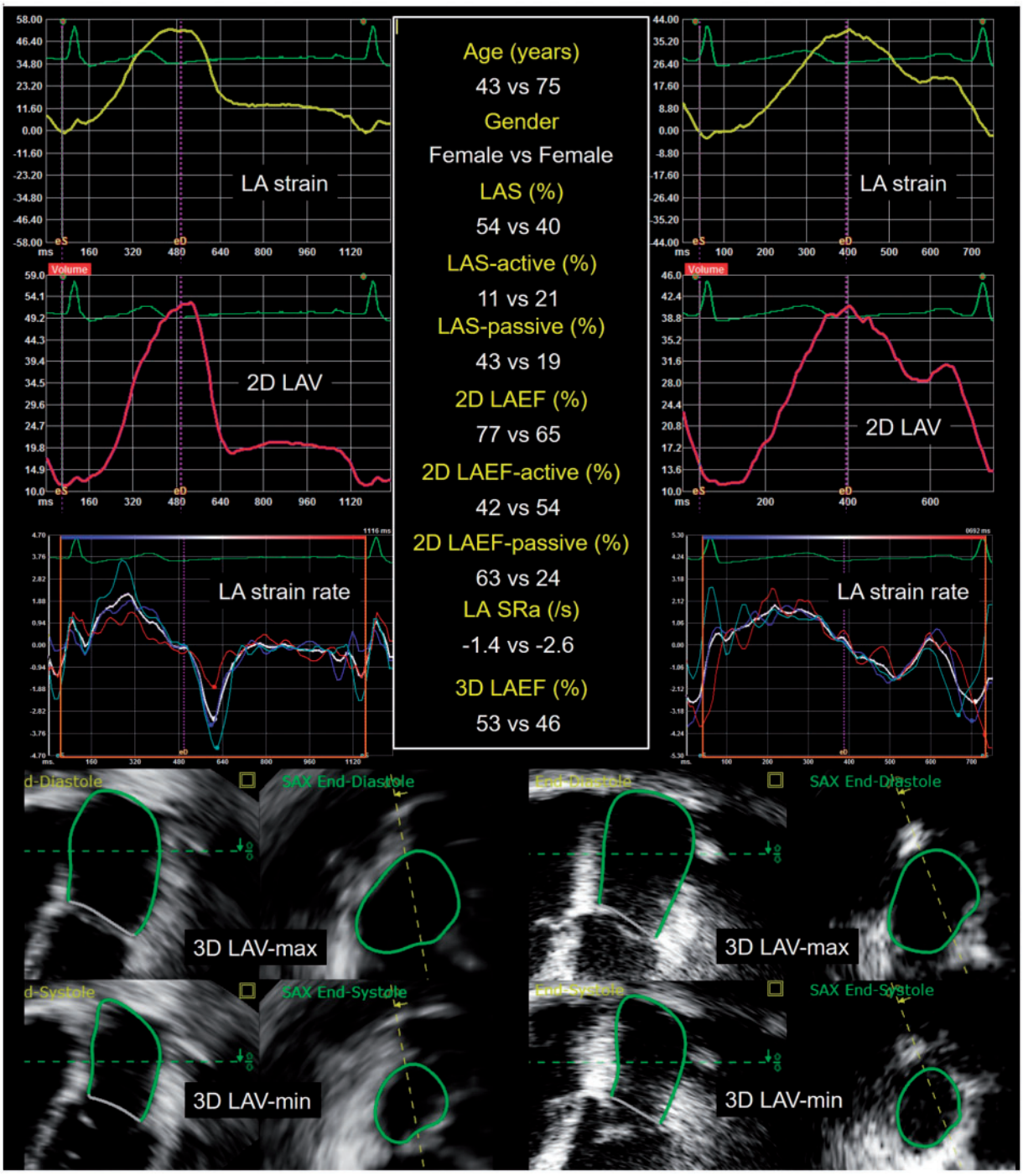

Figure 3 Two representative cases of LA functional assessments. LAV, left atrial volume; other abbreviations as in *Figure 2*.

Discussion

The present prospective, EACVI multicentre study provides contemporary normal reference values for LA function in a large cohort of healthy volunteers over a wide range of ages. 2DE analyses were performed using a VIS in order to obtain homogeneous measurements irrespective of the echocardiographic equipment used to acquire data. Left atrial reservoir and conduit function decreased with age while pump function increased. All indices of reservoir function and LA strains had no gender and vendor differences. Interestingly, inter-vendor differences were observed in 3D LA volume index and LA SRa despite the use of VIS software.

Previous single-centre studies with healthy subjects reported that the highest expected value for 3D LA volume index was: 33 mL/m^2 in

Table 4 Left atrial functions and stiffness according to age

	Age 20–40 ($n = 137$)		Age 40–60 ($n = 173$)		Age ≥60 ($n = 61$)	
	Medial (IQR)	Limits of normality ± SE[a,b]	Medial (IQR)	Limits of normality ± SE[a,b]	Medial (IQR)	Limits of normality ± SE[a,b]
Reservoir function						
LAS (%)	46.8 (42.3–52.4)	31.1 ± 2.6[b]	40.9 (35.4–46.1)	27.7 ± 1.5[b]	35.5 (30.9–41.9)	22.7 ± 2.0[b]
2D LAEF (%)	71.3 (67.3–74.9)	51.7 ± 2.0[b]	66.7 (62.8–72.4)	49.2 ± 2.7[b]	64.0 (58.1–69.5)	44.1 ± 1.7[b]
3D LAEF (%)	58.4 (53.1–63.1)	42.2 ± 2.3[b]	57.1 (52.2–61.3)	39.4 ± 1.9[b]	55.6 (50.6–60.4)	38.3 ± 2.5[b]
Pump function						
LAS-active (%)	15.6 (11.9–19.0)	7.2 ± 0.5[b]	16.3 (13.2–19.6)	9.3 ± 0.8[b]	16.8 (13.6–21.4)	7.7 ± 0.8[b]
Conduit function						
LAS-passive (%)	30.6 (26.8–36.5)	16.2 ± 1.6[b]	24.1 (19.7–29.3)	12.0 ± 1.0[b]	18.6 (14.7–22.6)	11.5 ± 0.1[b]
Stiffness						
E/e' divided by LAS	0.12 (0.10–0.15)	0.22 ± 0.01[a]	0.16 (0.13–0.22)	0.42 ± 0.04[a]	0.24 (0.18–0.29)	0.55 ± 0.09[a]
E/e' divided by 2D LAEF	0.08 (0.07–0.09)	0.15 ± 0.01[a]	0.10 (0.09–0.12)	0.23 ± 0.04[a]	0.14 (0.11–0.15)	0.24 ± 0.02[a]
E/e' divided by 3D LAEF	0.10 (0.08–0.12)	0.19 ± 0.01[a]	0.11 (0.10–0.15)	0.24 ± 0.04[a]	0.15 (0.13–0.17)	0.27 ± 0.02[a]
E/e'	5.6 (4.8–6.7)	9.0 ± 1.3[a]	6.8 (5.8–8.3)	13.4 ± 2.4[a]	8.3 (7.1–9.8)	13.3 ± 0.5[a]

CI, confidence interval; SD, standard deviation; other abbreviations as in *Figure 2*.
[a]Highest expected values.
[b]Lowest expected values.

the study of Wu *et al.*,[23] a Japanese cohort, using Philips equipment and software, 41 mL/m^2 in the study of Aune *et al.*,[24] a Norway cohort, using Philips equipment and software, and 43 mL/m^2 in the study of Badano *et al.*,[13] an Italian cohort, using GE equipment and TomTec software. This multicentre study with a Caucasian European population showed lower 3D LA volume index compared with single-centre studies in the Norway and Italy. The reasons for this difference may be caused by several factors: (i) lower temporal resolution of 3D echocardiographic data sets caused by a wide-angle 3D acquisition for the LA[13]; (ii) the difference in baseline blood pressure in male [systolic/diastolic blood pressure: 123/76 mmHg (mean) and 121/77 mmHg (median) in this study vs. 127/79 mmHg (mean) in the Norwegian cohort and 130/80 mmHg (median) in the Italian cohort]; and (iii) inter-vendor differences as demonstrated in this study.

A previous multicentre study reporting on a large cohort of healthy subjects showed that LAS and LAEF were negatively associated with age while having no differences in gender.[10] A meta-analysis of LA strain using 2D speckle tracking echocardiography has detected no difference in both gender and vendor.[11] These finding are consistent with the findings of this study. The mechanism of age-related changes in LA function, in particular pump function, may be explained by age-related changes in left ventricular diastolic performance from normal to diastolic dysfunction grade 1.[25] In fact, our data demonstrated the age-related increases in LA stiffness, an index that has been reported as a sensitive marker of diastolic dysfunction.[22,26] This study showed that the best concordance between the two major vendors was in LA strain whereas the major discordance was noted in 3D LA volume index and SRa. These data support the use of comparable values independently of the machine used to acquire LA images.

Limitations

This study presents several limitations. First, only half of the patients included in the study were available for LA function analysis indicating that dependency on image quality is one of the main limitations for strain analysis by speckle tracking. Second, the existence of inter-vendor differences in 3D LA volume index and LA SRa was not confirmed by the direct comparison in the same patients. Further study is warranted to investigate the cause of the inter-vendor differences. Last of all, whether the NORRE study results can be extrapolated to non-Caucasian European individuals is still unknown.

Conclusion

The NORRE study provides applicable reference ranges for LA functions. Multivariable analysis showed that age is independently associated with all LAS components irrespective of gender and vendor. Our data highlight the importance of age-specific assessment for LA function.

Supplementary data

Supplementary data are available at *European Heart Journal - Cardiovascular Imaging* online.

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Appendix of the list of contributors to the NORRE Study

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Echocardiographic reference ranges for normal left ventricular layer-specific strain: results from the EACVI NORRE study

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Aims To obtain the normal range for 2D echocardiographic (2DE) measurements of left ventricular (LV) layer-specific strain from a large group of healthy volunteers of both genders over a wide range of ages.

Methods and results A total of 287 (109 men, mean age: 46 ± 14 years) healthy subjects were enrolled at 22 collaborating institutions of the EACVI Normal Reference Ranges for Echocardiography (NORRE) study. Layer-specific strain was analysed from the apical two-, three-, and four-chamber views using 2DE software. The lowest values of layer-specific strain calculated as ±1.96 standard deviations from the mean were -15.0% in men and -15.6% in women for epicardial

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strain, -16.8% and -17.7% for mid-myocardial strain, and -18.7% and -19.9% for endocardial strain, respectively. Basal-epicardial and mid-myocardial strain decreased with age in women (epicardial; $P = 0.008$, mid-myocardial; $P = 0.003$) and correlated with age (epicardial; $r = -0.20$, $P = 0.007$, mid-myocardial; $r = -0.21$, $P = 0.006$, endocardial; $r = -0.23$, $P = 0.002$), whereas apical-epicardial, mid-myocardial strain increased with the age in women (epicardial; $P = 0.006$, mid-myocardial; $P = 0.03$) and correlated with age (epicardial; $r = 0.16$, $P = 0.04$). End/Epi ratio at the apex was higher than at the middle and basal levels of LV in men (apex; 1.6 ± 0.2, middle; 1.2 ± 0.1, base 1.1 ± 0.1) and women (apex; 1.6 ± 0.1, middle; 1.1 ± 0.1, base 1.2 ± 0.1).

Conclusion The NORRE study provides useful 2DE reference ranges for novel indices of layer-specific strain.

Keywords adult echocardiography • 2D echocardiography • deformation imaging • reference values

Introduction

Two-dimensional (2D) speckle tracking echocardiography (STE) enables quantitative evaluation of cardiac mechanics through image-based analysis of myocardial deformation.[1] Although left ventricular (LV) ejection fraction is the most commonly used parameter to assess LV mechanics, 2D-STE can detect latent LV dysfunction prior to a decline in LV ejection fraction by assessing mid-myocardial longitudinal strain.[2] Recently, technological advances in 2D-STE has enabled the assessment of layer-specific strain, thus allowing the measurement of epicardial, mid-myocardial, and endocardial longitudinal strain. The LV myocardium is divided into three myocardial layers consisting of circumferential fibres in the mid-myocardial layer and longitudinal fibres in the epicardial and endocardial layers.[3] In most heart diseases except some, such as sarcoidosis or hypertrophic cardiomyopathy, myocardial injury occurs predominantly in the endocardial fibres in the early stages of the disease.[4] Endocardial strain may have the potential to be more sensitive to assess myocardial function compared to epicardial or mid-myocardial strain in different cardiovascular diseases.[5–9] However, normal ranges for each type of layer-specific strain remain, to date, poorly defined.[10,11] The aim of this study was to establish the normal ranges of layer-specific strain from a large group of healthy volunteers of both genders over a wide range of ages.

The NORRE (Normal Reference Ranges for Echocardiography) study is the first European, large prospective, multicentre study performed in 22 laboratories accredited by the European Association of Cardiovascular Imaging (EACVI) and in one American laboratory, which has provided reference values for all 2D echocardiographic (2DE) measurements of all cardiac chambers,[12] Doppler parameters,[13] aortic dimensions,[14] 3D echocardiographic measurements of the LV volumes and strain,[15] 2DE measurements of LV strain,[16] 2D and 3D measurements of left atrial function,[17] and myocardial indices.[18] This study aimed to (i) establish normal reference limits for layer-specific strain in healthy adults and (ii) examine the influence of age and gender on these normal reference ranges.

Methods

Patient population

A total of 734 healthy European subjects constituted the final NORRE study population. The local ethics committees approved the study protocol. After the exclusion of patients that had incompatible image formats and/or poor image quality, the final study population consisted of 287 (39%) healthy subjects.

Echocardiographic examination

A comprehensive echocardiographic examination was performed using state-of-the-art echocardiographic ultrasound system (GE Vivid E9; Vingmed Ultrasound, Horten, Norway) following a recommended protocol approved by EACVI.[19,20] All echocardiographic images were recorded in a digital raw-data format (native DICOM format) and centralized for further analysis, after anonymization, at EACVI Central Core laboratory at the University of Liège, Belgium.

2D LV layer-specific strain

Quantification of layer-specific strain measurements were performed off-line with dedicated software (EchoPAC V.203, GE). For measuring layer-specific strain, attention was taken to cover the entire myocardial wall thickness with the region of interest (ROI) of each segment and to avoid to include the pericardium. Calculation of transmural variation of longitudinal strain across the entire myocardium was based on the assumption of linear distribution. Endocardial and epicardial strain were measured on the endocardial and epicardial ROI border, respectively, whereas the mid (centre line) of the ROI represented the average values of the transmural wall thickness. The layer-specific strain values were obtained by averaging the peak longitudinal strain of 17 segments (*Figure 1*). The ratio of endocardial to epicardial was calculated using the End/Epi ratio for the assessment of the strain gradient.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD). The 95% confidence interval was calculated as ±1.96 SDs from the mean. Differences between groups were analysed for statistical significance with the unpaired *t*-test for normally distributed continuous variables. Comparison of continuous variables according to age groups was done with one-way analysis of variance test. When a significant difference was found, *post hoc* testing with Bonferroni comparisons to identify specific group differences was used. Correlation between continuous variables was performed using the Pearson correlation test. Multivariable linear regression analyses were performed to examine the independent correlates between layer-specific strain and baseline parameters. Intra-observer and inter-observer variability were assessed in 20 randomly selected subjects using Bland–Altman analysis. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using JMP 11.0 statistical software (SAS Institute, Cary, NC, USA).

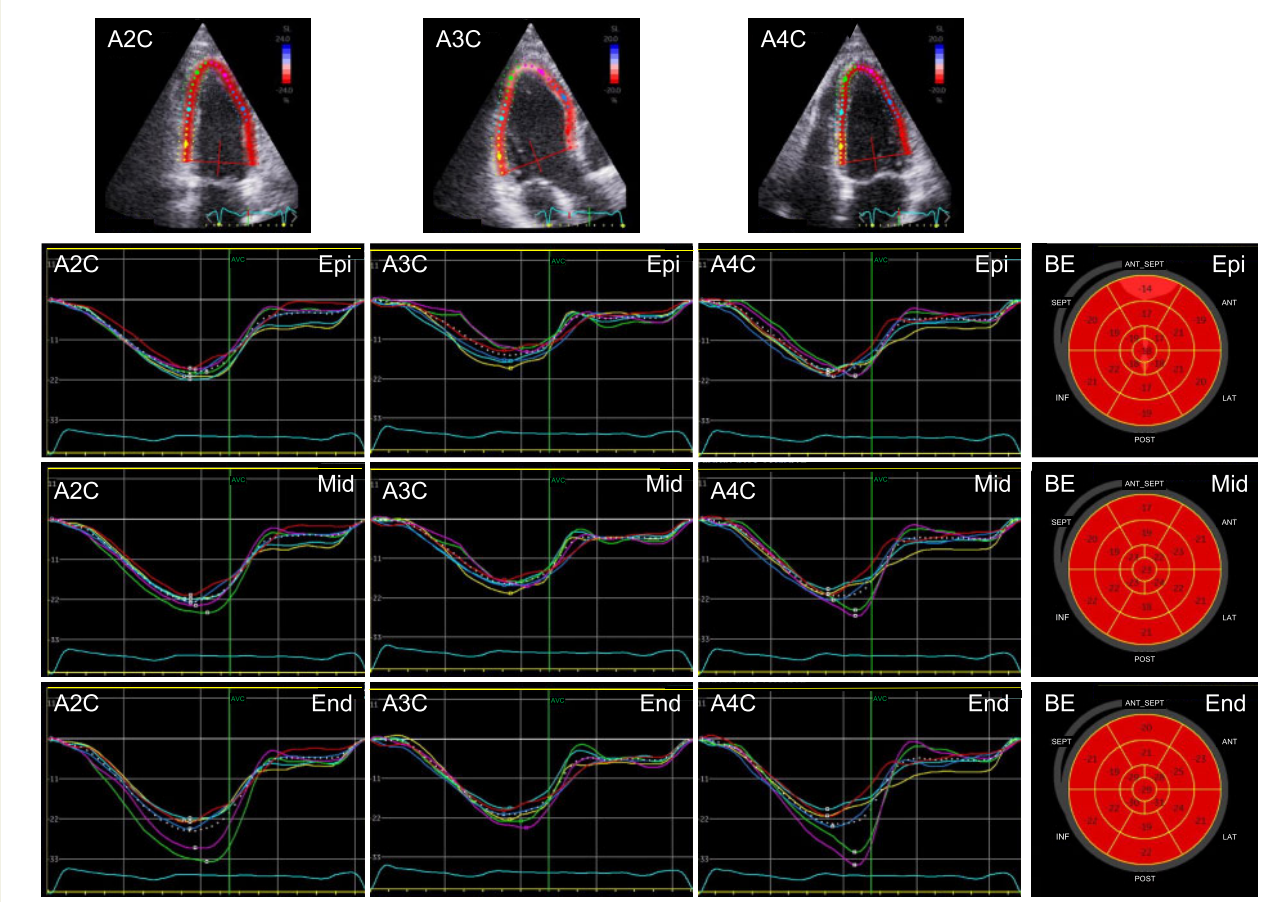

Figure 1 Layer-specific strain curves measurement by 2D speckle tracking echocardiography. A2C, apical two-chamber; A3C, apical three-chamber; A4C, apical four-chamber; Epi, epicardial strain; Mid, mid-myocardial strain; End, endocardial strain; BE, bull's eye of layer-specific strain.

Results

Demographic data

Table 1 summarizes the demographic data of the NORRE population analysed in the present study. A total of 109 men (mean age 46 ± 14 years) and 178 women (mean age 45 ± 14 years) were included. Systolic blood pressure was higher in men (mean age 123 ± 10 mmHg) than in women (116 ± 15 mmHg). Strain values may be affected by LV afterload. However, it remains to be clarified whether the strain values correlate with the LV afterload, and few studies have reported.[21] The mean frame rate was on the apical view were 63 ± 10/s (men 63 ± 11/s, women 64 ± 9/s, $P = 0.73$). Layer-specific strain results from the entire study population are depicted in *Table 2*. All average layer-specific strains were significantly higher in women than in men. The lowest values of layer-specific strains were -15.0% in men and -15.6% in women for epicardial strain, -16.8% and -17.7% for mid-myocardial strain, and -18.7% and -19.9% for endocardial strain, respectively. The highest values of layer-specific strain were -22.3% in men and -23.5% in women for epicardial strain, -25.1% and -26.0% for mid-myocardial strain, and -28.4% and -29.1% for endocardial strain, respectively.

Relationship between age, gender, and layer-specific strain

Relationships between gender and age with layer-specific strain in all apical views are shown in *Table 3* and *Figure 2*. No significant correlations were observed between age and layer-specific strains for all apical chamber views. In all age groups, layer-specific strain, including epicardial, mid-myocardial, and endocardial strain tended to be higher in women compared to men. In the age group between 20 and 40 years (epicardial, mid-myocardial, and endocardial strain) and in the age group >60 years, layer-specific epicardial and mid-myocardial strains were significantly higher in women than men.

Relationships between age and layer-specific strains in the apical, middle, and basal levels of the LV are shown in *Table 4* and *Figure 3*. No significant age dependency was observed with respect to layer-specific strain in all segments in men. However, the basal-epicardial and mid-myocardial strain decreased with age in women (epicardial; $P = 0.008$, mid-myocardial; $P = 0.003$) and correlated with age (epicardial; $r = -0.20$, $P = 0.007$, mid-myocardial; $r = -0.21$, $P = 0.006$, and endocardial; $r = -0.23$, $P = 0.002$). In contrast, theapical-epicardial and mid-myocardial strains increased with age in women (epicardial; $P = 0.006$ and mid-myocardial; $P = 0.03$) and correlated with age

Table 1 Characteristics of the population

Parameters	Total (n = 287)	Male (n = 109)	Female (n = 178)	P-value
Age (years)	46 ± 14	46 ± 14	45 ± 14	0.54
Height (cm)	170 ± 10	179 ± 8	165 ± 7	<0.001
Weight (kg)	69 ± 12	78 ± 10	63 ± 9	<0.001
Body surface area (m^2)	1.8 ± 0.2	2.0 ± 0.1	1.7 ± 0.1	<0.001
Systolic blood pressure (mmHg)	119 ± 14	123 ± 10	116 ± 15	<0.001
Diastolic blood pressure (mmHg)	74 ± 9	75 ± 8	73 ± 9	0.02
Glucose (mg/dL)	91 ± 11	95 ± 9	89 ± 11	<0.001
Cholesterol (mg/dL)	182 ± 30	186 ± 26	180 ± 32	0.17

Table 2 2DE parameters of layer-specific strain

	Total mean ± SD	Total 95% CI	Male mean ± SD	Male 95% CI	Female mean ± SD	Female 95% CI	P-value
Epicardial strain (%)							
Apical two-chamber	-19.8 ± 2.6	-14.7 to -24.8	-19.4 ± 2.4	-14.6 to -24.1	-20.0 ± 2.6	-14.9 to -25.1	0.03
Apical three-chamber	-18.8 ± 2.5	-13.8 to -23.7	-18.1 ± 2.3	-13.6 to -22.6	-19.2 ± 2.6	-14.1 to -24.3	<0.001
Apical four-chamber	-19.0 ± 2.4	-14.4 to -23.7	-18.5 ± 2.5	-13.6 to -23.4	-19.4 ± 2.3	-14.9 to -23.8	0.95
Average	-19.2 ± 2.0	-15.3 to -23.1	-18.7 ± 1.9	-15.0 to -22.3	-19.5 ± 2.0	-15.6 to -23.5	<0.001
Mid-myocardial strain (%)							
Apical two-chamber	-22.0 ± 2.7	-17.3 to -27.2	-21.6 ± 2.5	-16.6 to -26.5	-22.2 ± 2.8	-16.8 to -27.6	0.045
Apical three-chamber	-21.2 ± 2.8	-15.8 to -28.6	-20.5 ± 2.6	-15.5 to -25.6	-21.7 ± 2.8	-16.2 to -27.1	<0.001
Apical four-chamber	-21.1 ± 3.5	-14.2 to -28.1	-20.7 ± 2.7	-15.4 to -26.1	-21.3 ± 4.0	-13.6 to -29.1	0.11
Average	-21.5 ± 2.2	-17.3 to -25.7	-20.9 ± 2.1	-16.8 to -25.1	-21.8 ± 2.1	-17.7 to -26.0	<0.001
Endocardial strain (%)							
Apical two-chamber	-24.5 ± 3.0	-18.6 to -30.3	-24.1 ± 2.9	-18.5 to -29.7	-24.7 ± 3.0	-18.8 to -30.6	0.08
Apical three-chamber	-24.0 ± 4.2	-15.8 to -32.2	-23.4 ± 3.1	-17.3 to -29.5	-24.4 ± 4.7	-15.2 to -33.6	0.03
Apical four-chamber	-23.7 ± 2.9	-18.1 to -29.3	-23.2 ± 3.2	-17.0 to -29.4	-24.0 ± 2.6	-18.8 to -29.2	0.03
Average	-24.1 ± 2.4	-19.3 to -28.9	-23.6 ± 2.5	-18.7 to -28.4	-24.5 ± 2.3	-19.9 to -29.1	0.002
End/Epi ratio	1.3 ± 0.1	1.15 to 1.37	1.3 ± 0.1	1.15 to 1.38	1.3 ± 0.1	1.15 to 1.36	0.19

CI, confidence interval; SD, standard deviation.
P-value differences between genders.

(epicardial; r = 0.16, P = 0.04). Although all strain values tended to increase from the epicardium to the endocardium, this tendency was stronger at the apical compared to the basal LV. Therefore, End/Epi ratio at the apex was higher than at the middle or the basal LV levels in men (apex; 1.6 ± 0.2, middle; 1.2 ± 0.1, base 1.1 ± 0.1) and women (apex; 1.6 ± 0.1, middle; 1.1 ± 0.1, base 1.2 ± 0.1), and this relationship was preserved at all ages (*Table 4* and *Figure 3*).

Layer-specific strains determinants

Multivariable analysis for layer-specific strain showed that epicardial, mid-myocardial, and endocardial strain increased with body surface area (epicardial; β-coefficient = 0.32, P = 0.009, mid-myocardial; β-coefficient = 0.29, P = 0.02, endocardial; β-coefficient = 0.26, P = 0.03), whereas the End/Epi ratio was not related to body surface area. There was a significant increase in epicardial, mid-myocardial, and endocardial strain according to body surface area in univariable analysis but no association was observed after adjustment for confounders (*Table 5*).

Repeatability and reproducibility

Intra-observer and inter-observer variability for layer-specific strain are summarized in *Table 6*. Intra-observer and inter-observer analyses showed good repeatability and reproducibility in layer-specific strain (*Table 6* and *Figure 4*).

Discussion

The present prospective, EACVI multicentre study provides contemporary normal references values for 2DE measurements of layer-specific strain in a large cohort of healthy volunteers of both genders over a wide range of ages. Myocardial heterogeneity is characterized by higher deformation amplitude in the endocardial compared with

Table 3 Layer-specific strain at the apical two-chamber, apical three-chamber, and apical four-chamber according to gender and age

	Total ($n = 287$)		Age 20–40 ($n = 115$)		Age 40–60 ($n = 122$)		Age ≥60 ($n = 50$)		*P*-value		Male		Female	
	Male ($n = 110$), mean ± SD	Female ($n = 178$), mean ± SD	Male ($n = 39$), mean ± SD	Female ($n = 76$), mean ± SD	Male ($n = 50$), mean ± SD	Female ($n = 72$), mean ± SD	Male ($n = 20$), mean ± SD	Female ($n = 30$), mean ± SD	Male	Female	*R*	*P*-value	*R*	*P*-value
Epicardial longitudinal strain (%)														
Apical two-chamber	-19.4 ± 2.4	-20.0 ± 2.6[a]	-19.2 ± 2.5	-20.1 ± 2.6	-19.7 ± 2.5	-20.2 ± 2.6	-18.7 ± 2.0	-19.5 ± 2.6	0.23	0.59	-0.0002	1.00	0.08	0.28
Apical three-chamber	-18.1 ± 2.3	-19.2 ± 2.6[a]	-18.1 ± 2.1	-19.1 ± 2.8[a]	-18.5 ± 2.4	-19.4 ± 2.5	-17.3 ± 2.2	-18.9 ± 2.3[a]	0.14	0.38	0.06	0.53	0.06	0.45
Apical four-chamber	-18.5 ± 2.5	-19.4 ± 2.3[a]	-17.7 ± 2.2	-19.0 ± 2.5[a]	-19.3 ± 2.6	-19.9 ± 2.0	-18.2 ± 2.0	-19.1 ± 2.1	0.01	0.81	-0.17	0.08	-0.04	0.60
Average	-18.7 ± 1.9	-19.5 ± 2.0[a]	-18.3 ± 1.7	-19.4 ± 2.1[a]	-19.2 ± 2.1	-19.8 ± 1.9	-18.0 ± 1.5	-19.2 ± 1.8[a]	0.03	0.73	-0.05	0.63	0.05	0.48
Mid-myocardial longitudinal strain (%)														
Apical two-chamber	-21.6 ± 2.5	-22.2 ± 2.8[a]	-21.6 ± 2.5	-22.4 ± 2.7	-21.9 ± 2.6	-22.3 ± 2.8	-20.7 ± 2.2	-21.6 ± 2.9	0.19	0.32	0.06	0.53	0.12	0.12
Apical three-chamber	-20.5 ± 2.6	-21.7 ± 2.8	-20.6 ± 2.3	-21.6 ± 2.8	-20.8 ± 2.7	-21.9 ± 2.9[a]	-19.8 ± 2.6	-21.3 ± 2.6[a]	0.30	0.31	0.05	0.59	0.07	0.37
Apical four-chamber	-20.7 ± 2.7	-21.3 ± 4.0	-20.0 ± 2.4	-21.2 ± 2.7[a]	-21.4 ± 2.9	-21.5 ± 5.4	-20.4 ± 2.6	-21.3 ± 2.2	0.04	0.91	-0.14	0.14	0.02	0.78
Average	-20.9 ± 2.1	-21.8 ± 2.1[a]	-20.7 ± 1.8	-21.8 ± 2.2[a]	-21.4 ± 2.3	-22.1 ± 2.1	-20.3 ± 1.9	-21.4 ± 2.0[a]	0.11	0.57	-0.01	0.87	0.08	0.30
Endocardial longitudinal strain (%)														
Apical two-chamber	-24.1 ± 2.9	-24.7 ± 3.0	-24.4 ± 2.8	-25.0 ± 2.9	-24.3 ± 2.9	-24.6 ± 3.1	-22.9 ± 2.6	-24.0 ± 3.3	0.11	0.17	0.13	0.19	0.15	0.051
Apical three-chamber	-23.4 ± 3.1	-24.4 ± 4.7	-23.5 ± 2.8	-24.0 ± 6.1	-23.5 ± 3.3	-24.8 ± 3.4[a]	-22.7 ± 3.3	-24.3 ± 3.1	0.55	0.80	0.05	0.62	-0.0002	1.00
Apical four-chamber	-23.2 ± 3.2	-24.0 ± 2.6[a]	-22.6 ± 2.7	-23.5 ± 2.8	-23.9 ± 3.3	-24.5 ± 2.4	-22.9 ± 3.3	-23.9 ± 2.5	0.14	0.75	-0.11	0.24	-0.05	0.51
Average	-23.6 ± 2.5	-24.5 ± 2.3[a]	-23.5 ± 2.1	-24.4 ± 2.4[a]	-23.9 ± 2.8	-24.7 ± 2.3	-22.8 ± 2.4	-24.0 ± 2.3	0.25	0.45	0.02	0.83	0.09	0.21
End/Epi ratio	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	0.03	0.27	-0.15	0.12	-0.07	0.38

SD, standard deviation.
[a]$P < 0.05$ vs. male.

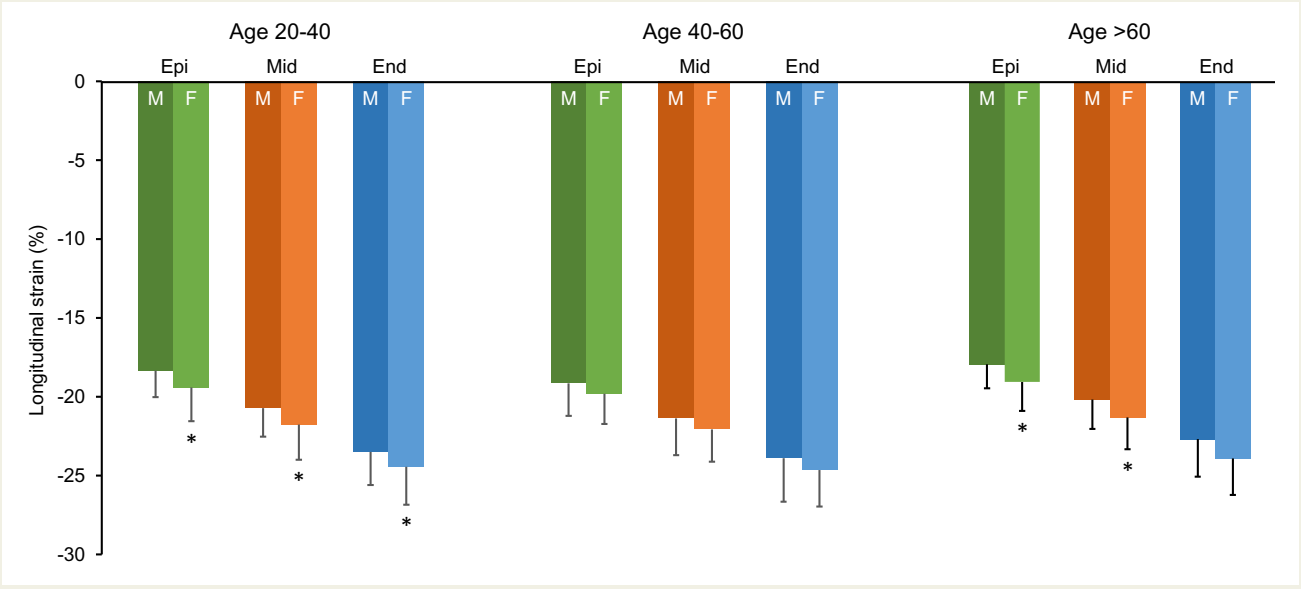

Figure 2 Bar graphs showing average layer-specific strain by 2D echocardiography analysis according to gender and age categories. End, endocardial strain; Epi, epicardial strain; Mid, mid-myocardial strain. *P-value differences between genders.

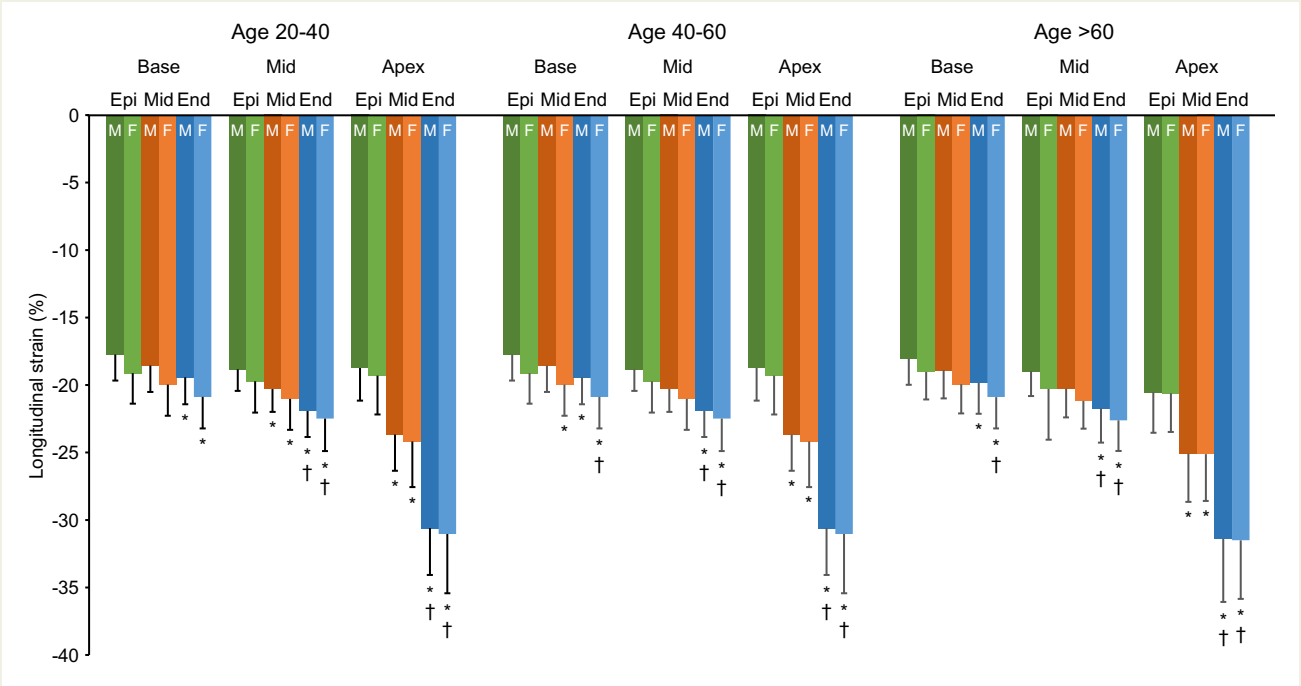

Figure 3 Bar graphs showing average layer-specific strain at the apical, the mid-ventricular, and the basal levels of the left ventricle by 2D echocardiography analysis according to gender and age categories. Epi, epicardial strain; Mid, mid-myocardial strain; End, endocardial strain. *$P < 0.05$ vs. epicardial strain. †$P < 0.05$ vs. mid-myocardial strain.

the epicardial layer.[22] Layer-specific strain is a novel method that is capable of assessing each layer of the myocardial function. Moreover, the absence of differences between vendors for layer-specific strain values makes this technique a useful tool for feasibility, accuracy, and reproducibility.[23]

Our results are consistent with previous studies showing good concordance with the absolute values of layer-specific strain and that all layer-specific strains in women were consistently higher than in men.[10,24,25] However, the relationship between layer-specific strain and age dependency is inconsistent. As reported by Nagata *et al.* and

Table 4 Layer-specific strain at the apical, mid-ventricular, and basal levels of left ventricle according to gender and age

	Total ($n = 287$)		Age 20–40 ($n = 115$)		Age 40–60 ($n = 122$)		Age ≥60 ($n = 50$)		*P*-value		Male		Female	
	Male ($n = 109$), mean ± SD	Female ($n = 178$), mean ± SD	Male ($n = 39$), mean ± SD	Female ($n = 76$), mean ± SD	Male ($n = 50$), mean ± SD	Female ($n = 72$), mean ± SD	Male ($n = 20$), mean ± SD	Female ($n = 30$), mean ± SD	Male	Female	*R*	*P*-value	*R*	*P*-value
Base														
Epi	17.8 ± 2.0	-18.9 ± 2.2[a]	-17.7 ± 1.9	-19.1 ± 2.3[a]	-18.1 ± 1.9	-19.0 ± 2.0[a]	-17.3 ± 2.0	-17.8 ± 1.8	0.32	0.008	-0.02	0.83	-0.20	0.007
Mid	18.6 ± 2.0	-19.7 ± 2.2[a]	-18.6 ± 2.0	-20.0 ± 2.3[a]	-18.9± 2.1	-19.9 ± 2.2[a]	-18.1 ± 2.0	-18.5 ± 1.8	0.30	0.003	-0.03	0.75	-0.21	0.006
End	-19.5 ± 2.2	-20.7 ± 2.6[a]	-19.4 ± 2.0	-20.9 ± 2.3[a]	-19.8 ± 2.3	-20.9 ± 2.3[a]	-18.9 ± 2.1	-19.7 ± 3.7	0.27	0.23	-0.04	0.67	-0.23	0.002
End/Epi ratio	1.1 ± 0.1	1.2 ± 0.1[a]	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.2 ± 0.4	0.95	0.06	-0.05	0.61	0.13	0.09
Middle														
Epi	-18.8 ±1.8	-19.8 ± 3.0[a]	-18.8 ± 1.6	-19.7 ± 2.3[a]	-19.0 ± 1.8	-20.3 ± 3.8[a]	-18.1± 1.8	-18.9 ± 1.8[a]	0.14	0.09	-0.10	0.31	-0.12	0.12
Mid	-20.1 ± 2.0	-20.9 ± 2.1[a]	-20.3 ± 1.7	-21.0 ± 2.3	-20.3 ± 2.1	-21.2 ± 2.1[a]	-19.3 ± 2.1	-20.1 ± 1.8	0.12	0.06	-0.12	0.23	-0.16	0.04
End	-21.7 ± 2.3	-22.4 ± 2.3[a]	-21.9 ± 1.9	-22.5 ± 2.4	-21.7 ± 2.5	-22.6 ± 2.3[a]	-21.0 ± 2.5	-21.5 ± 2.3	<0.001	0.08	-0.09	0.37	-0.14	0.07
End/Epi ratio	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.19	0.61	-0.02	0.85	-0.008	0.92
Apex														
Epi	-19.6 ± 2.8	-20.1 ± 2.9	-18.7 ± 2.4	-19.3 ± 2.9	-20.5 ± 3.0	-20.6 ± 2.9	-19.1 ± 2.2	-20.9 ± 2.8[a]	0.007	0.006	0.16	0.10	0.16	0.04
Mid	-24.3 ± 3,2	-24.9 ± 3.5	-23.7 ± 2.8	-24.2 ± 3.4	-25.1 ± 3.6	-25.1 ± 3.5	-23.8± 2.6	-26.0 ± 3.6[a]	0.06	0.03	0.12	0.22	0.13	0.08
End	-30.9 ± 4.1	-31.6 ± 4.5	-30.6 ± 3.5	-31.0 ± 4.4	-31.4 ± 4.7	-31.5 ± 4.3	-30.1 ± 3.9	-33.0 ± 4.9[a]	0.41	0.10	0.04	0.71	0.10	0.19
End/Epi ratio	1.6 ± 0.2	1.6 ± 0.1	1.6 ± 0.2	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	1.6 ± 0.1	0.01	0.002	-0.19	0.04	-0.05	0.61

SD, standard deviation.
[a]$P < 0.05$ vs. male.

Table 5 Univariable and multivariable analysis for layer-specific strain

Variables	Univariable analysis		Multivariable analysis	
	Coefficients	*P*-value	β-coefficients	*P*-value
Epicardial strain (%)				
Age (years)	0.02	0.70		
Male gender (=1)	0.21	<0.001		
Body mass index (kg/m^2)	0.07	0.25		
Body surface area (m^2)	0.24	<0.001	0.32	0.009
Systolic blood pressure (mmHg)	0.04	0.57		
Diastolic blood pressure (mmHg)	0.03	0.64		
Glycaemia (g/dL)	0.04	0.60		
Cholesterol (g/dL)	0.01	0.87		
Mid-myocardial strain (%)				
Age (years)	0.05	0.42		
Male gender (=1)	0.20	<0.001		
Body mass index (kg/m^2)	0.07	0.21		
Body surface area (m^2)	0.22	<0.001	0.29	0.02
Systolic blood pressure (mmHg)	0.04	0.58		
Diastolic blood pressure (mmHg)	0.06	0.32		
Glycaemia (g/dL)	0.07	0.33		
Cholesterol (g/dL)	0.04	0.57		
Endocardial strain (%)				
Age (years)	0.07	0.24		
Male gender (=1)	0.18	0.002		
Body mass index (kg/m^2)	0.07	0.25		
Body surface area (m^2)	0.19	<0.001	0.26	0.03
Systolic blood pressure (mmHg)	0.03	0.63		
Diastolic blood pressure (mmHg)	0.09	0.17	0.19	0.03
Glycaemia (g/dL)	0.10	0.19		
Cholesterol (g/dL)	0.07	0.38		
End/Epi ratio				
Age (years)	-0.12	0.04		
Male gender (=1)	0.08	0.19		
Body mass index (kg/m^2)	-0.02	0.80		
Body surface area (m^2)	0.10	0.08		
Systolic blood pressure (mmHg)	-0.0002	0.10		
Diastolic blood pressure (mmHg)	-0.14	0.03	-0.20	0.02
Glycaemia (g/dL)	-0.15	0.05		
Cholesterol (g/dL)	-0.12	0.11		

Shi *et al.*[10,24] no significant age dependency was observed concerning all layer-specific strains. In contrast, as reported by Alcidi *et al.* all layer-specific strains were progressively reduced with increasing age. The relationship between layer-specific strain and age dependence was inconsistent and different from the previous NORRE study of 2D strain.[16] This difference may be due to the smaller number of enrolled patients in this study than in previous NORRE study. Interestingly, the layer-specific strain gradient increased from the epicardial towards the endocardial layer. The mechanism underlying these findings remains unclear, but some considerations have been reported. The differences between epicardial and endocardial strain might be secondary to the ability of the endocardial fibres to stretch more potently compared to the epicardial fibres during end-diastole.[26] In addition, differences in coronary perfusion and metabolic demands between the epicardial and endocardial layers may also contribute to these differences.[27,28] In this context, the End/Epi ratio at the apex was higher than that at the middle or basal LV levels in both genders. (*Table 4* and *Figure 3*). The End/Epi ratio differs depending of the type of LV hypertrophic diseases, such as aortic stenosis[29] or hypertrophic cardiomyopathy,[30] and may have the potential to diagnose, not only these disease but also other forms of hypertrophic diseases. The hypertrophied myocardium may remodel differently in response to a variety of aetiologies, resulting in different epicardial and endocardial strains. Moreover, the results of our multivariable

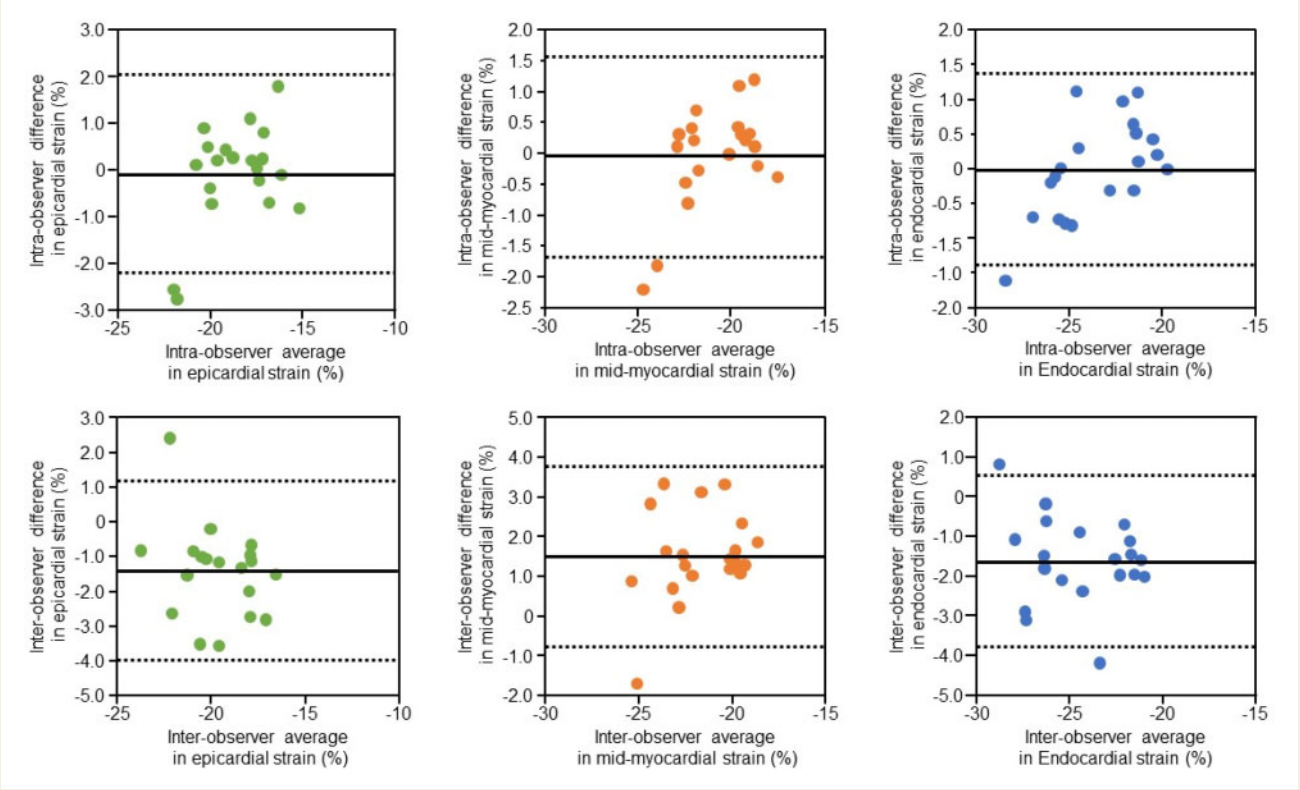

Figure 4 Bland–Altman analysis for assessing intra-observer and inter-observer variability of layer-specific strain. Dotted lines represent bias and 95% limits of agreement for measurements performed in 20 patients.

Table 6 Repeatability and reproducibility of 2D echocardiographic data

Variables	Mean ± SD	Mean ± SD	Bias	*P*-value	95% LOA
Intra-observer					
Average epicardial longitudinal strain (%)	-19.4 ± 1.9	-18.6 ± 1.6	-0.46	0.005	-19.7 to -19.1
Average mid-myocardial longitudinal strain (%)	-21.6 ± 2.1	-21.0 ± 2.0	-0.50	0.001	-22.1 to -21.5
Average endocardial longitudinal strain (%)	-24.4 ± 2.4	-23.5 ± 2.2	-0.60	0.003	-24.9 to -24.1
Inter-observer					
Average epicardial longitudinal strain (%)	-19.4 ± 1.9	-18.3 ± 1.7	1.05	<0.001	-18.2 to -17.4
Average mid-myocardial longitudinal strain (%)	-21.6 ± 2.1	-20.8 ± 1.9	0.82	0.001	-20.1 to -19.9
Average endocardial longitudinal strain (%)	-24.4 ± 2.4	-23.5 ± 2.2	0.90	<0.001	-23.6 to -23.1

LOA, lower limits of agreement; SD, standard deviation.

analysis (*Table 5*) suggest that the End/Epi ratio may have the potential to be a useful marker regardless of age, gender, or body surface area. Our data showed good reproducibility for the assessment of layer-specific strains, reinforcing the possibility of a promising application of this new advanced echocardiographic index in clinical practice.

Limitations

This study presents several limitations. First, only one-third of the patients included in the NORRE database could be analysed by the current available software. Second, since this study was conducted only on GE equipment, data on other equipment, such as Philips, is not available. However, in our previous study, we reported that no differences were noted between GE and Philips equipment with regard to longitudinal strain.[16] Third, the number of patients enrolled in this study was lower than in the previous NORRE study of LV 2D strain.[16] Therefore, the relationship between layer-specific strain and age dependency was inconsistent. The same tendency was observed for the basal and middle LV levels of all layer-specific strain. Fourth, whether the NORRE study results can be extrapolated to non-Caucasian European individuals is still unknown.

Conclusion

The NORRE study provides applicable 2DE reference ranges for layer-specific strain. Multivariable analysis did not show any significant association between layer-specific strain and age or gender.

Acknowledgements

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Echocardiographic reference ranges for normal non-invasive myocardial work indices: results from the EACVI NORRE study

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Aims To obtain the normal ranges for 2D echocardiographic (2DE) indices of myocardial work (MW) from a large group of healthy volunteers over a wide range of ages and gender.

Methods and results A total of 226 (85 men, mean age: 45 ± 13 years) healthy subjects were enrolled at 22 collaborating institutions of the Normal Reference Ranges for Echocardiography (NORRE) study. Global work index (GWI), global constructive work (GCW), global work waste (GWW), and global work efficiency (GWE) were estimated from left ventricle (LV) pressure–strain loops. Peak LV systolic pressure was non-invasively derived from brachial artery cuff pressure. The lowest values of MW indices in men and women were 1270 mmHg% and 1310 mmHg% for GWI, 1650 mmHg% and 1544 mmHg% for GCW, and 90% and 91% for GWE, respectively. The highest value for GWW

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was 238 mmHg% in men and 239 mmHg% in women. Men had significant lower values of GWE and higher values of GWW. GWI and GCW significantly increased with age in women.

Conclusion The NORRE study provides useful 2DE reference ranges for novel indices of non-invasive MW.

Keywords adult echocardiography • 2D echocardiography • myocardial work • reference values

Introduction

Myocardial strain analysis has emerged in the last decade as a reliable tool for studying myocardial mechanics, adding information on cardiac performance when compared with traditional parameters of left ventricle (LV) systolic function, such as ejection fraction (EF).[1–4] However, their relative load dependency makes the myocardial deformation indices unable to account for changes in pre- and afterload. Myocardial work (MW) is emerging as an alternative tool for studying LV myocardial systolic function, because it incorporates both deformation and load into its analysis. In this context, MW could be considered as an advancement of myocardial strain, allowing to investigate LV performance also in cases of changes in afterload that could lead to misleading conclusions if relying only on strain analysis. Conditions of increased afterload can in fact negatively impact on myocardial strain even if MW is normal.

MW assessment was initially calculated using invasive pressure measurements, which limited its widespread use in clinical practice.[5,6] Recently, Russell *et al.*[7] demonstrated that pressure–strain loops (PSLs) could estimate LV performance in a non-invasive manner, deriving LV pressure (LVP) curves from non-invasively acquired brachial artery cuff pressure. To date, the technique has been applied in myocardial ischaemia and in identification of cardiac resynchronization therapy (CRT)-responders with good results.[8–11]

The NORRE (Normal Reference Ranges for Echocardiography) study is the first European, large, prospective, multicentre study performed in 22 laboratories accredited by the European Association of Cardiovascular Imaging (EACVI) and in one American laboratory, which has provided reference values for all 2D echocardiographic (2DE) measurements of all cardiac chambers,[12] Doppler parameters,[13] aortic dimensions,[14] 3D echocardiographic measurements of the LV volumes and strain,[15] 2DE measurement of LV strains and twist,[16] and 2D and 3D measurement of left atrial function.[17] The present study aimed (i) to establish normal reference limits for MW indices in healthy adults and (ii) to examine the influence of age and gender on normal reference ranges.

Methods

Patient population

A total of 734 healthy European subjects constituted the final NORRE study population. The local ethics committees approved the study protocol. Only patients whose echocardiographic exams were acquired using

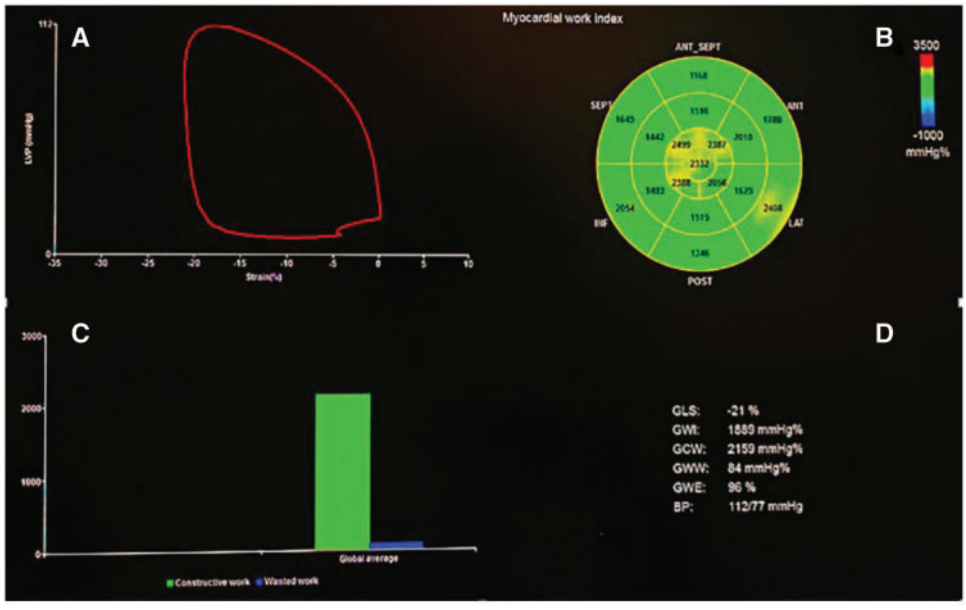

Figure 1 Measurement of myocardial work parameters by 2D echocardiography. (*A*) LV pressure–strain loop; (*B*) bull's eye of GWI; (*C*) bar graph representing GCW and GWW; and (*D*) results from myocardial work analysis. GCW, global constructive work; GWE, global work efficiency; GWI, global work index; GWW, global work waste; LV, left ventricle.

Table 1 Characteristics of the population

Parameters	Total ($n = 226$)	Male ($n = 85$)	Female ($n = 141$)	*P*-value
Age (years)	45 ± 13	45 ± 14	44 ± 13	0.6
Height (cm)	170 ± 10	178 ± 8	164 ± 7	<0.001
Weight (kg)	68 ± 12	78 ± 9	62 ± 9	<0.001
Body surface area (m^2)	1.8 ± 0.2	1.9 ± 0.1	1.7 ± 0.1	<0.001
Body mass index (kg/m^2)	23 ± 3	24 ± 2	23 ± 3	<0.001
Systolic blood pressure (mmHg)	116 ± 12	122 ± 9	113 ± 12	<0.001
Diastolic blood pressure (mmHg)	73 ± 8	75 ± 8	72 ± 9	0.01
Glucose (mg/dL)	91 ± 11	94 ± 7	89 ± 12	0.001
Cholesterol (mg/dL)	182 ± 31	187 ± 29	180 ± 32	0.019

Table 2 2DE parameters of myocardial work

	Total, mean ± SD or median (IQR)	Total, 95% CI or limits of normality ± SE[a,b]	Male, mean ± SD or median (IQR)	Male, 95% CI or limits of normality ± SE[a,b]	Female, mean ± SD or median (IQR)	Female, 95% CI or limits of normality ± SE[a,b]	*P*-value*
GWI (mmHg%)	1896 ± 308	1292–2505	1849 ± 295	1270–2428	1924 ± 313	1310–2538	0.07
GCW (mmHg%)	2232 ± 331	1582–2881	2228 ± 295	1650–2807	2234 ± 352	1543–2924	0.9
GWW (mmHg%)	78.5 (53–122.2)	226 ± 28[a]	94 (61.5–130.5)	238 ± 33[a]	74 (49.5–111)	239 ± 39[a]	0.013
GWE (mmHg%)	96 (94–97)	91 ± 0.8[b]	95 (94–97)	90 ± 1.6[b]	96 (94–97)	91 ± 1[b]	0.026

CI, confidence interval; GCW, global constructive work; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; SD, standard deviation; SE, standard error.
[a]Highest expected value.
[b]Lowest expected value.
**P*-value differences between genders.

GE echocardiographic ultrasound equipment ($n = 378$), which is the only one, that to date provides software for calculating MW, were included in the present study. After the exclusion of patients who had incompatible image formats and/or poor-image quality and/or no blood pressure measurements available at the time of echocardiographic examination, the final study population consisted of 226 (31%) normal subjects.

Echocardiographic examination

A comprehensive echocardiographic examination was performed using state-of-the-art echocardiographic ultrasound system (GE Vivid E9; Vingmed Ultrasound, Horten, Norway) following recommended protocols approved by the EACVI.[18,19] All echocardiographic images were recorded in a digital raw-data format and centralized for further analysis, after anonymization, at the EACVI Central Core Laboratory at the University of Liege, Belgium.

2D MW analysis

Quantification of MW was performed using commercially available software package (Echopac V.202, GE). It was measured from PSLs areas, which were constructed from non-invasive LVP curves combined with strain acquired with speckle tracking echocardiography (STE), as proposed by Russell *et al.*[7] Global Longitudinal Strain (GLS) was obtained as previously reported.[16] After calculating GLS, inserting values of brachial blood pressure and indicating the time of valvular events by echocardiography, the software derived non-invasive PSLs. Strain and pressure data were synchronized by aligning the valvular event times, which were set by pulse-wave Doppler recordings at mitral valve and aortic valve level and then confirmed by 2DE evaluation of the apical long-axis view. The area of the loop served as an index of regional and global MW (*Figure 1A*). Work was evaluated from mitral valve closure to mitral valve opening. A bull's eye with the segmental and global work index (GWI) values was also provided (*Figure 1B*). Moreover, additional indices of MW were obtained as follows (*Figure 1C* and *D*): global constructive work (GCW, work performed during shortening in systole adding negative work during lengthening in isovolumetric relaxation); global wasted work (GWW, negative work performed during lengthening in systole adding work performed during shortening in isovolumetric relaxation); and global work efficiency (GWE, constructive work divided by the sum of constructive and wasted work).

Statistical analysis

Normality of the distribution of continuous variables was tested by the Kolmogorov–Smirnov test. All data were expressed as mean ± standard deviation (SD) or median (interquartile range) as appropriate. The 95% confidence interval was calculated as ±1.96 SDs from the mean. The lowest (2.5th percentile) and highest (97.5th percentile) expected values for GWW and GWE were estimated in 1000 bootstrap samples to generate sampling distribution. Differences between groups were analysed for statistical significance with the unpaired *t*-test for normally distributed continuous variables and the Mann–Whitney *U* test for non-normally distributed continuous variables. Comparison of continuous variables

Table 3 2DE parameters of myocardial work and blood pressure values according to gender and age

	Age 20–40 years ($n = 95$)		Age 40–60 years ($n = 97$)		Age ≥60 years ($n = 34$)		*P*-value		Male		Female	
	Male, mean ± SD or median (IQR)	Female, mean ± SD or median (IQR)	Male, mean ± SD or median (IQR)	Female, mean ± SD or median (IQR)	Male, mean ± SD or median (IQR)	Female, mean ± SD or median (IQR)	Male	Female	*R*	*P*-value	*R*	*P*-value
SBP (mmHg)	120 ± 10	108 ± 10*	124 ± 8	115 ± 13*	121 ± 7	122 ± 12	0.1	<0.001	0.12	0.3	0.4	<0.001
DBP (mmHg)	73 ± 9	69 ± 8*	76 ± 6	74 ± 9	74 ± 8	76 ± 8	0.1	0.002	0.12	0.2	0.3	0.001
GWI (mmHg%)	1758 ± 270	1800 ± 251	1900 ± 317	2027 ± 341	1866 ± 286	2002 ± 270	0.2	<0.001	0.16	0.1	0.25	0.002
GCW (mmHg%)	2186 ± 240	2109 ± 289	2267 ± 327	2329 ± 365	2226 ± 328	2338 ± 386	0.5	0.001	0.09	0.3	0.22	0.007
GWW (mmHg%)	99 (68–144.5)	90 (48–145)*	89 (58–122.5)	76 (51–118)	85 (49–129)	90 (48–145)	0.5	0.6	-0.13	0.2	0.06	0.4
GWE (mmHg%)	95 (93–97)	95 (94–97)*	96 (95–97)	96 (95–97)	96 (94–97)	95 (94–97)	0.6	0.8	0.12	0.2	-0.03	0.7

CI, confidence interval; DBP, diastolic blood pressure; GCW, global constructive work; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; SBP, systolic blood pressure; SD, standard deviation.
*P-value < 0.05 vs. male.

according to age groups was done with the one-way analysis of variance test. When a significant difference was found, the *post hoc* testing with Bonferroni comparisons to identify specific group differences was used. Correlation between continuous variables was performed using Pearson's or Spearman's correlation coefficient. Multivariable linear regression analyses were performed to examine the independent correlates between MW indices and baseline parameters. Intra-observer and inter-observer variability was assessed in 20 randomly selected subjects using the Bland–Altman analyses. $P < 0.05$ was considered as statistically significant. All statistical analyses were carried out using SPSS version 20 (SPSS Inc., Chicago, IL, USA).

Results

Demographic data

Table 1 summarizes the demographic data of the NORRE population analysed in the present study. A total of 85 men (mean age 45 ± 14 years) and 141 women (mean age 44 ± 13 years) were included. 2DE MW indices obtained from the study population are displayed in *Table 2*. The lowest expected values of MW indices were 1270 mmHg% in men and 1310 mmHg% in women for GWI, 1650 mmHg% and 1544 mmHg% for GCW, and 90% and 91% for GWE, respectively. The highest expected value for GWW was 238 mmHg% in men and 239 mmHg% in women. GWW was higher in men than in women, while the opposite occurred for GWE.

Age and MW indices relationship

Relationships between age and MW indices are shown in *Table 3* and *Figure 2*. GWI and GCW increased with age in women ($R^2 = 0.06$, $P = 0.002$ and $R^2 = 0.04$, $P = 0.007$, respectively) along with systolic and diastolic blood pressure ($R^2 = 0.16$, $P < 0.001$ and $R^2 = 0.09$, $P = 0.001$, respectively). In the subgroup 20–40 years, GWW was higher in men than in women and the opposite occurred for GWE ($P = 0.01$ and $P = 0.04$, respectively), while no other gender differences were found in the different age subgroups.

Repeatability and reproducibility

Intra-observer and inter-observer variability for MW indices are summarized in *Table 4*. Intra-observer and inter-observer analyses showed good repeatability and reproducibility in MW indices (*Table 4*, *Figures 3* and *4*).

MW indices and baseline parameters relationship

Multivariable analysis for MW indices showed that GWI and GCW increased with systolic blood pressure (β-coefficient = 0.67, $P < 0.001$ and β-coefficient = 0.61, $P < 0.001$, respectively, *Table 5*). There was a significant increase in GWI and GCW according to age in univariable analysis but no association was observed after adjustment for confounders. Higher values of GWE in women than in men were observed only by univariable analysis (*Table 5*).

Discussion

The present prospective, EACVI, multicentre study provides contemporary normal references values for 2DE measurements of non-invasive MW indices in a large cohort of healthy volunteers over a

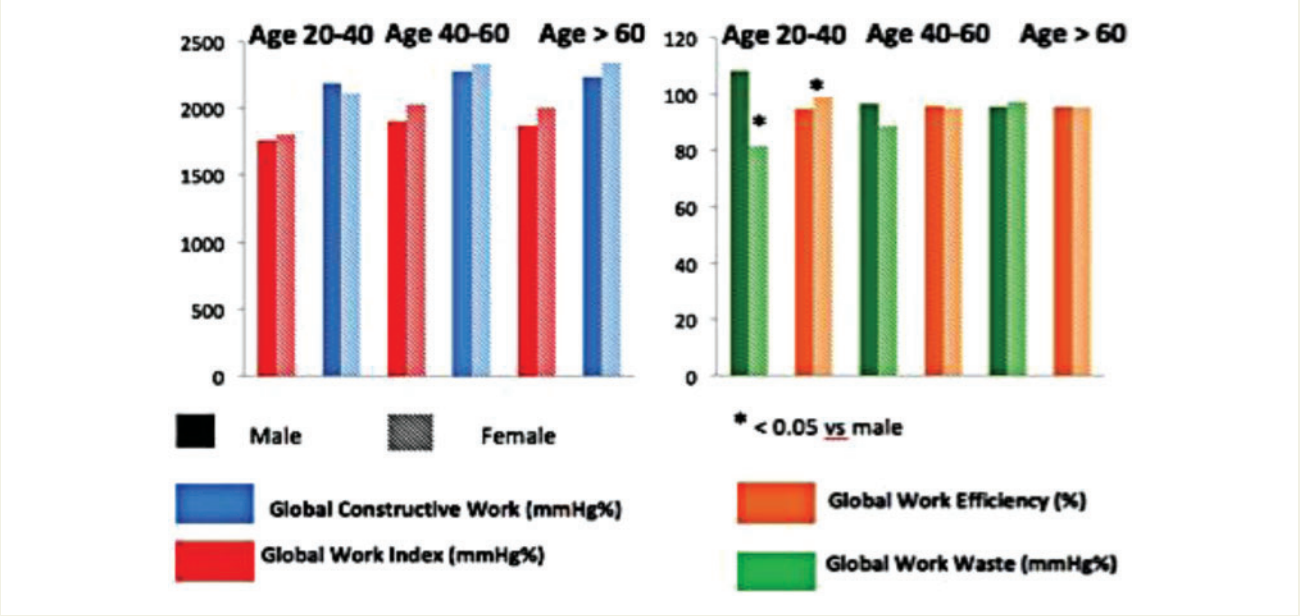

Figure 2 Bar graphs showing average MW parameters by 2D echocardiography analysis according to gender and age categories. **P*-value differences between genders.

Table 4 Repeatability and reproducibility of 2D echocardiographic data

Variables	Mean ± SD	Mean ± SD	Bias	*P*-value	95% LOA
Intraobserver					
GWI (mmHg%)	1760 ± 301	1802 ± 269	-42.1	0.1	215 to -299.3
GCW (mmHg%)	2128 ± 305	2178 ± 288	-49.7	0.07	179.2 to -278.7
GWW (mmHg%)	108 ± 62	89 ± 38	19.2	0.1	92.9 to -131.3
GWE (%)	94.4 ± 2.5	95.5 ± 1.7	-1	0.06	3.7 to -5.8
Interobserver					
GWI (mmHg%)	1798 ± 225	1833 ± 223	-34.6	0.1	155.3 to -224.5
GCW (mmHg%)	2167 ± 209	2156 ± 187	11.1	0.6	213.5 to -191.3
GWW (mmHg%)	109 ± 48	103 ± 65	6.6	0.6	116.8 to -103.6
GWE (%)	95 ± 1.7	95 ± 2.4	-0.2	0.7	5.1 to -4.7

GCW, global constructive work; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; LOA, lower limits of agreement; SD, standard deviation.

wide range of ages. 2DE analysis was performed using an EchoPAC workstation, which is the only system that currently provides software to calculate MW. The MW, derived from LVP/volume or pressure/length loops, has been investigated for almost 40 years,[20–23] and has been recently shown to also provide similar physiological information to pressure/strain loops.[6,7,24] Russell *et al.*,[7,11] more recently, introduced a method for calculating non-invasive MW, by STE and estimation of LVP from brachial artery cuff pressure. Moreover, these authors recently demonstrated a strong correlation of LV-PSLs area with regional glucose metabolism, assessed by fluorine 18-fluorodeoxyglucose-positron emission tomography.

The present NORRE sub-study is the first one, to date, to provide reference ranges for 2DE non-invasive MW in a multicentre study design. In our population of healthy individuals, univariable analysis denoted age-related changes in GWI and GCW. However, when analysing for gender-groups, both the previous indices increased with age in women, while no differences were found in men. This finding can be easily explained when considering the significant increase of both systolic and diastolic blood pressure, even if still in the normal range, according to age in women while no significant differences were found in men. Both GWI and GCW were in fact strongly correlated to blood pressure, as previously demonstrated. The increase in systolic blood pressure translates into an increase in afterload, which probably shifts LV work to a higher level of energy. Moreover, multivariable analysis revealed significant correlation only with systolic blood pressure for both GWI and GCW, with no gender and age-related changes. Univariable analysis for GWW and GWE showed lower and higher values in women than in men, respectively,

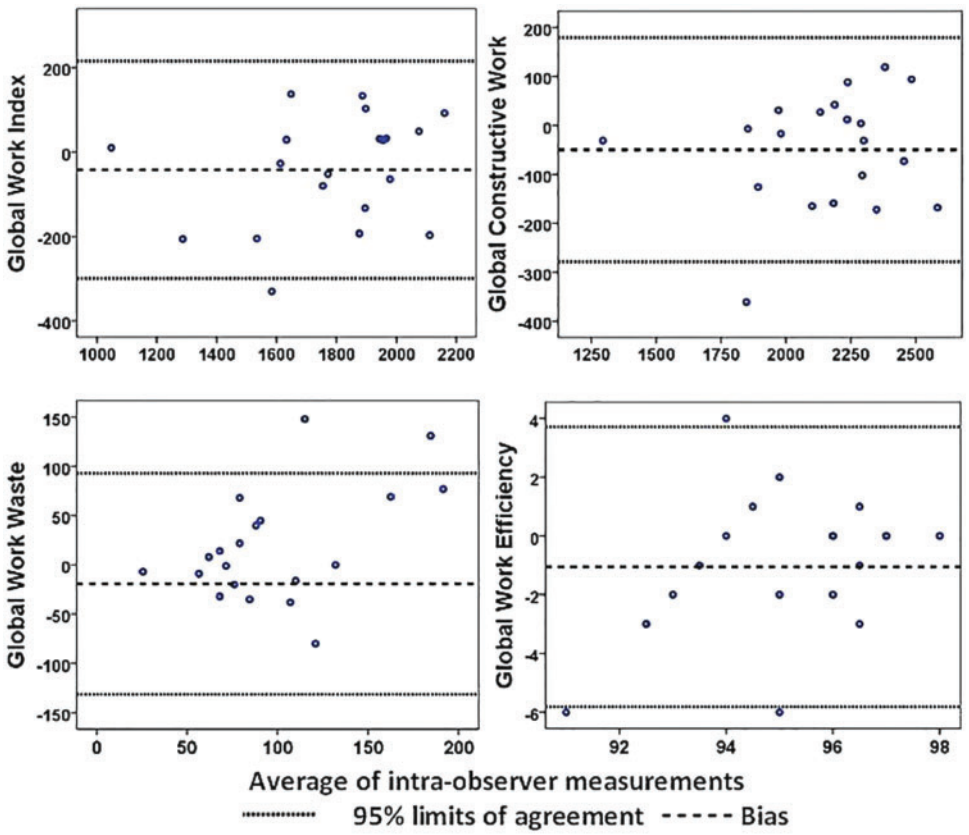

Figure 3 The Bland–Altman analysis for assessing intra-observer variability of global work index, global constructive work, global work waste, and global work efficiency. Dotted lines represent bias and 95% limits of agreement for measurements performed in 20 patients.

with no significant differences according to age. Specifically, when age and gender are considered, GWW and GWE were only different in the subgroup of 20–40 years olds. Again, this is highly related to the effect of blood pressure, which was higher in male, accounting for higher values of GWW. In the same sub-group, no differences were observed for GCW between men and women, while GWE was lower in men, as expected if considering that GWE is indirectly derived from the ratio of constructive and wasted MW. These results were, however, not confirmed in multivariable analysis.

Our data, thus, provide evidence of the absence of a strong dependence of MW on age and gender, while they highlight the association between GWI and GCW with systolic blood pressure. Moreover, MW takes into account deformation as well as afterload, potentially being superior to strain in assessing cardiac performance. As previously demonstrated, an increase in afterload may lead to reduction in systolic strain in the presence of preserved or even increased MW.[8]

To date, MW has been investigated in the field of CRT, showing promising results as a reliable predictor of response to CRT.[9–11] Preliminary interesting results have also been found in coronary artery disease. Boe *et al.*[8] showed increased sensitivity and specificity in identifying acute coronary occlusion in patients with non-ST-segment elevation myocardial infarction using regional cardiac work index, compared with all other echocardiographic parameters, including strain imaging. More recently, Chan *et al.*[25] reported the results of MW indices in three cardiovascular conditions, e.g. hypertension, ischaemic, and not-ischaemic dilated cardiomyopathy. Particularly, as in our study, they confirmed the high impact of blood pressure on MW indices by showing a significant increase in GWI in hypertensive patients when compared with controls, despite a normal global longitudinal strain. So, likely, in conditions of high arterial pressure, the LV works at higher energy level to compensate the increased afterload, as reflected by the higher GWI. Moreover, in the population of ischaemic and not-ischaemic dilated cardiomyopathy, they found a significant increase in GWW, with an impairment of myocardial performance, as expressed by reduced values of both GWI and GWE, along with global longitudinal strain. The prognostic significance of wasted work in dyssynchronous ventricles was described in previous studies, while the potential role of GWI and GWE in dilated cardiomyopathies with overt LV systolic dysfunction probably needs to be further investigated. However, it can be postulated that they could offer interesting results and additional information about cardiac performances at a very early stage of the disease, when LV is only mildly dilated and an overt systolic dysfunction is not observed, as well as in every condition of heart failure with preserved left ventricular EF. Therefore, in clinical practice, MW could play a promising role in the serial assessment of patients with or at risk of developing cardiovascular disease as in those with hypertension or cancer.[26] In particular, GWI and GCW could find more applications as indices of myocardial performance, being an expression of positive LV work. They provide complementary

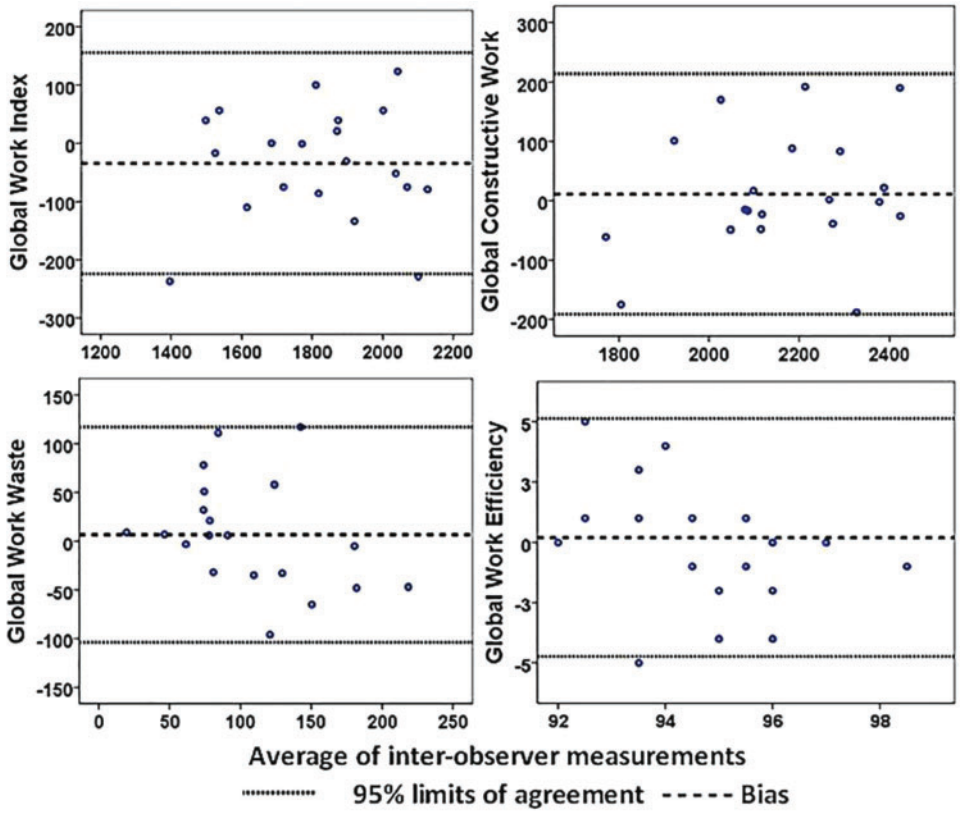

Figure 4 The Bland–Altman analysis for assessing inter-observer variability of global work index, global constructive work, global work waste, and global work efficiency. Dotted lines represent bias and 95% limits of agreement for measurements performed in 20 patients.

information to the one offered by EF and global longitudinal strain. Moreover, the assessment of GCW could play an important role in identifying responders to CRT, as an index of contractile reserve, fundamental for the success of the electrical therapy. On the contrary, but for the same purpose, GWW, which is an index of energy loss, as result of dyssynchronous and remodelled LV, could be an additional tool to identify possible responders to CRT. MW indices could also be helpful to examine the impact of treatment on LV function. Of note, our data showed a good reproducibility for the assessment of MW, reinforcing the possibility of a promising application of this new advanced echocardiographic parameter in clinical practice.

Limitations

This study presents several limitations. Only one-third of the patients included in the NORRE database were analysable by the current available software. Also, whether the NORRE study results can be extrapolated to non-Caucasian European individuals is still unknown.

Conclusion

The EACVI NORRE study provides applicable 2DE reference ranges for MW indices. Multivariable analysis did not show that age and gender were independently associated with MW indices.

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Table 5 Univariable and multivariable analysis for 2DE MW parameters

Variables	Univariable analysis Coefficient	*P*-value	Multivariable analysis β-coefficient	*P*-value
Global work index (mmHg%)				
Age (years)	0.20	0.002		
Male gender (=1)	-0.11	0.07		
Body mass index (kg/m^2)	0.12	0.05		
Systolic blood pressure (mmHg)	0.57	<0.001	0.67	<0.001
Diastolic blood pressure (mmHg)	0.37	<0.001		
Glycaemia (g/dL)	0.17	0.01		
Cholesterol (g/dL)	0.13	0.05		
Global constructive work (mmHg%)				
Age (years)	0.19	0.009		
Male gender (=1)	-0.008	0.9		
Body mass index (kg/m^2)	0.12	0.05		
Systolic blood pressure (mmHg)	0.63	<0.001	0.61	<0.001
Diastolic blood pressure (mmHg)	0.41	<0.001		
Glycaemia (g/dL)	0.25	<0.001		
Cholesterol (g/dL)	0.15	0.02		
Global work waste (mmHg%)				
Age (years)	-0.006	0.9		
Male gender (=1)	0.13	0.05		
Body mass index (kg/m^2)	-0.56	0.4		
Systolic blood pressure (mmHg)	0.11	0.07		
Diastolic blood pressure (mmHg)	0.05	0.4		
Glycaemia (g/dL)	0.04	0.5		
Cholesterol (g/dL)	0.03	0.6		
Global work efficiency (%)				
Age (years)	0.01	0.7		
Male gender (=1)	-0.14	0.03		
Body mass index (kg/m^2)	0.04	0.5		
Systolic blood pressure (mmHg)	0.01	0.8		
Diastolic blood pressure (mmHg)	0.02	0.7		
Glycaemia (g/dL)	0.02	0.7		
Cholesterol (g/dL)	-0.02	0.7		

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Correlation between non-invasive myocardial work indices and main parameters of systolic and diastolic function: results from the EACVI NORRE study

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Aims The present study sought to evaluate the correlation between indices of non-invasive myocardial work (MW) and left ventricle (LV) size, traditional and advanced parameters of LV systolic and diastolic function by 2D echocardiography (2DE).

Methods and results A total of 226 (85 men, mean age: 45 ± 13 years) healthy subjects were enrolled at 22 collaborating institutions of the Normal Reference Ranges for Echocardiography (NORRE) study. Global work index (GWI), global constructive work (GCW), global work waste (GWW), and global work efficiency (GWE) were estimated from LV pressure-strain loops using custom software. Peak LV pressure was estimated non-invasively from brachial artery cuff pressure. LV size, parameters of systolic and diastolic function and ventricular-arterial coupling were measured by echocardiography. As advanced indices of myocardial performance, global longitudinal strain (GLS), global circumferential strain (GCS), and global radial strain (GRS) were obtained. On multivariable analysis, GWI was significantly correlated with GLS (standardized beta-coefficient = -0.23, $P < 0.001$), ejection fraction (EF) (standardized beta-coefficient = 0.15, $P = 0.02$), systolic blood pressure (SBP) (standardized beta-coefficient = 0.56, $P < 0.001$) and GRS (standardized beta-coefficient = 0.19, $P = 0.004$), while GCW was correlated with GLS (standardized beta-coefficient = -0.55, $P < 0.001$), SBP (standardized beta-coefficient = 0.71, $P < 0.001$), GRS (standardized beta-coefficient = 0.11, $P = 0.02$), and GCS (standardized beta-coefficient = -0.10, $P = 0.01$). GWE was directly correlated with EF and inversely correlated with Tei index (standardized beta-coefficient = 0.18, $P = 0.009$ and standardized beta-coefficient = -0.20, $P = 0.004$, respectively), the opposite occurred for GWW (standardized beta-coefficient =--0.14, $P = 0.03$ and standardized beta-coefficient = 0.17, $P = 0.01$, respectively).

Conclusion The non-invasive MW indices show a good correlation with traditional 2DE parameters of myocardial systolic function and myocardial strain.

Keywords adult echocardiography • speckle tracking echocardiography • myocardial work • myocardial strain

Introduction

Myocardial deformation analysis, by tissue Doppler imaging (TDI) and/or speckle tracking echocardiography (STE), developed in the last decade as a reliable tool for assessing left ventricle (LV) systolic function. In addition to traditional parameters, such as ejection fraction (EF),[1,2] myocardial strain (MS) allows the detection of early subclinical LV dysfunction in a variety of cardiac diseases.[3–9] However, its relative load-dependency makes it unable for MS to account for changes in pre- and afterload. Recently, non-invasive myocardial work (MW) was proposed as a new tool to study LV performance, which takes into account myocardial deformation and afterload. Russell *et al.*,[10] indeed, developed a non-invasive method to calculate MW using LV pressure-strain loops (PSLs) obtained from STE. These authors demonstrated that regional differences in MW assessed by PSLs have a strong correlation with myocardial glucose metabolism as evaluated with fluorodeoxyglucose positron emission tomography. The application of these concepts to myocardial ischaemia and the assessment of cardiac resynchronization therapy (CRT)-responders have been evaluated, showing good results.[11–17]

The NORRE (Normal Reference Ranges for Echocardiography) study is the first European, large, prospective, multicentre study performed in 22 laboratories accredited by the European Association of Cardiovascular Imaging (EACVI) and in one American laboratory, which has provided reference values for all 2D echocardiographic (2DE) measurements of the four cardiac chambers,[18] Doppler parameters,[19] aortic dimensions,,[20] 3D echocardiographic measurements of LV volumes and strain,[21] 2DE measurement of LV strains and twist,[22] 2D and 3D measurement of left atrial function,[23] and, more recently, 2D measurement of MW indices.[24] The present study aimed to evaluate the correlation between indices of non-invasive MW and LV size, traditional and advanced parameters of LV systolic and indices of diastolic function by 2DE.

Methods

Patient population

A total of 734 healthy European subjects constituted the final NORRE study population. The local ethics committees approved the study protocol. Since GE echocardiographic system is the only equipped with a software package to calculate MW, only patients scanned with this system ($n = 378$) were included. After the exclusion of patients that had incompatible image format and/or poor-image quality and/or whose blood pressure at the time of echocardiographic examination was not available, the final study population consisted of 226 (31% of the total NORRE population, 58% of all patients scanned with GE ultrasound system) normal subjects. All the 23 laboratories involved in the NORRE studies contributed to the final population.

Echocardiographic examination

A comprehensive echocardiographic examination was performed using a state-of-the-art echocardiographic ultrasound system (GE Vivid E9; Vingmed Ultrasound, Horten, Norway) following recommended protocols approved by the EACVI.[25,26] All echocardiographic images were recorded in a digital raw-data format (native DICOM format) and centralized for further analysis, after anonymization, at the EACVI Central Core Laboratory at the University of Liege, Belgium.

LV end-diastolic and end-systolic volumes (EDV and ESV, respectively) were measured and indexed to body surface area (BSA), and EF was calculated using biplane Simpson's method.[27] LV mass was calculated from linear measurements obtained from parasternal views and indexed to BSA. Mitral annular plane systolic excursion was measured by the use of M-mode echocardiography in an apical view at the septal and lateral mitral annuli.

The left ventricle outflow tract (LVOT) diameter was measured at the aortic valve annulus, 0.5–1 cm below the aortic cups from a zoomed parasternal long-axis acoustic window. LVOT velocity-time integral was measured in the apical five-chamber view using pulsed-wave Doppler just proximal to the aortic valve. Stroke volume (SV) by Doppler ($LVOT_{area}$

Table 1 Standard and advanced echocardiographic characteristics of study population

	Total ($n = 226$), mean ± SD or medial (IQR)	Male ($n = 85$), mean ± SD or medial (IQR)	Female ($n = 141$), mean ± SD or medial (IQR)	*P*-value[a]
LVEDV (mL)	93 ± 24	107 ± 25	84 ± 19	**<0.001**
LVESV (mL)	34 ± 10	39 ± 11	31 ± 8	**<0.001**
LVEDV (mL/m^2)	52 ± 11	55 ± 12	50 ± 10	**0.002**
LVESV (mL/m^2)	19 ± 5	20 ± 5	19 ± 5	**0.02**
LVEF (%)	63 ± 5	63 ± 5	63 ± 5	0.6
LV mass indexed (g/m^2)	71 ± 17	76 ± 16	67 ± 16	**<0.001**
SV indexed (mL/m^2)	39 (35–44)	40 (36–47)	38 (34–43)	**0.03**
CO (mL/min)	4.6 (3.9–5.3)	4.9 (4.3–5.9)	4.4 (3.8–5.1)	**<0.001**
CI (mL/min/m^2)	2.6 ± 0.5	2.6 ± 0.6	2.7 ± 0.6	0.5
Septal MAPSE (mm)	15 (14–17)	16 (15–17.7)	15 (14–18)	**<0.001**
Lateral MAPSE (mm)	17 (15–18)	17 (15.2–19)	16 (15–19)	**0.004**
Septal s' wave (m/s)	8 (7–9)	8 (8–10)	8 (7–8)	**<0.001**
Lateral s' wave (m/s)	10 (8–12)	11 (9–12)	9 (8–11)	**0.002**
LAV (mL)	45.1 (38.3–54.7)	50.5 (42.9–59)	42.4 (36.5–50)	**<0.001**
LAV indexed (mL/m^2)	25.4 (22–30.1)	25.4 (22.3–30.5)	25.4 (21.8–29.9)	0.7
E wave (cm/s)	0.76 ± 0.16	0.72 ± 0.16	0.79 ± 0.16	**0.003**
A wave (cm/s)	0.58 (0.48–0.68)	0.55 (0.46–0.58)	0.59 (0.50–0.68)	0.09
Deceleration time (ms)	173 (159–202)	180 (160–210)	172 (157–198)	0.2
E/*A* ratio	1.3 (1–1.6)	1.3 (0.99–1.6)	1.3 (1–1.6)	0.5
Septal e' wave (m/s)	10 (9–12)	10 (9–12)	10 (9–12)	0.9
Lateral e' wave (m/s)	14 (11–16)	14 (11–17)	14 (11–16)	0.3
E/e' ratio	6.2 (5.3–7.6)	5.8 (5–6.9)	6.5 (5.7–7.9)	**0.001**
PASP (mmHg)	18 ± 5	17.5 ± 5.2	18.6 ± 4.9	0.2
Tei index	0.45 (0.39–0.51)	0.47 (0.42–0.55)	0.42 (0.38–0.49)	**<0.001**
Ea (mmHg/mL)	1.4 (1.3–1.7)	1.4 (1.2–1.5)	1.5 (1.3–1.8)	**<0.001**
Ees (mmHg/mL)	1.5 (1.3–1.8)	1.5 (1.3–1.6)	1.6 (1.4–1.9)	**<0.001**
Ea/Ees	0.94 (0.93–0.94)	0.94 (0.93–0.94)	0.93 (0.93–0.94)	**0.03**
GLS (%)	-21 ± 3.3	-20.5 ± 1.9	-21.3 ± 3.9	0.08
GCS (%)	-32.7 ± 4.5	-33.1 ± 5.1	-32.4 ± 4	0.3
GRS (%)	34.1 ± 8.8	33 ± 9.7	35 ± 8.1	0.1
GWI (mmHg%)	1896 ± 308	1849 ± 295	1924 ± 313	0.07
GCW (mmHg%)	2232 ± 331	2228 ± 295	2234 ± 352	0.9
GWW (mmHg%)	78.5 (53–122.2)	94 (61.5–130.5)	74 (49.5–111)	**0.013**
GWE (mmHg%)	96 (94–97)	95 (94–97)	96 (94–97)	**0.026**

P-values <0.05 are set in bold.
CI, cardiac index; CO, cardiac output; Ea, arterial elastance; EDV, end-diastolic volume; Ees, end-systolic elastance; EF, ejection fraction; ESV, end-systolic volume; GCS, global circumferential strain; GCW, global constructive work; GLS, global longitudinal strain; GRS, global radial strain; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; LAV, left atrial volume; LV, left ventricle; MAPSE, mitral annular plane systolic excursion; PASP, pulmonary arterial systolic pressure; SD, standard deviation; SV, stroke volume.
[a]*P*-value is differences between gender.

× LVOT velocity–time integral), cardiac output (CO) (SV × heart rate), and cardiac index (CI) (CO/BSA) were calculated. Transmitral flow pattern with E and A wave velocities was obtained with the sample volume positioned at mitral leaflet tips. Systolic (s') and early diastolic mitral annular velocity (e'), at both the septal and lateral side, were obtained using pulse wave (PW) TDI; moreover, isovolumetric contraction time (IVCT), isovolumetric relaxation time (IVRT), and ejection time (ET) were measured by PW TDI in order to calculate the Tei index.[28] Biplane left atrial volume (LAV) was calculated using Simpson's biplane method and indexed to BSA. Arterial elastance (Ea) and end-systolic elastance (Ees) were calculated according to Chen *et al.*[29]; subsequently, Ea/Ees ratio was obtained and used as an index of ventricular-arterial coupling (VAC).

2D LV strain and MW analysis

Quantification of 2D strain was performed using commercially available software (Echopac V.202, GE). Analysis was performed in all three apical views (LV four-, two-, and three-chamber views) as well as three short-axis views (LV basal, mid, and apical views). The reference point was set at the onset of the QRS complex. End-systole was identified as the time in which the LV cavity was the smallest. The endocardial border was

Table 2 Univariable and multivariable analysis for GWI

Variables	Univariable analysis		Multivariable analysis	
	Coefficient	P	Standardized β-coefficient	P
SBP (mmHg)	**0.57**	**<0.001**	**0.56**	**<0.001**
EDV (mL)	0.09	0.1		
ESV (mL)	-0.07	0.2		
EDV indexed (mL/m^2)	0.11	0.1		
ESV indexed (mL/m^2)	-0.08	0.2		
EF (%)	**0.32**	**<0.001**	**0.15**	**0.02**
LV mass indexed (g/m^2)	**0.15**	**0.02**		
SV indexed (mL/m^2)	**0.26**	**<0.001**		
CO (mL/min)	**0.14**	**0.03**		
CI (mL/min/m^2)	**0.19**	**0.004**		
Septal MAPSE (mm)	-0.012	0.7		
Lateral MAPSE (mm)	-0.015	0.8		
Septal s' wave (cm/s)	-0.06	0.3		
Lateral s' wave (cm/s)	**-0.13**	**0.04**		
LAV (mL)	0.12	0.08		
LAV indexed (mL/m^2)	**0.19**	**0.006**		
E wave (cm/s)	0.12	0.07		
A wave (cm/s)	**0.17**	**0.009**		
Deceleration time (ms)	-0.05	0.3		
E/A ratio	-0.06	0.3		
Septal e' wave (cm/s)	-0.13	0.05		
Lateral e' wave (cm/s)	-0.03	0.05		
E/e' ratio	**0.23**	**0.001**		
PASP (mmHg)	0.06	0.4		
Tei index	-0.07	0.2		
Ea (mmHg/mL)	0.08	0.2		
Ees (mmHg/mL)	0.09	0.1		
Ea/Ees	**0.29**	**<0.001**		
GLS (%)	**-0.51**	**<0.001**	**-0.23**	**<0.001**
GCS (%)	-0.15	0.05		
GRS (%)	**0.22**	**0.006**	**0.19**	**0.004**

P-values <0.05 are set in bold.
CI, cardiac index; CO, cardiac output; Ea, arterial elastance; EDV, end-diastolic volume; Ees, end-systolic elastance; EF, ejection fraction; ESV, end-systolic volume; GCS, global circumferential strain; GCW, global constructive work; GLS, global longitudinal strain; GRS, global radial strain; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; LAV, left atrial volume; LV, left ventricle; MAPSE, mitral annular plane systolic excursion; PASP, pulmonary arterial systolic pressure; SD, standard deviation; SBP, systolic blood pressure; SV, stroke volume.

traced in end-systole and the region of interest was adjusted to exclude the pericardium by attentively aligning the epicardial border. The integrity of tracking was visually confirmed as well as ascertained from the credibility of the strain curves, in addition to the automated tracking detection in the software. If necessary, the region of interest was readjusted. Peak systolic circumferential and peak systolic radial strain were measured at the basal, midventricular, and apical levels in each wall and averaged into a global value for each short-axis level and type of strain.

MW was obtained using a vendor-specific module by PSLs areas, which were constructed from non-invasive LV pressure (LVP) curves combined with strain acquired with STE, as previously reported.[10,24] Peak systolic LVP was assumed to be equal to brachial systolic blood pressure (SBP) measured by cuff manometer. Therefore, a LVP curve was obtained using an empiric, normalized reference curve that was adjusted according to the duration of the LV isovolumetric and ejection phases, defined by the mitral and aortic event times, as set by echocardiography.

Strain and pressure data were synchronized by aligning the valvular event times. Global work index (GWI) was obtained as total work within the area of the LV PSLs, calculated from mitral valve closure to mitral valve opening. Moreover, additional indices of MW were calculated as follows: global constructive work (GCW), work performed during shortening in systole adding negative work during lengthening in isovolumetric relaxation; global wasted work (GWW), negative work performed during lengthening in systole adding work performed during shortening in isovolumetric relaxation; global work efficiency (GWE), constructive work divided by the sum of constructive and wasted work.

Statistical analysis

Normality of the distribution of continuous variables was tested by the Kolmogorov–Smirnov test. Continuous variables were expressed as means ± standard deviation (SD) or median (interquartile range) as appropriate. Differences between groups were analysed for statistical significance with the unpaired *t*-test for normally distributed continuous variables and the Mann–Whitney *U* test for non-normally distributed continuous variables. Correlation between continuous variables was performed using Pearson's or Spearman's correlation coefficient as appropriate. Multivariable linear regression analyses were performed to examine the independent correlates between MW indices and standard and advanced echocardiographic parameters. For multiple linear regression models, multicollinearity was also examined by computation of variance inflation factor. In case of collinear variables, the variable with the highest correlation coefficient was included. *P*-value <0.05 was considered as statistically significant. All statistical analyses were carried out using SPSS version 21 (SPSS Inc., Chicago, IL, USA).

Results

A total of 85 men (mean age 45 ± 14 years) and 141 women (mean age 44 ± 13 years) were included. Other demographic data of the population analysed in the present study were previously reported.[24] Standard and advanced 2DE parameters of the study population are displayed in *Table 1*. LV mass and volumes were greater in men compared with women, even after normalization for BSA; the same was observed for SV, CO, and CI. No significant differences were found for EF and all average strain components. Indices of VAC were slightly higher in women.

Correlations between GWI and 2DE parameters

As expected, GWI showed a good correlation with SBP and global longitudinal strain (GLS) ($r = 0.57$, $P < 0.0001$ and $r = -0.51$, $P < 0.001$, respectively), a moderate correlation with EF and Ea/Ees ($r = 0.32$, $P < 0.001$ and $r = 0.29$, $P < 0.001$) and a weak correlation with LV mass indexed to BSA, SV indexed to BSA, CO, CI, lateral s' wave, *E*/e' ratio and global radial strain (GRS) (*Table 2*). On multivariable analysis, GWI was significantly correlated with GLS (standardized beta-coefficient = -0.23, $P < 0.001$), EF (standardized beta-coefficient = 0.15, $P = 0.02$), SBP (standardized beta-coefficient = 0.56

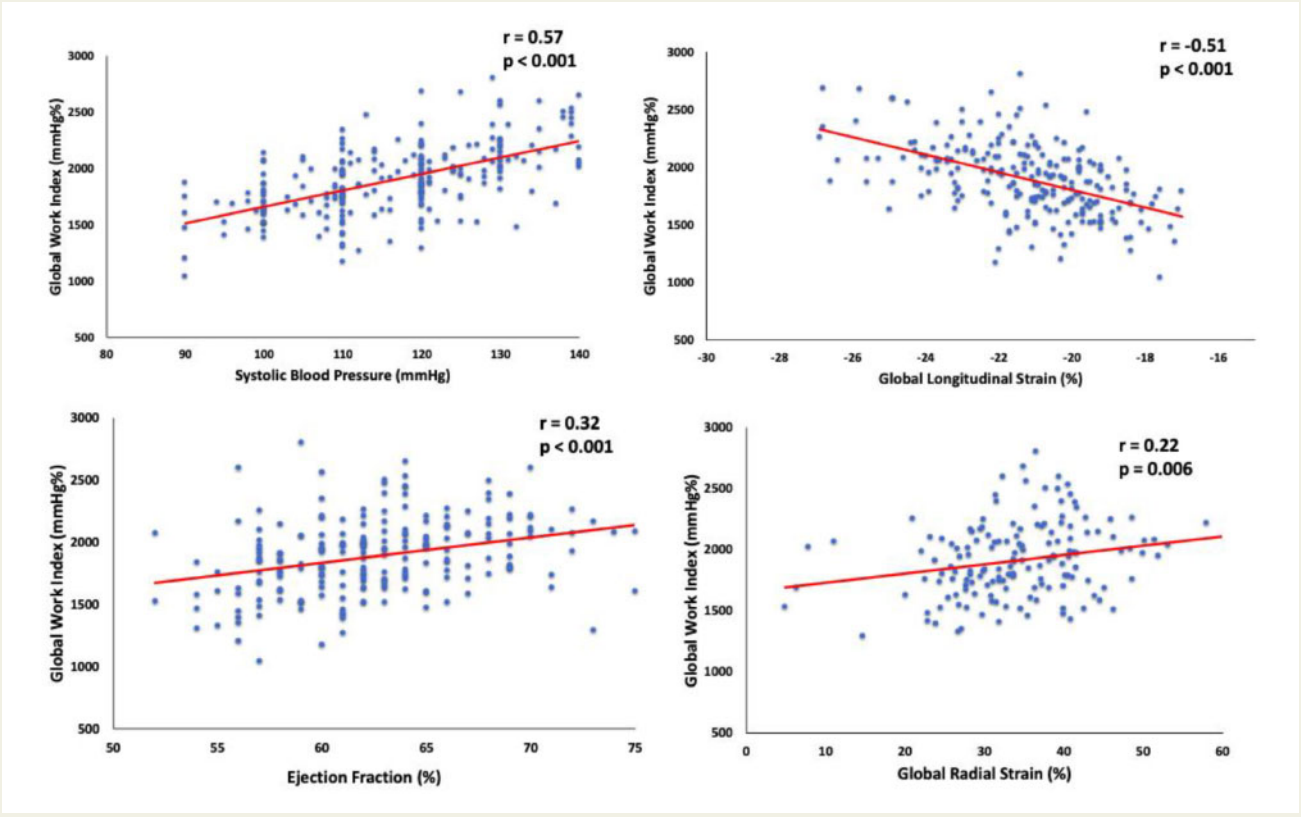

Figure 1 Main relations of global work index.

$P < 0.001$) and GRS (standardized beta-coefficient = 0.19, $P = 0.004$) (*Figure 1* and *Table 2*).

Correlations between GCW and 2DE parameters

GCW showed a good correlation with SBP and GLS ($r = 0.64$, $P < 0.001$ and $r = -0.51$, $P < 0.001$, respectively), a moderate correlation with EF and Ea/Ees ($r = 0.26$, $P < 0.001$ and $r = 0.29$, $P < 0.001$) and a weak correlation with LV mass indexed to BSA, EDV indexed to BSA, SV indexed to BSA, CO, CI, lateral s' wave, LAV, and LAV indexed to BSA, *E*/e' ratio, GRS ,and global circumferential strain (GCS) (*Table 3*). On multivariable analysis, GCW was significantly correlated with GLS (standardized beta-coefficient = -0.55, $P < 0.001$), SBP (standardized beta-coefficient = 0.71, $P < 0.001$), GRS (standardized beta-coefficient = 0.11, $P = 0.01$) and GCS (standardized beta-coefficient = -0.10, $P = 0.02$) (*Figure 2* and *Table 3*).

Correlations between GWW and GWE and 2DE parameters

On multivariable analysis, GWW was significantly correlated with the Tei index (standardized beta-coefficient = 0.17, $P = 0.01$) and inversely correlated with EF (standardized beta-coefficient = -0.14, $P = 0.03$). The opposite occurred for GWE (standardized beta-coefficient = -0.20, $P = 0.004$ and standardized beta-coefficient = 0.18, $P = 0.009$, respectively, *Tables 4* and *5*).

Discussion

Reference ranges for MW indices have been recently provided by the previous NORRE study.[24] Correlations between MW and demographical variables have been also investigated, showing the absence of a strong dependence of MW indices on age, gender, and body mass index.[24] Hence, due to the growing interest in MW, the present NORRE sub-study sought to evaluate the correlations existing between the new indices of MW and LV dimensions, standard and advanced 2DE parameters of LV systolic function, and indices of diastolic function.

We did not find a strong correlation between MW indices and LV size. On univariable analysis, GWW and GWE were indeed weakly correlated with ESV, whereas GWI and GCW were weakly correlated with LV mass indexed to BSA. The latter finding could be due to the fact of a major contractile mass being involved in the production of positive work.[30] However, in pathological cardiac hypertrophy, a reduction of MW indices was recently reported.[31] Despite the physiological interest, we have to acknowledge that all these associations are not strong, not observed for all MW indices, and not confirmed in multivariable analysis; so their real clinical significance is doubtful. Probably, these data could be explained when considering that the study population was entirely composed of healthy subjects, leading to restricted LV size values ranges. In cardiac disease, such as cardiomyopathies and heart valve disease, instead, changes in both LV size and function are often observed[32–34] Thus, LV remodelling and dysfunction are usually strictly correlated, the one affected by

Table 3 Univariable and multivariable analysis for GCW

Variables	Univariable analysis		Multivariable analysis	
	Coefficient	P	Standardized β-coefficient	P
SBP (mmHg)	**0.64**	**<0.001**	**0.71**	**<0.001**
EDV (mL)	0.13	0.06		
ESV (mL)	-0.01	0.8		
EDV indexed (mL/m^2)	**0.14**	**0.04**		
ESV indexed (mL/m^2)	-0.02	0.6		
EF (%)	**0.26**	**<0.001**		
LV mass indexed (g/m^2)	**0.17**	**0.008**		
SV indexed, mL/m^2	**0.25**	**<0.001**		
CO (mL/min)	**0.16**	**0.01**		
CI (mL/min/m^2)	**0.19**	**0.005**		
Septal MAPSE (mm)	-0.02	0.7		
Lateral MAPSE (mm)	-0.006	0.9		
Septal s' wave (cm/s)	-0.05	0.4		
Lateral s' wave (cm/s)	**-0.14**	**0.03**		
LAV (mL)	**0.17**	**0.01**		
LAV indexed (mL/m^2)	**0.23**	**0.001**		
E wave (cm/s)	0.05	0.4		
A wave (cm/s)	0.11	0.09		
Deceleration time (ms)	-0.02	0.7		
E/A ratio	-0.06	0.3		
Septal e' wave (cm/s)	**-0.15**	**0.01**		
Lateral e' wave (cm/s)	-0.07	0.2		
E/e' ratio	**0.2**	**0.003**		
PASP (mmHg)	0.03	0.6		
Tei index	-0.03	0.5		
Ea (mmHg/mL)	0.08	0.2		
Ees (mmHg/mL)	0.08	0.2		
Ea/Ees	**0.29**	**<0.001**		
GLS (%)	**-0.51**	**<0.001**	**-0.55**	**<0.001**
GCS (%)	**-0.16**	**0.04**	**-0.10**	**0.02**
GRS (%)	**0.19**	**0.01**	**0.11**	**0.01**

P-values <0.05 are set in bold.
CI, cardiac index; CO, cardiac output; Ea, arterial elastance; EDV, end-diastolic volume; Ees, end-systolic elastance; EF, ejection fraction; ESV, end-systolic volume; GCS, global circumferential strain; GCW, global constructive work; GLS, global longitudinal strain; GRS, global radial strain; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; LAV, left atrial volume; LV, left ventricle; MAPSE, mitral annular plane systolic excursion; PASP, pulmonary arterial systolic pressure; SD, standard deviation; SBP, systolic blood pressure; SV, stroke volume.

the other and vice versa, especially in advanced cardiovascular diseases. On the contrary, in normal subjects, it is not really surprising to find only a mild association between LV size and indices of MW, being both in a normal range.

Regarding LV systolic function, we tested correlations with traditional parameters and with MS, which is an established advanced index to study LV systolic function. While associations with GLS were obviously expected, we also found an intriguing significant correlation between both GWI and GCW with GRS. Furthermore, GCW was significantly correlated even with GCS. As known, due to the complex architecture of myocardial fibres, the LV systolic motion is the result of three principal components: base to apex longitudinal shortening, epicardium towards endocardium radial thickening and circumferential rotation and shortening.[1] Our findings, thus, highlights as likely all the components of myocardial deformation contribute to generate MW, so it, and in particular GCW, could be supposed to globally reflect LV mechanics and performance. In our analysis, GWI and GCW were also significantly correlated with parameters that traditionally reflect LV systolic performance, namely EF, SV, CO, and CI. These data are perfectly in accordance with the physiological substrate of GWI and GCW. In a normal heart, indeed, all myocardial segments contract in a synchronized manner resulting in positive work, the constructive work, which by definition is the work contributing towards LV ejection.[12] Accordingly, GCW, as index of contractile and viable myocardium, has been proposed as a potential parameter to identify CRT responders by Galli *et al.*[12,13] The same authors showed preliminary results of GCW' s application even in non-obstructive hypertrophic cardiomyopathy, as a reliable tool to estimate LV performance and functional capacity.[31]

Among diastolic parameters, GWI and GCW correlated with LA size and E/E' ratio, though only on univariable analysis. Probably this finding should be interpreted in the context of normal ranges of both the diastolic parameters. In our population, in fact, increasing values of LA size and E/e' were not an expression of diastolic dysfunction, being both in the normal range. Besides, this association was not confirmed in multivariable analysis; so according to our data correlation of MW with parameters of diastolic function was really poor. However, an interesting exception was the Tei index. A significant association between Tei index and both GWW and GWE was found. It is a combined index of global systolic and diastolic function, which relies on measure of the same part of cardiac cycle analysed by MW: from mitral valve closure to mitral valve opening, namely mechanical systole including isovolumetric relaxation time. Higher values of Tei index are secondary to prolonged IVCT and/or IVRT respect to ET; it could be translated in a higher wasted work, due mainly to myocyte s' shortening in a prolonged IVRT, and consequent lower efficiency.

Finally, as MW has been recently proposed as a potential new method of estimation of VAC,[35] we aimed to test its correlation with the main index of VAC, Ea/Ees ratio, calculated by echocardiography.[29,36] It is the result of complex formulas including SV, EF, SBP, and diastolic blood pressure (all parameters correlated with GWI and GCW) and accounting for time too.[29] So, the significant correlation with Ea/Ees ratio and its easier measurement could reinforce its application also as an alternative index of VAC. However, more studies are needed to evaluate the performance of MW and its role as an established tool for studying VAC needs to be further investigated and validated.

Our data, hence, support the role of MW as a reliable parameter of myocardial systolic performance, in addition to traditional ones and MS. MW, indeed, adjusting myocardial deformation for LVP dynamics, could offer further information for the evaluation of cardiac performance in conditions of subclinical LV dysfunction as well as in heart failure with preserved EF (HFpEF). In this field preliminary data have been recently obtained, depicting the superiority of GCW respect to GLS as a better determinant of exercise capacity in patients

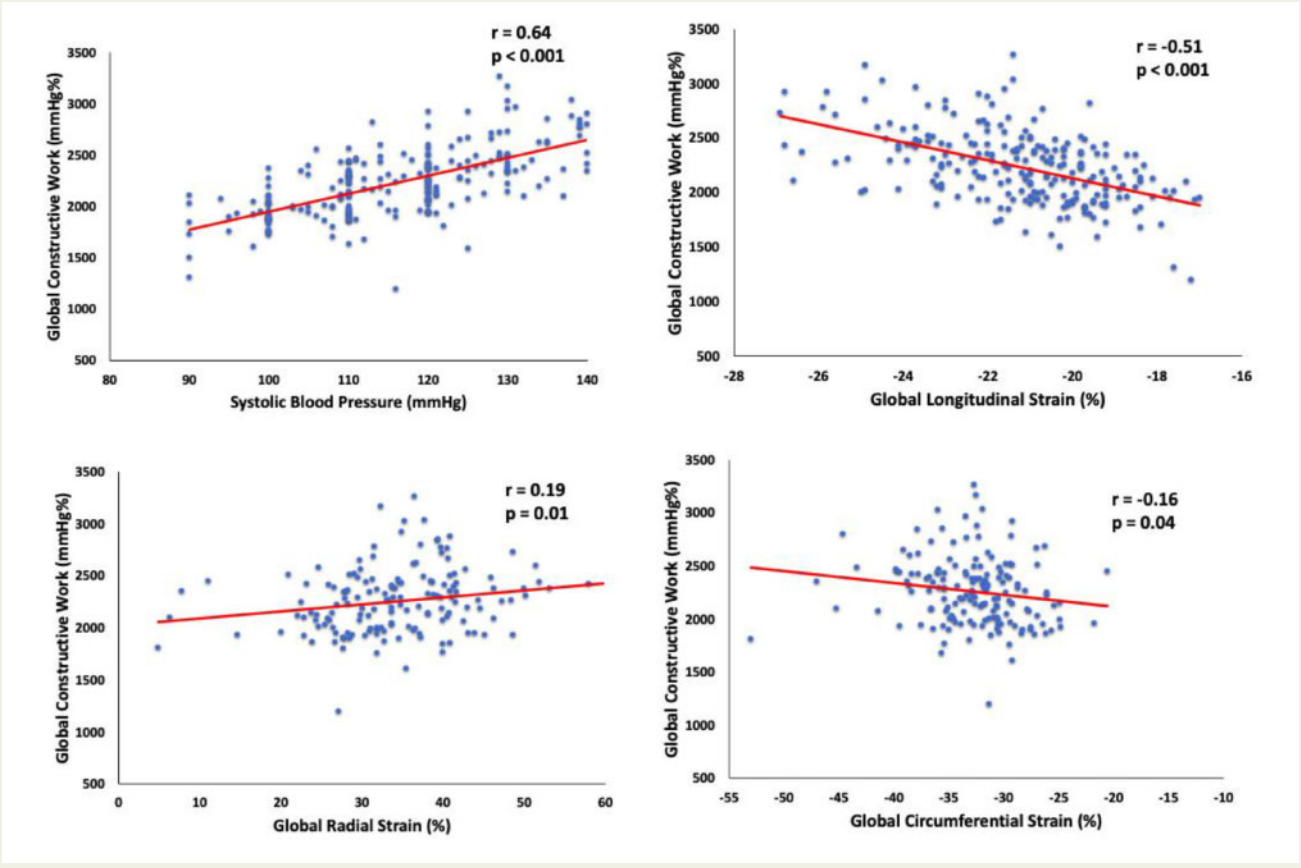

Figure 2 Main relations of global constructive work.

with HFpEF.[37] Therefore, besides its promising application in patients candidates to CRT, MW could be investigated in the subset of patients at risk of development or at an early stage of cardiovascular disease, for example patients under cardiotoxic treatment.

Limitations

Only 31% of the patients included in the NORRE study have been available for MW analysis, due mainly to the possibility of application of MW only to exams acquired through GE echocardiographic ultrasound system, adding the dependency on image quality and blood pressure availability. Moreover, whether the NORRE study results can be extrapolated to non-Caucasian European individuals is still unknown.

Non-invasive LVP estimation by brachial cuff pressure is imprecise, representing a limitation of LV PSLs as obtained by Russell *et al.* Nevertheless, it was recently demonstrated that, despite discrepancies between cuff pressure and invasive pressure, MW analysis was accurate, due to temporal integration and less pressure differences from aortic valve opening to closure.[38]

Based on our findings the current software is indeed promising, but further studies in larger populations with various forms of heart diseases, comparing the results of this software against

invasively obtained PV loops and calculations of cardiac work parameters, are required before introducing it into daily clinical use.

Conclusion

The NORRE study shows good correlations of GWI with EF and GRS, and of GCW with GRS and GCS, as well as with GLS. Weak correlations are observed between MW indices and LV size. MW is a promising tool to study myocardial systolic performance; however, further investigations are needed before introducing it in routine clinical practice.

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Conflict of interest: none declared.

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Table 4 Univariable and multivariable analysis for GWW

Variables	Univariable analysis		Multivariable analysis	
	Coefficient	P	Standardized β-coefficient	P
SBP (mmHg)	0.12	0.07		
EDV (mL)	0.04	0.5		
ESV (mL)	**0.14**	**0.03**		
EDV indexed (mL/m^2)	-0.008	0.9		
ESV indexed (mL/m^2)	0.12	0.06		
EF (%)	**-0.17**	**0.01**	**-0.14**	**0.03**
LV mass indexed (g/m^2)	0.03	0.6		
SV indexed (mL/m^2)	0.05	0.4		
CO (mL/min)	0.04	0.5		
CI (mL/min/m^2)	-0.02	0.7		
Septal MAPSE (mm)	0.01	0.8		
Lateral MAPSE (mm)	-0.01	0.8		
Septal s' wave (cm/s)	-0.08	0.2		
Lateral s' wave (cm/s)	-0.01	0.8		
LAV (mL)	0.11	0.1		
LAV indexed (mL/m^2)	0.06	0.3		
E wave (cm/s)	-0.11	0.1		
A wave (cm/s)	-0.03	0.5		
Deceleration time (ms)	0.07	0.2		
E/A ratio	-0.05	0.4		
Septal e' wave (cm/s)	-0.12	0.05		
Lateral e' wave (cm/s)	-0.07	0.9		
E/e' ratio	-0.03	0.6		
PASP (mmHg)	-0.04	0.6		
Tei index	**0.24**	**<0.001**	**0.17**	**0.01**
Ea (mmHg/mL)	-0.05	0.4		
Ees (mmHg/mL)	-0.05	0.4		
Ea/Ees	-0.04	0.5		
GLS (%)	0.09	0.1		
GCS (%)	0.03	0.6		
GRS (%)	-0.4	0.6		

P-values <0.05 are set in bold.
CI, cardiac index; CO, cardiac output; Ea, arterial elastance; EDV, end-diastolic volume; Ees, end-systolic elastance; EF, ejection fraction; ESV, end-systolic volume; GCS, global circumferential strain; GCW, global constructive work; GLS, global longitudinal strain; GRS, global radial strain; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; LAV, left atrial volume; LV, left ventricle; MAPSE, mitral annular plane systolic excursion; PASP, pulmonary arterial systolic pressure; SD, standard deviation; SBP, systolic blood pressure; SV, stroke volume.

Table 5 Univariable and multivariable analysis for GWE

Variables	Univariable analysis		Multivariable analysis	
	Coefficient	P	Standardized β-coefficient	P
SBP (mmHg)	0.004	0.9		
EDV (mL)	-0.02	0.6		
ESV (mL)	**-0.15**	**0.03**		
EDV indexed (mL/m^2)	0.01	0.8		
ESV indexed (mL/m^2)	**-0.14**	**0.04**		
EF (%)	**0.20**	**0.004**	**0.18**	**0.009**
LV mass indexed (g/m^2)	0.01	0.8		
SV indexed (mL/m^2)	-0.03	0.6		
CO (mL/min)	-0.02	0.7		
CI (mL/min/m^2)	0.03	0.6		
Septal MAPSE (mm)	0.009	0.9		
Lateral MAPSE (mm)	0.02	0.7		
Septal s' wave (cm/s)	0.08	0.2		
Lateral s' wave (cm/s)	-0.008	0.9		
LAV (mL)	-0.07	0.3		
LAV indexed (mL/m^2)	-0.02	0.7		
E wave (cm/s)	0.11	0.9		
A wave (cm/s)	0.02	0.7		
Deceleration time (ms)	-0.09	0.1		
E/A ratio	0.05	0.4		
Septal e' wave (cm/s)	0.12	0.07		
Lateral e' wave (cm/s)	0.03	0.6		
E/e' ratio	0.02	0.7		
PASP (mmHg)	0.03	0.7		
Tei index	**-0.26**	**<0.0001**	**-0.20**	**0.004**
Ea (mmHg/mL)	0.07	0.2		
Ees (mmHg/mL)	0.07	0.3		
Ea/Ees	0.08	0.2		
GLS (%)	**-0.019**	**0.003**		
GCS (%)	-0.06	0.4		
GRS (%)	0.06	0.4		

P-values <0.05 are set in bold.
CI, cardiac index; CO, cardiac output; Ea, arterial elastance; EDV, end-diastolic volume; Ees, end-systolic elastance; EF, ejection fraction; ESV, end-systolic volume; GCS, global circumferential strain; GCW, global constructive work; GLS, global longitudinal strain; GRS, global radial strain; GWE, global work efficiency; GWI, global work index; GWW, global work waste; IQR, interquartile range; LAV, left atrial volume; LV, left ventricle; MAPSE, mitral annular plane systolic excursion; PASP, pulmonary arterial systolic pressure; SD, standard deviation; SBP, systolic blood pressure; SV, stroke volume.

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Part II

Multiparametric approach in the diagnosis, prognosis and treatment of aortic stenosis

JAMA Cardiology | Original Investigation

Outcomes of Patients With Asymptomatic Aortic Stenosis Followed Up in Heart Valve Clinics

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IMPORTANCE The natural history and the management of patients with asymptomatic aortic stenosis (AS) have not been fully examined in the current era.

OBJECTIVE To determine the clinical outcomes of patients with asymptomatic AS using data from the Heart Valve Clinic International Database.

DESIGN, SETTING, AND PARTICIPANTS This registry was assembled by merging data from prospectively gathered institutional databases from 10 heart valve clinics in Europe, Canada, and the United States. Asymptomatic patients with an aortic valve area of 1.5 cm^2 or less and preserved left ventricular ejection fraction (LVEF) greater than 50% at entry were considered for the present analysis. Data were collected from January 2001 to December 2014, and data were analyzed from January 2017 to July 2018.

MAIN OUTCOMES AND MEASURES Natural history, need for aortic valve replacement (AVR), and survival of asymptomatic patients with moderate or severe AS at entry followed up in a heart valve clinic. Indications for AVR were based on current guideline recommendations.

RESULTS Of the 1375 patients included in this analysis, 834 (60.7%) were male, and the mean (SD) age was 71 (13) years. A total of 861 patients (62.6%) had severe AS (aortic valve area less than 1.0 cm^2). The mean (SD) overall survival during medical management (mean [SD] follow up, 27 [24] months) was 93% (1%), 86% (2%), and 75% (4%) at 2, 4, and 8 years, respectively. A total of 104 patients (7.6%) died under observation, including 57 patients (54.8%) from cardiovascular causes. The crude rate of sudden death was 0.65% over the duration of the study. A total of 542 patients (39.4%) underwent AVR, including 388 patients (71.6%) with severe AS at study entry and 154 (28.4%) with moderate AS at entry who progressed to severe AS. Those with severe AS at entry who underwent AVR did so at a mean (SD) of 14.4 (16.6) months and a median of 8.7 months. The mean (SD) 2-year and 4-year AVR-free survival rates for asymptomatic patients with severe AS at baseline were 54% (2%) and 32% (3%), respectively. In those undergoing AVR, the 30-day postprocedural mortality was 0.9%. In patients with severe AS at entry, peak aortic jet velocity (greater than 5 m/s) and LVEF (less than 60%) were associated with all-cause and cardiovascular mortality without AVR; these factors were also associated with postprocedural mortality in those patients with severe AS at baseline who underwent AVR (surgical AVR in 310 patients; transcatheter AVR in 78 patients).

CONCLUSIONS AND RELEVANCE In patients with asymptomatic AS followed up in heart valve centers, the risk of sudden death is low, and rates of overall survival are similar to those reported from previous series. Patients with severe AS at baseline and peak aortic jet velocity of 5.0 m/s or greater or LVEF less than 60% have increased risks of all-cause and cardiovascular mortality even after AVR. The potential benefit of early intervention should be considered in these high-risk patients.

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Editor's Note
Supplemental content

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In the western world, calcific aortic stenosis (AS), the most common valvular heart disease, represents a major public health burden.[1] Currently, there is no pharmacological treatment that prevents or slows the progression of AS.[2] Surgical aortic valve replacement (SAVR) and transcatheter aortic valve replacement (TAVR) are the only therapies to significantly improve both survival and symptoms[3,4] and are recommended in symptomatic patients with severe AS.[5,6] The management of patients with asymptomatic severe AS, particularly the choice between early intervention vs watchful waiting, continues to be a matter of debate.[7,8] Current guidelines advocate delaying AVR until symptoms or left ventricular (LV) systolic dysfunction develop.[5,6] However, observational studies in 2010[9] and 2015[10] have suggested that early elective AVR might improve outcomes in patients with severe asymptomatic AS. This approach has been reinforced by continued advances in surgical techniques and aortic valve prostheses, the advent of TAVR, and the low perioperative mortality and morbidity rates achieved in valve centers of reference.[11,12] However, preemptive surgery before onset of symptoms or LV systolic dysfunction is considered in only a selected group of patients after careful risk stratification. This is at least in part because the evidence for intervention in asymptomatic severe AS is derived from small, heterogeneous, retrospective, single-center studies, which have generally included the need for AVR (not always motivated by the development of symptoms or LV dysfunction) in the composite study end point.[13-20] Moreover, decision making for AVR remains particularly difficult in older patients in whom it is sometimes unclear if the benefits of intervention outweigh the risk.[5,6]

In recent years, the establishment of multidisciplinary services delivered by experts in valvular heart disease has become the basis for the implementation of heart valve clinics.[21] These clinics provide standardized care based on international evidence-based norms and facilitate large clinical registries, which may be used to further refine guideline recommendations and quality improvement. The Heart Valve Clinic International Database (HAVEC) is a multicenter registry created for prospective data collection of patients with echocardiographic confirmation of AS and other valve diseases.[22] The objective of the present study was to determine the natural history and outcomes of patients with moderate or severe AS who are followed up in a heart valve clinic.

Key Points

Question What is the outcome of patients with asymptomatic aortic stenosis (AS) followed up in a specialized heart valve clinic?

Findings In this study using data from the Heart Valve Clinic International Database including 1375 patients from 10 heart valve clinics, left ventricular ejection fraction less than 60% and peak aortic jet velocity greater than 5 m/s were independent factors associated with all-cause and cardiovascular mortality in patients with asymptomatic severe AS. The adverse association of these factors with survival remains significant following aortic valve replacement, suggesting the need for earlier intervention.

Meaning Taking into consideration the low procedural risk associated with aortic valve replacement, the potential benefit of earlier intervention should be considered in high-risk patients with asymptomatic severe AS.

Methods

The data, analytic methods, and study materials can be made available to other researchers for purposes of reproducing the results or replicating the procedure after approval of the HAVEC group. Data are centrally collected at the Department of Cardiology, Centre Hospitalier Universitaire du Sart Tilman, Liège, Belgium. This retrospective analysis of clinically acquired data was approved by the respective institutional review boards of each participating center, and informed consent was waived because collected data were deidentified and retrospective.

Study Population

The HAVEC registry was assembled by merging data from prospectively gathered electronic institutional databases of 10 heart valve clinics, as defined by the European Society of Cardiology Working Group in Valvular Heart Diseases,[13] collected between 2001 and 2014. The analyses were then performed retrospectively. Patients were eligible for this registry if they had AS diagnosed with the use of 2-dimension echocardiography at 1 of the participating centers and were followed-up according to available guidelines on a regular basis. Exclusion criteria included aortic valve area (AVA) greater than 1.5 cm^2; class I indications for AVR (rest AS-related or exercise AS-related symptoms [ie, angina, syncope, and dyspnea] or LV ejection fraction [EF] less than 50%); concomitant congenital heart valve disease more than mild mitral, tricuspid, or pulmonic valve disease; or prior valve surgery. The study was conducted in accordance with the respective institutional guidelines, national legal requirements, and the revised Helsinki declaration.[23]

Doppler Echocardiography

Transthoracic echocardiography was performed as part of routine clinical practice using commercially available systems. The severity of AS was evaluated according to standard methods. Peak aortic jet velocity was derived from transaortic flow, recorded with continuous wave Doppler using a multiwindow approach. Peak and mean gradients were calculated using the simplified Bernoulli equation. The continuity equation was used to calculate AVA. Moderate and severe AS were defined as an AVA between 1.0 and 1.5 cm^2 and less than 1.0 cm^2, respectively. Left ventricular EF was estimated by the Simpson biplane method.

Follow-up

Follow-up was organized within each participating center according to available guidelines (every 6-12 months in patients with severe AS) (eTable 1 in Supplement 1). Data collection started after baseline evaluation until last available contact or death. Follow-up data were obtained by direct patient interview and clinical examination; telephone calls with physicians, patients, or next of kin; or review of autopsy records and

Table 1. Comparison of Patients With Moderate vs Severe Aortic Stenosis (AS) at Baseline

Variable	Mean (SD)			P Value
	All (N = 1375)	Moderate AS (n = 514)	Severe AS (n = 861)	
Age, y	71 (13)	68 (13)	72 (12)	<.001
Male, No. (%)	834 (60.7)	337 (65.6)	497 (57.7)	.004
Height, cm	167 (9)	168 (9)	166 (9)	.04
Weight, kg	75 (15)	78 (15)	73 (16)	<.001
Body surface area, m^2	1.8 (0.2)	1.9 (0.2)	1.8 (0.2)	<.001
Systolic blood pressure, mm Hg	140 (19)	140 (18)	140 (20)	.97
Diastolic blood pressure, mm Hg	78 (11)	78 (10)	77 (11)	.41
Hypertension, No. (%)	833 (60.6)	327 (63.6)	506 (58.8)	.07
Diabetes, No. (%)	245 (17.8)	95 (18.4)	150 (17.4)	.74
Smoker, No. (%)	415 (30.1)	180 (35.0)	235 (27.3)	.002
Dyslipidemia, No. (%)	722 (52.5)	299 (58.1)	423 (49.1)	<.001
Chronic obstructive pulmonary disease, No. (%)	104 (7.6)	48 (9.3)	56 (6.5)	.03
β-Blockers, No. (%)	482 (35.1)	150 (29.2)	332 (38.6)	<.001
Angiotensin-converting enzyme inhibitor, No. (%)	447 (32.5)	177 (34.4)	270 (31.4)	.31
LV mass, g/m^2	207 (73)	209 (58)	206 (81)	.51
LVESV, mL	39 (21)	40 (22)	39 (20)	.53
LVEDV, mL	103 (34)	110 (35)	100 (33)	<.001
SV index, mL/m^2	44 (11)	46 (11)	42 (11)	<.001
LV ejection fraction, %	65.5 (7.4)	66 (6.9)	65 (7.3)	.003
Peak aortic velocity, m/s	3.8 (0.8)	3.3 (0.7)	4.1 (0.7)	<.001
Mean aortic pressure gradient, mm Hg	37 (17)	26 (12)	44 (16)	<.001
Aortic valve area, cm^2	0.94 (0.3)	1.20 (0.2)	0.78 (0.1)	<.001
Mitral E wave velocity, cm/s	87 (28)	84 (22)	88 (31)	.02
Mitral E/A ratio	1 (0.6)	1 (0.4)	1 (0.6)	.80
E/e' ratio	10.8 (5.7)	10.6 (4.6)	10.9 (6.4)	.28

Abbreviations: EDV, end-diastolic volume; ESV, end-systolic volume; LV, left ventricular; SV, stroke volume.

death certificates. Information was collected regarding development of cardiac symptoms, subsequent AVR (performed for development of guideline indications: symptom onset, abnormal exercise test, peak aortic velocity greater than 5.5 m/s, or rapid progression of AS severity), and death.[24] Exercise testing was performed in selected patients (572 of 1375 patients [41.6%]), especially when the symptomatic status was unclear. Cardiac deaths were classified as directly related to AS (ie, sudden death or heart failure) or to other cardiac pathology (ie, fatal myocardial infarction). All-cause mortality was the primary end point of the study; cardiovascular-related mortality was the secondary end point. Follow-up echocardiography data were obtained in all patients who underwent AVR to confirm the progression of moderate to severe AS (ie, AVA less than 1 cm^2).

Statistical Analysis

Data are reported as means with standard deviations for continuous variables or numbers and percentages of individuals for categorical variables. Group comparisons for categorical variables were obtained with χ^2 test and for continuous variables with Mann-Whitney *U* test if the normality of data was violated based on a Shapiro-Wilk test. Analyses of overall and cardiovascular mortality were performed by censoring data at the time of AVR. Multivariable analysis was then performed by including covariates selected on the basis of their known link to outcome in patients with AS (ie, age, sex, comorbidities, AS severity, and LVEF) into a Cox proportional hazard model. Peak aortic jet velocity (greater than or equal to 5 m/s) and LVEF (less than 60%) were also expressed as categorical variables.[6,25] Survival curves were computed based on the Kaplan-Meier method. Regarding the prediction of all-cause and cardiovascular death, receiver operating characteristic curve analyses were performed, and areas under the curve (AUCs) were reported. The most accurate cutoff values (ie, best compromise between sensitivity and specificity) were obtained using Youden index. A *P* value less than .05 was considered statistically significant, and all *P* values were 2-tailed. Statistical analyses were performed using SPSS version 23 (IBM).

Results

A total of 1763 patients were included in the present registry, of whom 388 (22.0%) were excluded because of missing data regarding LVEF or AS severity. The characteristics of the remaining 1375 patients who fulfilled inclusion criteria are described in **Table 1**. The mean (SD; range) AVA was 0.94 (0.3; 0.30-1.50) cm^2 and was less than 1 cm^2 in 861 patients (62.6%) (Table 1).

Outcome During Medical Management

Clinical follow-up information for patients in the 10 centers is shown in eTable 1 in Supplement 1. Echocardiographic data regarding the rate of progression of initially moderate to severe AS were not routinely available, although severe AS was documented in all patients with moderate AS at baseline who underwent AVR during follow-up. The mean (SD; range) follow-up time was 27 (24; 2-224) months. A total of 542 patients (39.4%) required AVR (SAVR, 429 [79.2%]; TAVR, 113 [20.8%]). The 2-year, 4-year, and 8-year overall survival rates for the entire cohort during medical management were 93% (1%), 86% (2%), and 75% (4%), respectively. The cardiovascular death-free survival rates were 96% (1%) at 2 years, 90% (1%) at 4 years, and 83% (3%) at 8 years. Of the 104 deaths during medical management, 57 (54.8%) were from a cardiovascular cause, including 38 from heart failure and 7 from sudden cardiac death. The incidence rate of sudden death was 2.5 cases per 1000 patient-years.

Patients With Severe AS at Entry

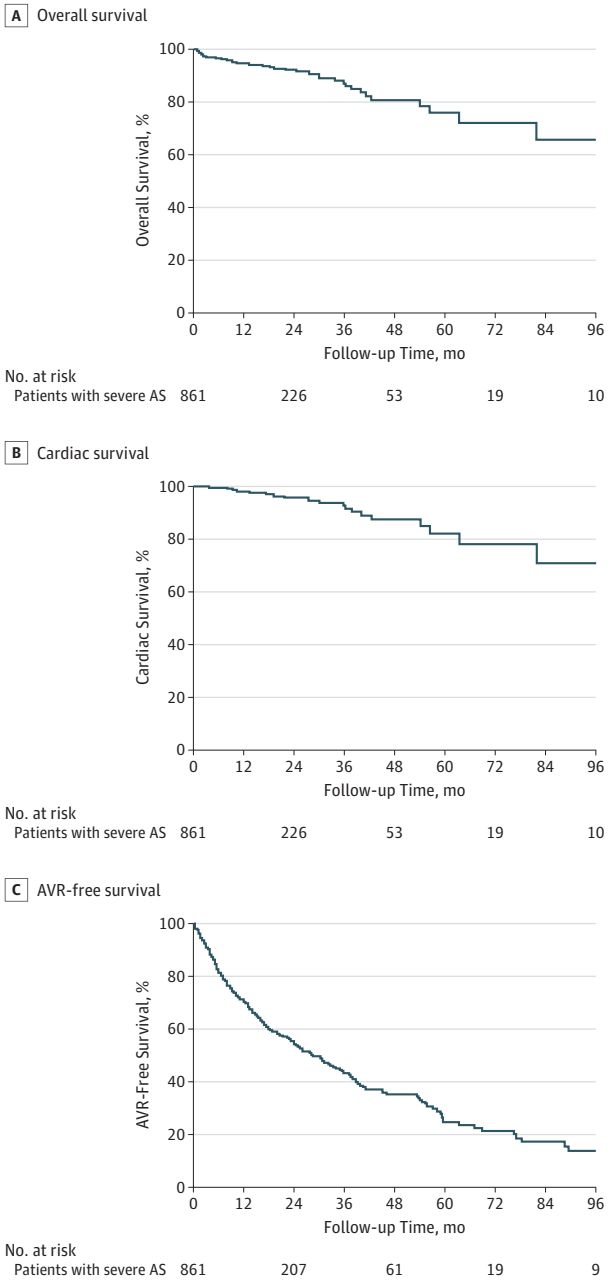
Among the 861 patients with severe AS at entry, the 2-year, 4-year, and 8-year overall survival rates were 92% (1%), 80% (3%), and 65% (8%), respectively (**Figure 1**A); the cardiovascular death-free survival rates at 2 years, 4 years, and 8 years were 96% (1%), 87% (3%), and 71% (9%), respectively (Figure 1B); and the 2-year, 4-year, and 8-year AVR-free survival rates were 54% (2%), 32% (3%), and 12% (3%), respectively (Figure 1C). Of the 64 deaths during medical management in patients with severe AS, 32 (50%) were from a cardiovascular cause, including 23 from heart failure, 4 from sudden cardiac death, 2 from myocardial infarction, 2 from stroke, and 1 from pulmonary embolism.

Aortic valve replacement was performed in 388 of 861 patients with severe AS (45.1%), with SAVR performed in 310 (79.9%) (**Table 2**). Indications for AVR were development of a class I indication in 366 patients (94.3%), a class IIa indication in 18 (4.6%), and a class IIb indication in 4 (1.0%). In these patients, the mean (SD) time between inclusion and AVR was 14.4 (16.6) months, and the median (range) time was 8.7 (0-133) months. Combined coronary artery revascularization was performed in 82 patients (26.5%) at the time of SAVR.

Patients With Moderate AS at Entry

Among the 514 patients with moderate AS at baseline, 154 (30.0%) underwent AVR (SAVR, 110 [71.4%]; TAVR, 44 [28.6%]); 128 patients (83.1%) developed class I indications, 22 (14.3%) developed class IIa indications, and 4 (2.6%) developed class IIb indications. Echocardiography preceding AVR confirmed that the stenosis had progressed to the severe stage (AVA less than 1.0 cm^2) in all patients. Combined coronary artery revascularization was performed in 34 patients at the time of AVR. The mean (SD) time between inclusion and AVR was 29.9 (24.4) months, and the median (range) time was 22.6 (0-98) months. The mean (SD) overall survival rate was 94% (1%) at 2 years, 89% (2%) at 4 years, and 78% (4%) at 8 years follow-up (eFigure 1 in Supplement 1). In these patients with moderate AS at baseline, AVR-free survival rates are provided in eFigure 2 in Supplement 1. Of the 40 deaths during medical management, 25 were cardiovascular in nature, including heart failure in 14 and sudden death in 3. Of note, 2 of 3 patients who died suddenly had confirmed severe AS on echocardiography.

Figure 1. Kaplan-Meier Estimates for Events in Patients With Severe Aortic Stenosis (AS)



Kaplan-Meier analyses of overall survival (A), cardiovascular death–free survival (B), and aortic valve replacement (AVR)–free survival (C) for patients with severe AS at entry to the registry.

Predictors of Outcome

For the entire cohort, age, dyslipidemia, chronic obstructive pulmonary disease, higher systolic blood pressure, peak aortic jet velocity, and LVEF were associated with all-cause

Table 2. Comparison of Survivors vs Nonsurvivors in Patients With Severe Aortic Stenosis at Baseline

Variable	Mean (SD)			P Value
	Survivor (n = 738)	Death Under Medical Treatment (n = 64)	Death After AVR (n = 59)	
Age, y	72 (12)	78 (7)[a]	72 (10)[b]	<.001
Male, No. (%)	425 (57.6)	37 (58)	35 (59)	.97
Height, cm	166 (9)	167 (10)	169 (8)	.11
Weight, kg	73 (16)	73 (15)	73 (12)	.94
Body surface area, m^2	1.81 (0.2)	1.81 (0.2)	1.82 (0.2)	.84
Systolic blood pressure, mm Hg	139 (19)	149 (23)[a]	142 (19)	.001
Diastolic blood pressure, mm Hg	77 (11)	80 (10)	78 (11)	.12
Hypertension, No. (%)	436 (59.1)	43 (67)	27 (46)	.04
Diabetes, No. (%)	119 (16.1)	17 (27)	14 (24)	.06
Smoker, No. (%)	194 (26.3)	24 (37)	17 (29)	.18
Dyslipidemia, No. (%)	381 (51.6)	27 (42)	15 (25)	<.001
Chronic obstructive pulmonary disease, No. (%)	42 (5.7)	9 (14)	5 (9)	.03
β-Blockers, No. (%)	282 (38.2)	26 (41)	24 (41)	.94
Angiotensin-converting enzyme inhibitor, No. (%)	225 (30.5)	25 (39)	20 (34)	.39
LV mass, g/m^2	202 (83)	227 (67)	218 (67)	.06
LVESV, mL	39 (21)	39 (14)	40 (16)	.96
LVEDV, mL	101 (34)	95 (27)	102 (29)	.49
SV index, mL/m^2	42 (11)	41 (11)	42 (11)	.72
LV ejection fraction, %	66 (7)	60 (5)[a]	64 (9)[b]	<.001
Peak aortic velocity, m/s	4.1 (0.7)	4.2 (0.9)	4.4 (0.8)[a]	.001
Mean aortic pressure gradient, mm Hg	43 (16)	42 (17)	49 (18)[a,b]	.02
Aortic valve area, cm^2	0.78 (0.15)	0.77 (0.15)	0.77 (0.16)	.72

Abbreviations: AVR, aortic valve replacement; EDV, end-diastolic volume; ESV, end-systolic volume; LV, left ventricular; SV, stroke volume.

[a] Significant difference with survivors.

[b] Significant difference with death under medical treatment.

Table 3. Multivariable Predictors of Mortality (Aortic Valve Replacement Censored) With Echocardiographic Data as Continuous and Categorical Variables in Patients With Severe Aortic Stenosis at Baseline

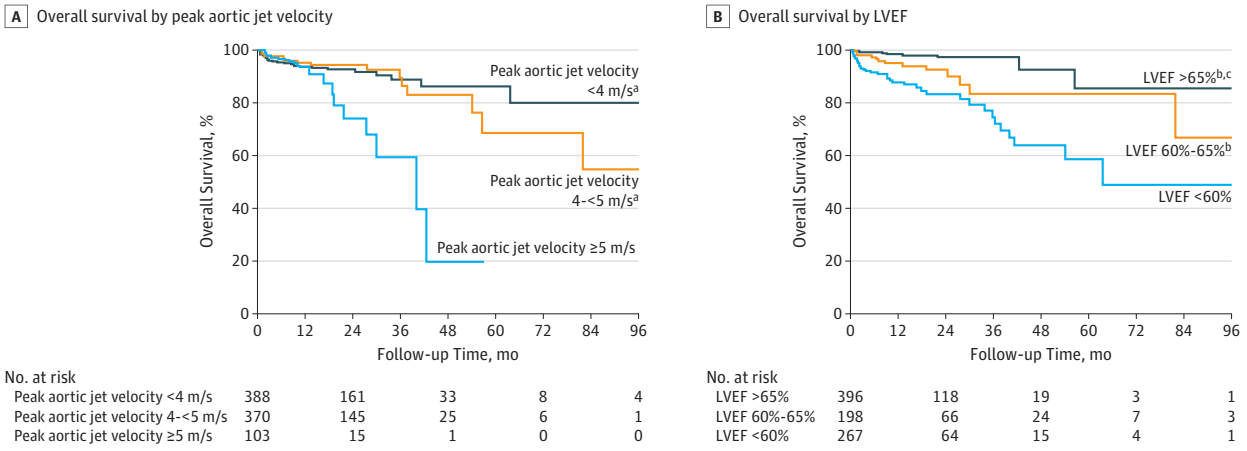
Predictor	All-Cause Mortality		Cardiovascular Mortality	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Continuous Variables				
Age, per 1 y	1.05 (1.02-1.08)	.002	1.05 (1.00-1.10)	.03
Systolic blood pressure, per mm Hg	1.02 (1.01-1.03)	.004	NA	NA
Diabetes	1.34 (0.73-2.44)	.35	2.84 (1.24-6.55)	.01
Dyslipidemia	0.65 (0.38-1.12)	.12	NA	NA
Chronic obstructive pulmonary disease	2.47 (1.14-5.34)	.02	NA	NA
Peak aortic velocity, per 0.1 m/s	1.03 (0.99-1.07)	.11	1.01 (1.03-1.14)	.001
LVEF, per 1%	0.90 (0.86-0.94)	<.001	0.90 (0.85-0.96)	.002
Categorical Variables				
Age, per 1 y	1.05 (1.02-1.09)	.001	1.06 (1.01-1.11)	.02
Systolic blood pressure, per mm Hg	1.02 (1.01-1.03)	.003	NA	NA
Diabetes	1.38 (0.76-2.50)	.29	2.95 (1.26-6.90)	.01
Dyslipidemia	0.58 (0.34-1.00)	.051	NA	NA
Chronic obstructive pulmonary disease	2.56 (1.19-5.48)	.02	NA	NA
Peak aortic velocity ≥5 m/s	2.05 (1.01-4.16)	.046	6.31 (2.51-15.9)	<.001
LVEF <60%	5.01 (2.93-8.57)	<.001	4.47 (2.06-9.70)	<.001

Abbreviations: HR, hazard ratio; LVEF, left ventricular ejection fraction; NA, not applicable.

mortality. Age, peak aortic jet velocity, and LVEF were also associated with cardiovascular death (eTable 2 in Supplement 1).

In patients with severe AS (**Table 3**), echocardiographic determinants of all-cause mortality identified in the multivariable analysis were peak aortic jet velocity greater than 5 m/s and LVEF. Independent determinants of cardiovascular mortality were age, diabetes, peak aortic jet velocity greater than 5 m/s, and LVEF. When peak aortic jet velocity and LVEF

Figure 2. Kaplan-Meier Estimates for Events in Patients With Severe Aortic Stenosis According to Left Ventricular Ejection Fraction (LVEF) and Peak Aortic Jet Velocity



Kaplan-Meier analyses of overall survival in patients with severe aortic stenosis at baseline as a function of peak aortic jet velocity (A) and LVEF (B).

[a] Significant difference with peak aortic jet velocity of 5 m/s or greater.

[b] Significant difference with LVEF less than 60%.

[c] Significant difference with LVEF of 60% to 65%.

were taken as continuous variables, both were independently associated with cardiovascular mortality (Table 3). Using receiver operating characteristic curve analysis, the best cutoff values regarding the prediction of overall death were 59.6% for LVEF (AUC, 0.73; sensitivity, 81%; specificity, 56%) and 4.7 m/s for peak aortic jet velocity (AUC, 0.50; sensitivity, 30%; specificity, 80%). The AUCs for LVEF and peak aortic jet velocity were 0.68 and 0.59, respectively, for the prediction of cardiovascular death. Of note, there was a graded association of reduced survival with increased peak aortic jet velocity and with decreased LVEF (**Figure 2**). No EF threshold higher than 65% further affected survival. For peak aortic jet velocity, no additional prognostic information was obtained for velocities between 4 and 5 m/s. Similar data were obtained for AVA (eTable 3 and eFigure 3 in Supplement 1) for both total and cardiovascular mortality. In patients with initially moderate AS, the best cutoffs associated with the outcomes were 64% for LVEF and 3.0 m/s for peak aortic jet velocity (eTable 4 in Supplement 1).

Post-AVR Outcomes

Thirty-day mortality following AVR was very low (n = 13 [0.9%]; SAVR, 7; TAVR, 6). During follow-up, a total of 69 patients who underwent AVR died (SAVR, 49; TAVR, 20), including 22 from a cardiovascular cause, of which 17 were from heart failure and 2 were from sudden death. The mean (SD) 2-year, 4-year, and 6-year postprocedural overall survival rates were 83% (2%), 75% (4%), and 68% (6%), respectively. Patients with severe AS at baseline and peak aortic velocity greater than 5 m/s had significantly lower mean (SD) postoperative survival rates than those with peak aortic velocity less than 5 m/s (2 years: 73% [8%] vs 84% [2%]; 4 years: 65% [10%] vs 78% [4%]; 6 years: 54% [13%] vs 70% [6%]; $P = .03$). Similarly, patients with severe AS at entry with reduced baseline LVEF less than 60% also had lower mean (SD) postoperative survival rates than those with baseline LVEF of 60% or greater (2 years: 67% [7%] vs 87% [5%]; 4 years: 63% [8%] vs 78% [4%]; 6 years: 63% [8%] vs 69% [7%]; $P = .02$). In multivariable analysis, age (hazard ratio [HR], 1.03; 95% CI, 1.01-1.06; $P = .003$), diabetes (HR, 2.62; 95% CI, 1.90-4.95; $P = .003$), dyslipidemia (HR, 0.2; 95% CI, 0.10-0.37; $P < .001$), and peak aortic velocity greater than 5 m/s (HR, 2.20; 95% CI, 1.16-4.18; $P = .02$) were independently associated with postoperative survival. Of note, LVEF less than 60% was not associated with reduced postoperative survival in multivariable analysis.

Discussion

The management of patients with asymptomatic AS has continued to challenge clinicians.[6,21] A randomized clinical trial (Evaluation of Transcatheter Aortic Valve Replacement Compared to Surveillance for Patients With Asymptomatic Severe Aortic Stenosis; NCT03042104) has been initiated to compare outcomes of asymptomatic patients with severe AS who are randomized to transfemoral TAVR vs clinical and echocardiographic follow-up (ie, active surveillance). To our knowledge, a randomized surgical trial has not been performed, and current practice patterns vary widely. In the present registry, for patients with asymptomatic moderate or severe AS and preserved LVEF greater than 50% at baseline followed up in heart valve clinics over the intermediate term, the mean 2-year and 4-year overall survival rates under medical management were 93% and 86%, respectively. The crude rate of sudden death over the follow-up interval was low (0.65%) and represented approximately one-tenth of all cardiovascular deaths.

In patients with severe AS at entry, age, systolic blood pressure level, comorbidities (eg, chronic obstructive pulmonary disease), peak aortic jet velocity greater than 5 m/s, and LVEF less than 60% were associated with all-cause mortality.

Age, peak aortic jet velocity of 5 m/s or greater, and LVEF less than 60% were also independently associated with cardiovascular death.

During follow-up, 34% of patients required AVR, and this rate rose to 59% at 4 years. Most AVRs were dictated by a class I indication (ie, symptom development), and most cardiovascular deaths were related to heart failure. The 30-day mortality following AVR in this series was very low (0.9%). After AVR, the negative effect of peak aortic jet velocity remained significant, while LVEF less than 60% was no longer associated with cardiovascular death. Interestingly, in patients with moderate AS at entry who progressed to severe AS and were referred for AVR, the baseline variables predicting worse outcomes were directionally similar (peak aortic jet velocity of 3.0 m/s or greater and LVEF less than 60%). Two of 3 patients with moderate AS at entry who had sudden cardiac death during follow-up had confirmed severe AS on surveillance echocardiography.

Approximately one-half of patients diagnosed with moderate or severe AS do not report symptoms.[8,15] The clinically silent phase of severe AS is associated with a risk of sudden death ranging from 0.25% to 1.7% per year.[18,19,25] Given the current low periprocedural mortality rates for SAVR and transfemoral TAVR, earlier intervention has been advocated, and to our knowledge, the current strategy of watchful waiting has not been examined in a large cohort of patients with asymptomatic moderate or severe AS monitored in specialized heart valve clinics. Delay in reporting symptoms is common in patients with AS.[12] Considering an annual mortality rate of approximately 30% for patients with severe AS, once symptoms develop, early recognition of symptoms and timely referral to intervention are critical.[3,4] It has been shown that when patients are regularly followed up within a heart valve clinic program, symptoms are recognized at an earlier and less severe stage, thus optimizing timing of AVR.[12,26] Compared with previous studies, the low rate of sudden death, the good overall midterm survival rates, and the very low rate of 30-day mortality following AVR observed in the HAVEC registry likely reflect appropriate monitoring, planning, and high adherence to guidelines.[13-18] However, our data highlight the need for additional efforts with probably closer follow-up in these patients, since the occurrence of overt heart failure remains a significant problem even in heart valve centers of excellence.

Comorbidities are frequent in elderly individuals with AS, and AS increases the mortality from myocardial infarction, stroke, trauma, or emergency noncardiac surgery.[27-31] The HAVEC registry data highlighted that age and chronic obstructive pulmonary disease significantly worsen patients' prognosis. Age has not been consistently reported as an outcome predictor in the literature. However, many older adults with severe AS are not candidates for surgical AVR because of high surgical risk, advanced age, frailty, or comorbid conditions.[32] Some complications after transcatheter AVR (eg, vascular injuries) are more common in very elderly patients.[3,4]

Although supportive data are limited, an LVEF less than 50% is considered the appropriate threshold for defining LV systolic dysfunction in AS.[5,6,33] In the HAVEC registry, patients with EF between 50% and 59% had less favorable outcomes and experienced more heart failure-related deaths than those with EF greater than 60%. These data reinforce observations from previous retrospective studies[33,34] and provide support for adjusting the cutoff for LVEF (less than 60% instead of less than 50%) to define dysfunction and consider AVR in asymptomatic severe AS.

Despite limited evidence with a class IIa indication, asymptomatic patients with very severe AS (peak aortic jet velocity greater than 5 to 5.5 m/s) are often referred for AVR.[5,6,14] Peak aortic jet velocity is recorded directly with the use of continuous Doppler interrogation and, unlike AVA, does not require calculations and has high reproducibility. Peak aortic jet velocity is a robust prognostic parameter in AS, with increasingly worse outcome from patients with mild to very severe (greater than 5 m/s) stenosis.[14,15,19] This gradual effect of stenosis severity was challenged in a 2015 large multicenter retrospective Japanese study.[10] However, the main limitations of this study were the inclusion of patients with LVEF less than 50% and the absence of standardized follow-up and treatment strategy. By contrast, the HAVEC registry confirmed previous observations regarding stenosis severity in a very large population of patients with asymptomatic AS evaluated and monitored in heart valve clinics. In fact, very severe obstruction (peak aortic jet velocity of 5 m/s or greater) was predictive of all-cause mortality and cardiovascular death regardless of treatment strategy in asymptomatic patients with AS.[14] Although AVA encompassed a broad range of values from 0.3 to 1.50 cm^2, it was also associated with outcomes in these patients (eFigure 3 in Supplement 1). An AVA less than 0.8 cm^2 was associated with markedly increased risk of all-cause and cardiovascular mortality.

Limitations

Our study had limitations. Data from a centralized database (clinical and echocardiographic data at baseline and clinical data at follow-up) were obtained from each center. However, because of incomplete echocardiographic data at baseline and/or during follow-up, a total of 22% of the initially included patients were not included in the final study analysis. Follow-up echocardiographic data were also collected from all patients who underwent AVR to confirm the progression from moderate to severe AS (ie, AVA greater than 1 cm^2). However, in the context of this study, we did not collect the echocardiographic parameters of AS severity and LV function at follow-up visits. This precluded the analysis of the rate of progression from moderate to severe AS. Although exercise testing was commonly performed (572 patients), some patients were considered asymptomatic based solely on questionnaire on symptom status (not available in all centers). The assessment of myocardial strain, which could identify patients with subclinical LV dysfunction,[20] was not systematically performed. The reasons for which symptomatic patients died under medical management could not be ascertained.

Conclusions

This study shows that asymptomatic patients with severe AS followed up in heart valve clinics have a low risk of sudden death and good midterm survival. Asymptomatic patients with very severe AS (peak aortic jet velocity of

5 m/s or greater) or with LVEF less than 60% have higher all-cause and cardiovascular mortality even after successful AVR. These findings provide support for consideration of early elective AVR in these patients. Closer and more frequent (every 6 to 12 months) clinical and echocardiographic follow-up might be implemented in patients with moderate AS and a peak aortic jet velocity of 3.0 m/s or greater or LVEF less than 60%.

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Continuing Cardiology Education

REVIEW ARTICLE

Exercise echocardiography in valve disease

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Abstract

Evidence supporting the use of exercise echocardiography to identify the true hemodynamic consequences of valvular heart disease (VHD) is progressively accumulating. From a clinical standpoint, the evaluation of VHD limited to resting conditions often underestimates the full clinical impact of the lesion. Exercise echocardiography has proved to be an important clinical tool in the risk stratification and the decision making of patients with VHD. It is very useful in case of discrepancy between symptoms and severity of valve lesion. Moreover, the evaluation of dynamic components of VHD, ventricular function, and exercise capacity provides clinician additive prognostic value that can be really helpful in the management planning of these patients.

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Exercise echocardiography is a key exam in the evaluation of symptoms and hemodynamic consequences of valvular heart disease (VHD). The importance of this technique is related to the fact that valve disease assessment at rest can miss some dynamic components that are influenced by loading conditions, heart rate, blood pressure, or myocardial dysfunction. Moreover, stress evaluation reflects more accurately the hemodynamics of the valve during patient's daily activity, thus improving patients' risk stratification and helping in clinical decision making, especially in circumstances of discrepancy between symptoms and echocardiographic parameters. Evidence accumulated over the last decade has led to the incorporation of stress echocardiography into the current guidelines. The aim of this review is to sum up the role of the exercise echocardiographic test in the evaluation and management of patients with VHD.

Exercise Protocols

Exercise echocardiography can be performed using either a treadmill or bicycle ergometer, with a symptom-limited protocol in which the workload is gradually increased in stages. The most common treadmill protocol used is Bruce protocol, in which the expected exercise level for a given age and sex can be expressed as functional aerobic capacity. The modified Bruce protocol has two warm-up stages, each lasting 3 min. The first is at 1.7 mph and a 0% inclination, and the second is at 1.7 mph and a 5%. A disadvantage of the treadmill use is that scanning during exercise is not feasible, thus imaging is performed at rest and immediately after completion of exercise. This problem is overcome with bicycle stress echocardiography, which can be performed with either supine or upright ergometer and has the advantage of allowing continuous assessment of many echocardiographic parameters during exercise, not only after peak exercise.

Usually, semisupine bicycle exercise is preferred in the evaluation of VHD because it is technically easier than upright bicycle or treadmill exercise and allows the assessment of multiple stress parameters during each step of exercise testing. The patient pedals at a constant cadence against an increasing workload, which starts at 25 watts

(50 watts for younger patients) with increments of 25 watts every 2 or 3 min [1]. The main disadvantage of semisupine bicycle exercise is that the patient has to have a certain amount of training to be able to reach the maximum workload or heart rate. Appearance of symptoms should be assessed regularly, and blood pressure, heart rate and 12-lead electrocardiography should be monitored continuously during the examination. Criteria for interruption are the target heart rate achievement, or onset of typical chest pain, ≥2 mm horizontal or downsloping ST segment depression, limiting breathlessness, dizziness, hypotension (systolic blood pressure drop below baseline), complex ventricular arrhythmia, and muscular exhaustion. During each step of the exercise test, at peak and early phases of the recovery period, valvular and subvalvular gradients, regurgitant flows, and hemodynamic consequences such as pulmonary artery pressure should be assessed. Relevant is also the evaluation of left ventricle (LV) global and regional function.

Indication for Stress Test in Native Valve Disease

The purpose of the stress echocardiography is to discriminate if patients are candidate to an intervention or if they can be closely followed up. In particular, it can guide the decision making in two specific circumstances: (1) nonsevere valve disease in "symptomatic" patients; (2) severe valve disease in asymptomatic patients. In the first case, patients with exertional breathlessness, chest pain, or unexplained acute pulmonary edema with an apparent non-critical valve disease at rest require revaluation of valve disease severity based on flow-dependent changes (dynamic component assessment with exercise), but also investigation of coexistence abnormalities, such as inducible ischemia. In such patients, exercise stress echocardiography is very helpful. The European Society of Cardiology/European Association of Cardiothoracic Surgery (ESC/EACTS) valve disease management guidelines [2] recommend stress echocardiography and intervention in patients with abnormal exercise stress tests response and severe aortic stenosis (AS) or significant mitral stenosis (MS) (Class I). On the other hand, exercise testing can be used to unmask symptoms such as limiting dyspnea, dizziness, syncope or near syncope, especially in elderly patients that reduce their daily activity level as a mechanism to avoid symptoms. The ESC/EACTS guidelines recommend exercise testing and intervention in cases of demonstrated symptoms related to AS, MS and mitral regurgitation (MR) (Class I) [2]. In addition, only exercise echocardiography provides information regarding dynamic systolic pulmonary artery pressure (SPAP) changes, which represent an important predictor of worse prognosis in patients with valve disease [3].

Aortic Stenosis

AS is a highly prevalent disease in developed country, especially among elderly. According to the ESC/EACTS guidelines, the onset of symptoms and/or LV systolic dysfunction in patients with severe AS is a clear indication for aortic valve replacement (AVR) (Class I), since once symptom develop mortality rate goes from 3% within 6 months, until 50% over 2 years [2]. The risk of sudden death in asymptomatic patients with severe AS is considered to be low (<1% per year), which does not justify recommending early prophylactic surgery in such patients (in which case operative mortality rate is usually >1%). However, it has been shown that during an exercise test, approximately one-third of patients exhibit exercise-limiting symptoms; these patients also have worse outcomes [4, 5]. Hence, the role of the exercise testing in the identification of symptomatic patients with severe AS that could benefit from an AVR has been strongly recommended by the ESC/EACTS guideline (Class I) [2].

Beside its important role in unmasking symptoms, exercise Doppler echocardiographic findings have also demonstrated to provide incremental prognostic value over resting echocardiographic and exercise electrocardiographic parameters [6, 7]. An increase in mean transaortic gradient (MG) by >20 mmHg has been associated with an increased risk of cardiac-related events. This increase in MG most likely reflects the presence of a very calcified, non-compliant valve, unable to increase its opening area during exercise, thus a more severe AS [6] (Figure 1). Hence, a MG rise >20 mmHg in patients with asymptomatic severe AS is indicative of a "high risk" patient and has been retained as an indication for AVR in the ESC Guidelines (Class IIb) [2]. Other determinants of abnormal response to exercise stress testing and also indicators of poor prognosis are an abnormal blood pressure response during exercise (Systolic blood pressure drop during exercise is a Class IIa indication for AVR) and an inadequate increase in ejection fraction with exercise. However, due to limited available data, (mainly small observational studies) exercise-induced LV systolic dysfunction has not been included in the current guidelines as a parameter that may be used to indicate the need for aortic valve surgery. Exercise-induced left ventricular (LV) dysfunction—decrease or no change in LV ejection fraction (EF)—may be explained by afterload mismatch and/or exercise-exhausted coronary flow reserve leading to subendocardial ischemia and/or more extensive myocardial fibrosis [8]. Interestingly, the evaluation of LV longitudinal function using two-dimensional speckle tracking analysis, that is global longitudinal strain (GLS) analysis, could be more sensitive to detect the early form of myocardial dysfunction and to predict outcome in AS

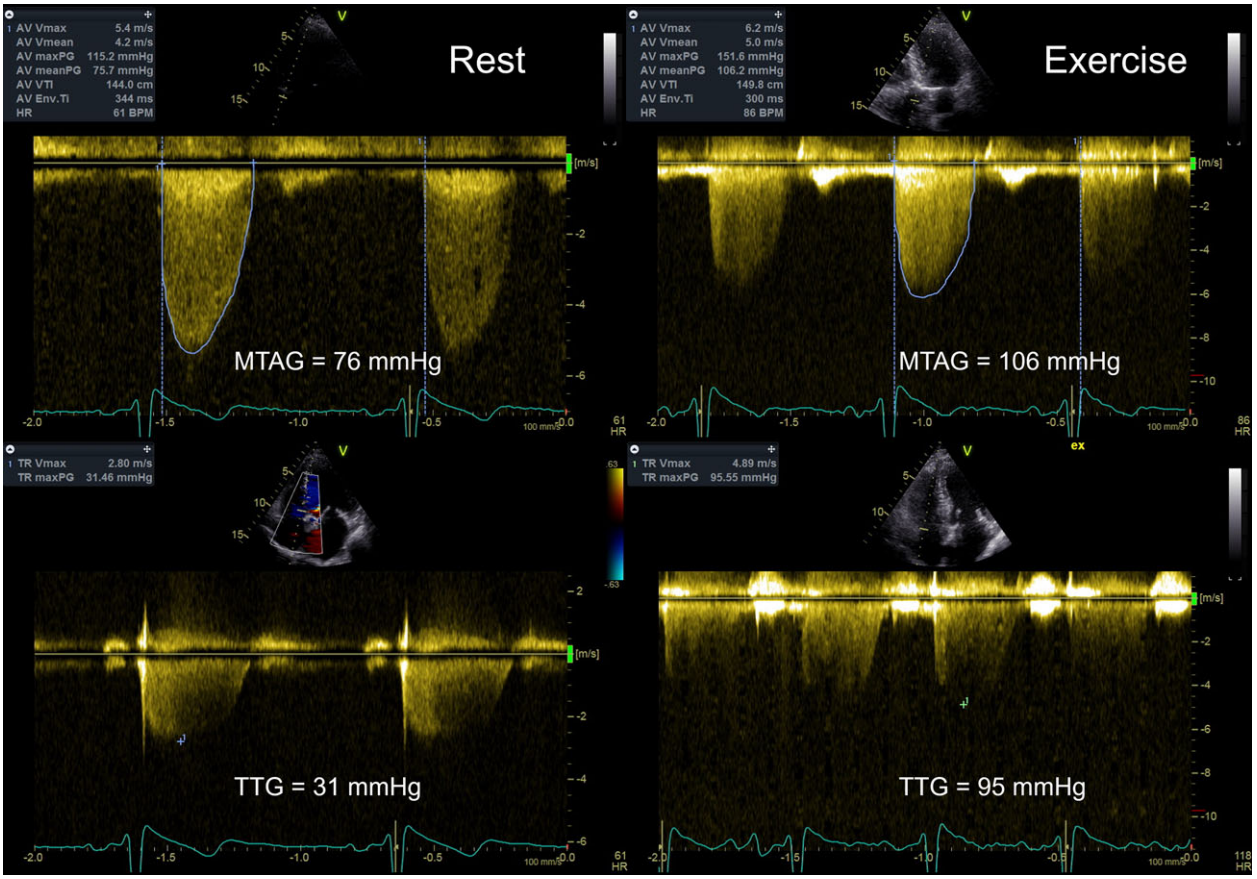

Figure 1. Exercise echocardiography in aortic stenosis. Doppler assessment of an asymptomatic patient with severe aortic stenosis at rest (left panels). At exercise (right panels), significant increase of mean transaortic gradient and development of systolic pulmonary hypertension. AV, aortic valve; AV Env.Ti, aortic time interval; HR, heart rate; maxPG, maximal pressure gradient; meanPG, mean pressure gradient; MG, mean gradient; TR, tricuspid regurgitation; TTG, transtricuspid gradient; V_{max}, maximal velocity; *V*mean, mean velocity; VTI, velocity time integral.

[9]. In particular, patients with abnormal exercise response seem to have lower GLS values at rest [9] and after exercise [10], than those with normal exercise test. Exercise-induced pulmonary hypertension (PHT), defined as a systolic pulmonary artery pressure (SPAP) >60 mmHg, is another recognized independent predictor of cardiac events and reduced survival [11, 12]. To date, in spite of their prognostic role from several observational studies, exercise-induced echocardiographic changes, such as limited LV contractile reserve (either assessed by changes in LVEF or changes in GLS with exercise) or PHT are not yet accepted indications for AVR in asymptomatic patients with AS. This is mainly due to the small number of patients included in the studies, to the fact that most of the studies come from well-known tertiary centers with high experience in exercise stress echocardiography, which deems widespread clinical implementation doubtful. In our experience, exercise stress echocardiography is of real clinical value in risk stratification of asymptomatic patients with severe AS and centers should be encouraged to perform the test so that more evidence to be gathered to support the role of exercise stress echocardiography in the management of asymptomatic severe AS patients.

Aortic Regurgitation

In symptomatic patients with severe aortic regurgitation (AR), current guidelines recommend promptly AVR, given the high mortality implied once symptoms occurred (Class I) [2]. Thus, exercise testing appears to be really useful to uncover symptoms in subjects with severe AR who report being asymptomatic or, otherwise, in patients with non-severe AR, to confirm equivocal symptoms and reveal other causes, such as dynamic MR, diastolic dysfunction, or PH [13]. Exercise stress echocardiography has no proven value to assess AR severity, because AR is reduced with increasing heart rate, but proved to be very useful for the assessment of the long-term hemodynamic consequences of the AR on the LV through the evaluation

of LV contractile reserve. Some studies have indeed demonstrated that the lack of contractile reserve (<5% increase in LVEF) better predicts LV systolic dysfunction development and lower survival at follow-up or after AVR than resting indices of LV function [14, 15]. Also longitudinal function assessment with tissue Doppler imaging at rest and during exercise may reveal early signs of LV systolic dysfunction [16]. Vinereanu et al. have, in fact, correlated reduced LV long axis contraction, assessed by medial mitral annulus systolic excursion and peak systolic velocity, with poor exercise responses in asymptomatic patients with severe aortic regurgitation [16]. Thus, although exercise stress echography is not recommended for the routine management of AR, the assessment of LV contractile reserve and the identification of subclinical LV dysfunction could improve significantly the timing of aortic valve surgery, especially in patients with borderline values of LVEF or end-systolic values.

Mitral Stenosis

A non-compliant mitral valve may be moderately stenotic at rest but hemodynamically severely stenotic during stress, as it fails to open further to accommodate the exercise-induced increase in flow. In addition, because indexed valve area thresholds are not defined, stress echocardiography may be useful for grading MS in patients with a large body surface area [17]. A mean transvalvular gradient of >15 mmHg with exercise or >18 mmHg during dobutamine infusion have been associated with worse outcomes in asymptomatic patients with MS. In addition, SPAP >60 mmHg on exertion [18], especially when it occurs at low-level exercise [19] is suggestive of a hemodynamically significant MS, at higher risk of hemodynamic decompensation, which may benefit from percutaneous valvotomy if anatomy is suitable. (Figure 2). According to the ESC guidelines, in patients with

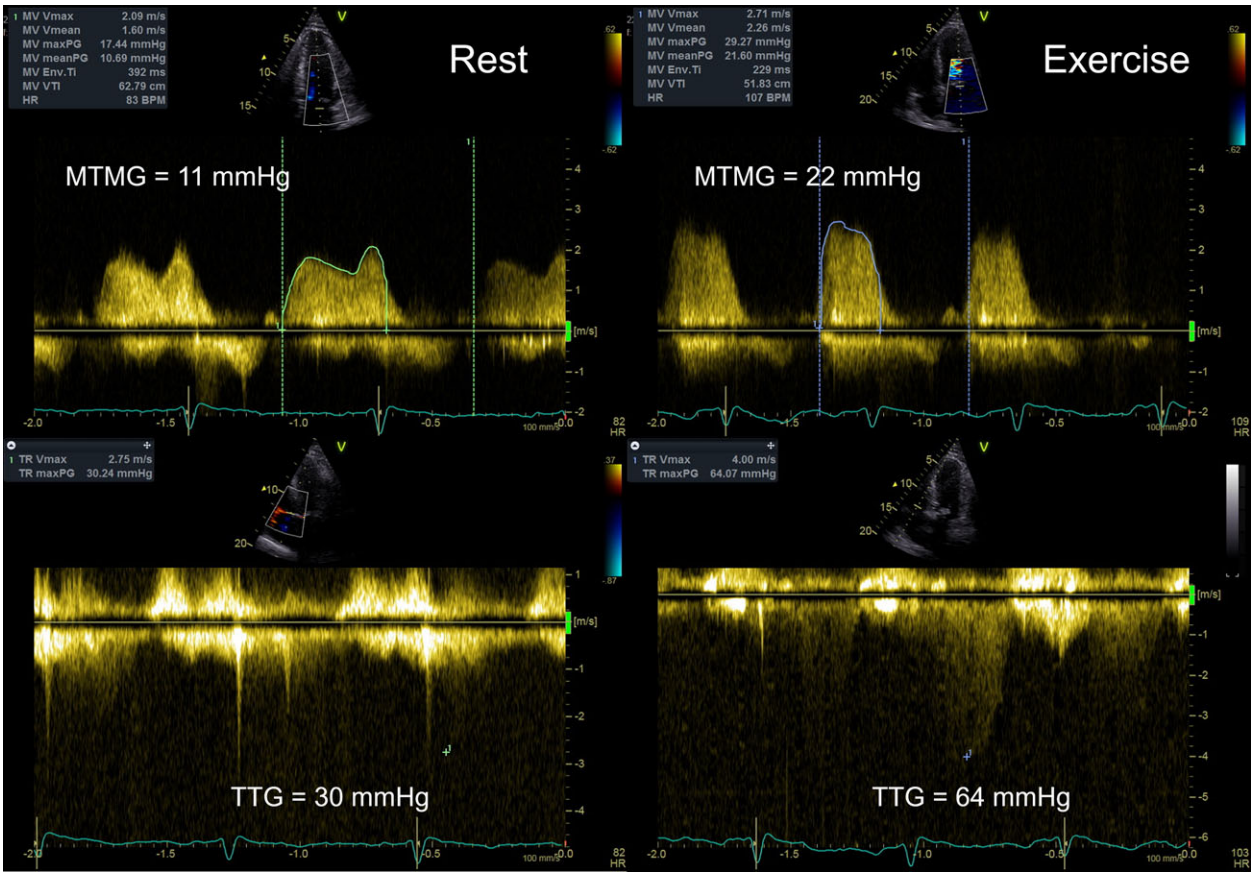

Figure 2. Exercise echocardiography in mitral stenosis. Doppler measurement of transmitral (upper panels) and transtricuspid pressure gradient (lower panels) in an asymptomatic patient with severe mitral stenosis. The stress test demonstrated a significant exercise-induced increasing of MG and SPAP (right panels), which represent important predictors of risk. HR, heart rate; maxPG, maximal pressure gradient; meanPG, mean pressure gradient; MG, mean gradient; MV, mitral valve; SPAP, systolic pulmonary artery pressure; TR, tricuspid regurgitation; TTG, transtricuspid gradient; V_{max}, maximal velocity; V_{mean}, mean velocity; VTI, velocity time integral.

moderate but significant MS (valve area <1.5 cm^2 but >1 $cm^{2)}$, stress echocardiography is indicated before major non-cardiac surgery or pregnancy planning with the aim of identifying these high-risk patients for acute hemodynamic decompensation [2]. During routine surveillance, stress echocardiography can be considered in

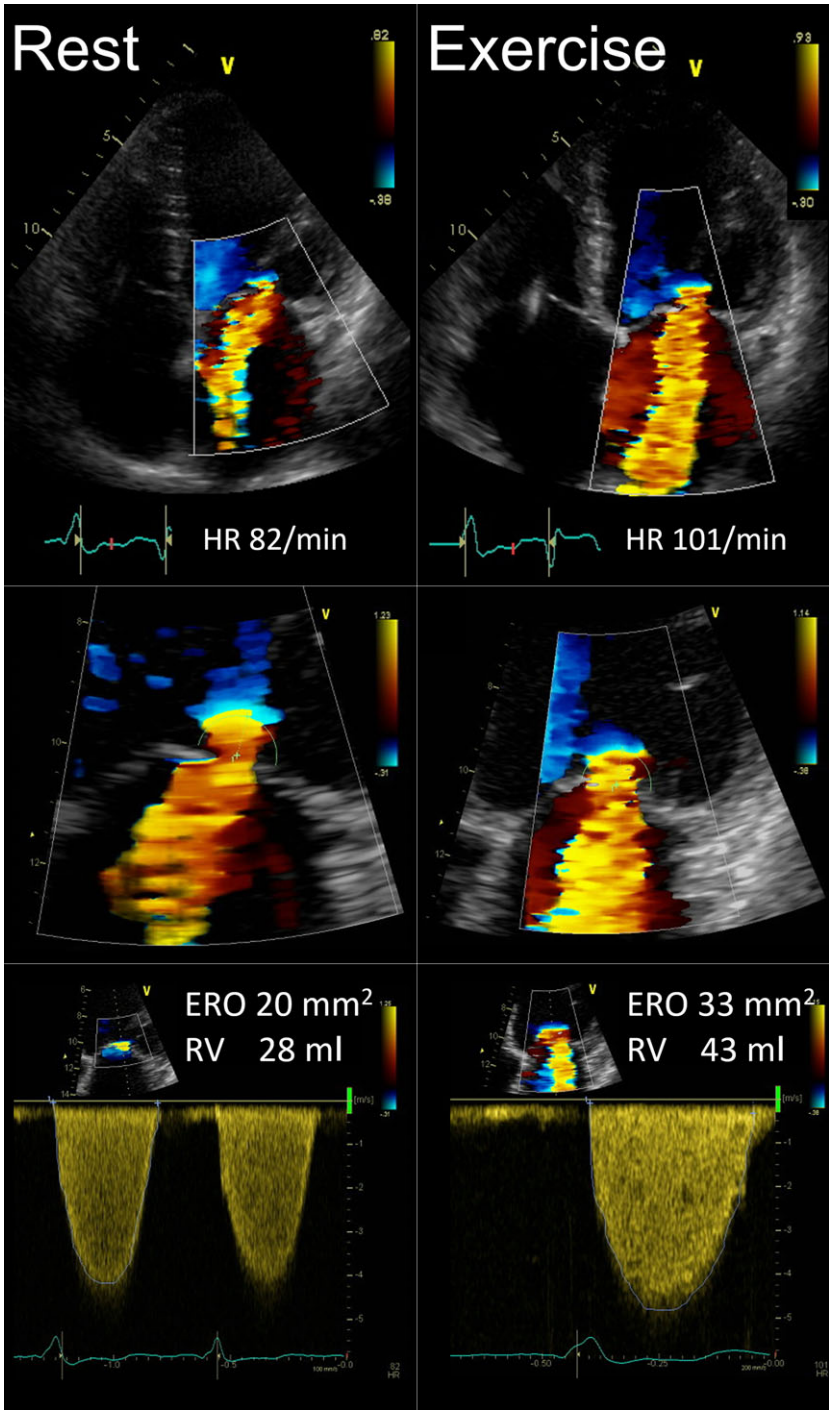

Figure 3. Exercise echocardiography in mitral regurgitation. Apical 4-chamber views of color-flow Doppler, proximal flow-convergence region and Doppler measurement in a patient with secondary mitral regurgitation at rest (left panels) and at exercise (right panels). The acute increase in MR during exercise (increase in ERO ≥13 mm^2) is predictor of worse prognosis. ERO, effective regurgitant orifice; HR, heart rate; R Vol, regurgitant volume.

asymptomatic severe MS with a valve area <1 cm^2 if the valve is unsuitable for valvotomy.

Mitral Regurgitation

MR is classified as primary (organic/structural) or secondary (functional/non-structural). The clinical value of exercise echocardiography has been extensively demonstrated in patients with MR. Exacerbation of MR severity (≥1 grade), exercise-induced PH, impaired LV contractile reserve, inducible ischemia, dynamic LV dyssynchrony, and altered exercise capacity, together with the development of symptoms during exercise echocardiography, provide the clinician clear prognostic information, therefore enabling a more accurate definition of the optimal timing of intervention. In asymptomatic patients with ≥moderate primary MR, exercise stress echocardiography may reveal dynamic MR in approximately one-third of cases [20]. Dynamic MR is often associated with exercise-induced pulmonary hypertension because of the close relationship between the increase in MR and increase in SPAP. In the ESC/EACTS guideline, exercise pulmonary hypertension (SPAP ≥60 mmHg) is now considered as an indication for mitral valve repair in asymptomatic patients without LV dysfunction/dilation (Class IIb) [2]. In addition, because the lack of LV contractile reserve is independently associated with reduced cardiac event-free survival [20], elective surgery could also be contemplated in asymptomatic patients with severe MR.

Rarely, exercise-induced increase in MR occurs as a consequence of acute transient ischemia. This type of dynamic MR is often clinically revealed by a flash pulmonary edema and may be easily identified using exercise stress echocardiography. Identification of a wall motion abnormality during exercise accompanied by an increase in MR severity should trigger patient assessment with coronary angiography to demonstrate the presence of a significant right or circumflex coronary artery stenosis. In such a scenario, myocardial revascularization alone may solve exercise-induced increase in MR severity, without any need for mitral valvuloplasty. In chronic secondary MR, although there is a correlation between the rise in MR during exercise and the increase in SPAP, the degree of MR at rest is unrelated to the magnitude of MR changes during exercise [21, 22]. Dynamic MR is strongly related to exercise-induced changes in mitral valve configuration at both ends of the tethered leaflets and to intermittent changes in LV synchronicity. Characteristically, dynamic secondary MR occurs independently of detectable myocardial ischemia. The increase in MR is more pronounced in patients with exercise-limiting dyspnea and in those hospitalized for acute pulmonary edema, and the acute increase in MR during exercise (increase in ERO ≥13 mm^2) (Figure 3) independently predicts cardiac death and heart failure admission [23]. Considering the adverse prognostic implications of dynamic exercise MR in patients with moderate secondary MR, the development of dyspnea secondary to increased severity of MR and PHT (SPAP ≥60 mmHg) during exercise echocardiography is considered as a further incentive to perform a combined mitral valve repair at the time of surgical coronary revascularization (Class IIa).

Prosthetic Valves

Patients with prosthetic valves have usually higher transvalvular pressure gradient than native valve, mainly related to the type and dimension of the prosthesis implanted. Actually, a pathological increase in transprosthetic gradient can be related to a prosthetic-patient mismatch (PPM) or an acquired stenosis, caused by a pannus overgrowth, thrombus formation or leaflet calcification [24]. Exercise stress echocardiography is really useful in patients with exertional symptoms to diagnose hemodynamically significant prosthetic obstruction or PPM [13]. Indeed during exercise, a stenotic prosthetic valve or PPM is associated with a marked increase in transprosthetic gradient, pulmonary arterial hypertension and reproduction of symptoms, while a normal functioned prosthetic valve have minimal increase in gradient [25, 26]. In the presence of aortic prosthetic valves, an increase in mean gradient >20 mmHg is suggestive for obstruction, together with a calculated functional valve area failure to rise [25, 27]. In patients with prosthetic mitral valve, a mean gradient rise >12 mmHg or exertion-induced SPAP increase to >60 mmHg are suggestive of significant stenosis [13, 26].

Conclusions

Exercise echocardiography proved to be an important clinical tool in the risk stratification and the decision making of patients with VHD. It is very useful in case of discrepancy between symptoms and severity of valve lesion. Moreover, the evaluation of dynamic components of VHD, ventricular function and exercise capacity provide clinician additive prognostic value that can be really helpful in the management planning of these patients. However, prospective large-scale or randomized clinical trials are needed to validate the improved outcomes in stress imaging-guided decision making.

Conflict of Interest

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Epicardial Adipose Tissue and Myocardial Fibrosis in Aortic Stenosis Relationship With Symptoms and Outcomes

A Study Using Cardiac Magnetic Resonance Imaging

The onset of symptoms is a critical point in the natural history of aortic stenosis (AS) that often occurs late in the disease course, and it represents the cardinal indication for aortic valve replacement (AVR) (1). To date, the clinical outcome significance of myocardial replacement fibrosis in asymptomatic AS remains controversial (2,3). Other local and systemic factors might contribute to the severity of left ventricular (LV) remodeling, symptoms, and the outcome of the patient. Among them, epicardial adipose tissue (EAT) represents an inflammatory visceral fat depot confined within the pericardium that might unfavorably affect the heart through paracrine or vasocrine actions.

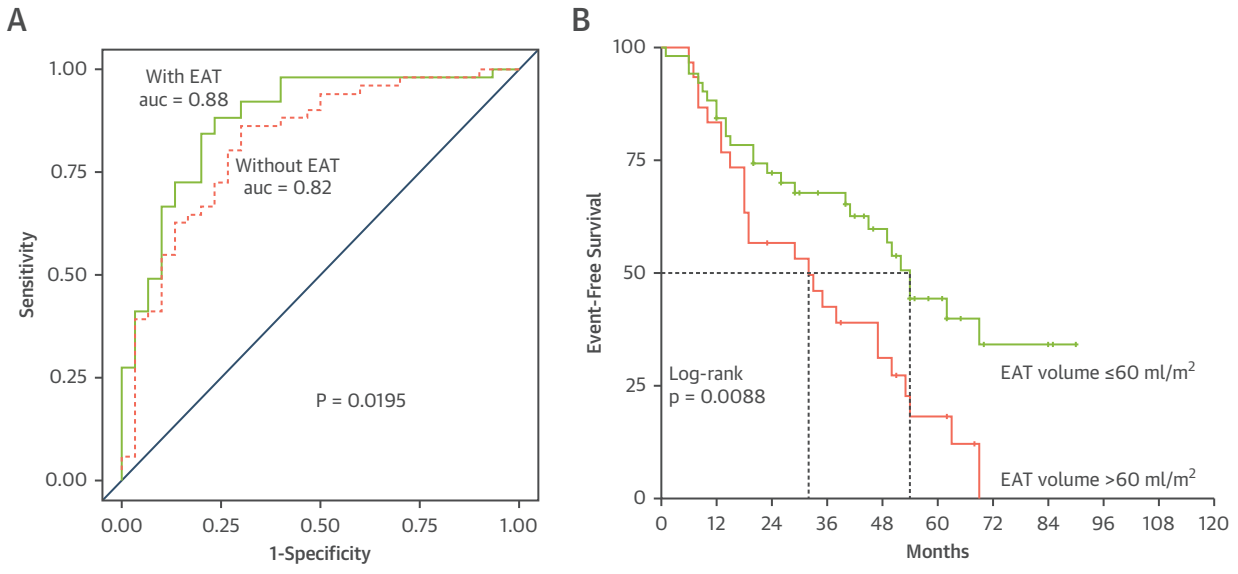
In this single-center study, we sought to investigate the respective contribution of EAT and late gadolinium-enhancement fibrosis, quantified by cardiac magnetic resonance (CMR), to ascertain the symptomatic status and outcome of patients with AS.

A total of 118 patients with moderate or severe AS (mean aortic valve area: 0.9 ± 0.3 cm^2) were enrolled between March 2008 and October 2016. Mean age was 71 ± 13 years. At baseline, 81 patients were asymptomatic, and 37 presented with symptoms. Mean fibrosis was 7.5 ± 5.0% (percentage of the LV mass), and mean EAT volume was 97.4 ± 67.3 ml. CMR parameters were indexed to body surface area.

In multivariable logistic regression, after adjustment for sex, creatinine, atrial fibrillation, coronary artery disease, LV ejection fraction, and blood pressure, the aortic mean pressure gradient ($p = 0.014$), brain natriuretic peptide (BNP) levels ($p = 0.001$), body mass index ($p = 0.032$), and the LV fibrosis index ($p = 0.043$) emerged as independent cofactors associated with symptoms. Linear regression identified valvulo-arterial impedance ($p < 0.0001$), waist circumference ($p = 0.0002$), and low-density lipoprotein cholesterol ($p = 0.014$) as independent predictors of fibrosis ($r^2 = 0.45$).

After a median follow-up of 34 months (interquartile range: 18 to 54 months), 51 asymptomatic patients experienced events, including 6 cardiovascular deaths (2 after AVR) and 45 patients who needed AVR, which was driven by symptom onset. EAT volume was higher in asymptomatic patients who developed symptoms ($p = 0.015$). In multivariable Cox regression, aortic valve area ($p = 0.007$), relative wall thickness ($p = 0.008$), triglycerides ($p = 0.02$), creatinine ($p = 0.011$), and the EAT volume index ($p = 0.006$) were independently associated with the occurrence of events. The addition of EAT

FIGURE 1 Added Value of EAT Volume to Predict Events in Asymptomatic AS Patients



(A) Receiver-operating characteristic curves and area under the curve (auc) values are shown for predictors with or without epicardial adipose tissue (EAT) volume. **(B)** Kaplan-Meier curves of event-free survival for 51 asymptomatic patients with aortic stenosis with a low EAT volume index compared with 30 patients with a high EAT volume.

volume to the other predictors improved the predictive accuracy of events (Figure 1A). The cumulative event rates were higher in patients with high EAT volume (>60 ml/m^2) compared with patients with low EAT volume (≤60 ml/m^2) (Figure 1B). In linear regression, waist circumference ($p = 0.003$) and sex ($p = 0.034$) were the only 2 variables independently associated with EAT volume ($r^2 = 0.18$).

As previously reported, we showed that symptoms were associated with the degree of myocardial remodeling (i.e., fibrosis and BNP release). In addition, the extent of myocardial fibrosis was related to the valvulo-arterial impedance, which is an estimate of global LV afterload. However, focal fibrosis and BNP level did not predict outcome of asymptomatic patients. The main novel finding of our study was that the outcome of asymptomatic patients was predicted by the severity of AS, the relative wall thickness, and the EAT volume. Hence, the 2 CMR parameters might have distinct, complementary clinical diagnostic significance.

On the one hand, fibrosis may complement the clinical evaluation of symptomatic status. It is related to local, likely irreversible "replacement" fibrosis, which is driven by the degree of valve stenosis and the systemic arterial compliance (4), visceral adiposity (waist circumference), and metabolic profile (low-density lipoprotein cholesterol).

On the other hand, evaluating EAT volume may allow better identification of patients with asymptomatic AS who are at higher risk of events during follow-up. Excess EAT could contribute to the inflammatory burden of AS by producing proatherogenic cytokines, which may promote valve calcification, and also to myocardial steatosis, which may lead to LV strain impairment (5). EAT could thus represent a new therapeutic target in asymptomatic patients with AS.

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A Hyperdynamic RV Is an Early Marker of Clinical Decompensation and Cardiac Recovery in Aortic Stenosis With Normal LV Ejection Fraction

The right ventricle (RV) has been poorly studied in patients with aortic stenosis (AS) and normal left ventricular (LV) function, due to the belief that it is only affected by pulmonary hypertension (PH) secondary to advanced LV dysfunction. Despite this, increased RV afterload is frequent in AS (1), difficult to assess noninvasively (2), and may be present without detectable PH. The RV is extremely sensitive to afterload changes and may provide an earlier marker of progression than pulmonary pressures. Although RV dysfunction is the typical response to an increase in acute pulmonary pressure, RV adaptation to chronic afterload involves increasing contractility (3,4). Therefore, we hypothesized that in progressive AS with chronic afterload elevation, RV function would increase before detectable PH, which would affect symptoms and normalize after aortic valve replacement (AVR).

Eighty patients with isolated AS (23 with moderate, 24 with severe asymptomatic, 33 with severe symptomatic AS) and 28 control subjects of similar age and sex distribution (18 normotensive; 10 hypertensive)

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Impact of aortic stenosis on layer-specific longitudinal strain: relationship with symptoms and outcome

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Aims The present study sought to assess the impact of aortic stenosis (AS) on myocardial function as assessed by layer-specific longitudinal strain (LS) and its relationship with symptoms and outcome.

Methods and results We compared 211 patients (56% males, mean age 73 ± 12 years) with severe AS and left ventricular ejection fraction (LVEF) ≥50% (114 symptomatic, 97 asymptomatic) with 50 controls matched for age and sex. LS was assessed from endocardium, mid-myocardium, and epicardium by 2D speckle-tracking echocardiography. Despite similar LVEF, multilayer strain values were significantly lower in symptomatic patients, compared to asymptomatic and controls [global LS: 17.9 ± 3.4 vs. 19.1 ± 3.1 vs. 20.7 ± 2.1%; endocardial LS: 20.1 ± 4.9 vs. 21.7 ± 4.2 vs. 23.4 ± 2.5%; epicardial LS: 15.8 ± 3.1 vs. 16.8 ± 2.8 vs. 18.3 ± 1.8%; $P < 0.001$ for all]. On multivariable logistic regression analysis, endocardial LS was independently associated to symptoms ($P = 0.012$), together with indexed left atrial volume ($P = 0.006$) and LV concentric remodelling ($P = 0.044$). During a mean follow-up of 22 months, 33 patients died of a cardiovascular event. On multivariable Cox-regression analysis, age ($P = 0.029$), brain natriuretic peptide values ($P = 0.003$), LV mass index ($P = 0.0065$), LV end-systolic volume ($P = 0.012$), and endocardial LS ($P = 0.0057$) emerged as independently associated with cardiovascular death. The best endocardial LS values associated with outcome was 20.6% (sensitivity 70%, specificity 52%, area under the curve = 0.626, $P = 0.022$). Endocardial LS (19.1 ± 3.3 vs. 20.7 ± 3.3, $P = 0.02$) but not epicardial LS (15.2 ± 2.8 vs. 15.9 ± 2.5, $P = 0.104$) also predicted the outcome in patients who were initially asymptomatic.

Conclusion In patients with severe AS, LS impairment involves all myocardial layers and is more prominent in the advanced phases of the disease, when the symptoms occur. In this setting, the endocardial LS is independently associated with symptoms and patient outcome.

Keywords aortic stenosis • multilayer strain • endocardial longitudinal strain • speckle-tracking echocardiography

Introduction

Aortic stenosis (AS) is currently the most common valvular heart disease, and its prevalence is increasing as the population ages.[1] Symptomatic patients with severe AS have a high mortality rate and require prompt aortic valve replacement (AVR).[2,3] Although asymptomatic patients are at increased risk for untoward events, their management remains controversial. Current guidelines consider AVR as reasonable in asymptomatic patients with reduced (<50%) left ventricular ejection fraction (LVEF) and in patients who exhibit

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symptoms during an exercise test.[4,5] However, symptoms are subjective and LVEF can remain normal for long despite markedly impaired myocardial function. We previously demonstrated that 2D LV global longitudinal strain (GLS) could detect early subtle myocardial dysfunction in AS patients.[6–9] The impairment of global LV longitudinal function is associated with myocardial fibrosis, which is, in turn, a potential prognostic marker in patients with AS.[10] However, longitudinal function is actually largely governed by the subendocardial myocardial fibres, which are affected first by the pathological changes (hypertrophy, increased wall stress, and reduced arterial compliance) associated with AS.[11,12] Recent 2D strain software allows separate evaluation of endocardial, mid-myocardial, and epicardial myocardial deformation. To date, little is known about the impact of AS on the different myocardium layers. The present study sought to investigate the relationship between changes in layer-specific strain and the clinical outcome of patients with severe AS and preserved LVEF.

Methods

Patient population

A total of 249 patients with severe AS who were prospectively examined in our heart valve clinic between January 2007 and February 2018 were evaluated. Inclusion criteria were severe AS defined by an aortic valve area ≤0.6 cm^2/m^2 by echocardiography, normal LVEF (≥50%) as calculated by 2D echocardiography, no more than mild associated cardiac valve lesion, sinus rhythm, and good images quality. Thirty-nine patients were excluded for suboptimal quality of speckle-tracking image analysis. The final study population consisted of 211 patients, which were divided into two groups, according to the symptomatic status. The control group included 50 patients matched for age and sex. All patients gave written informed consent and the hospital ethics committee approved the study.

Echocardiographic measurements

Transthoracic echocardiograms were performed using a Vivid ultrasound (7, E9 or E95) System (GE Healthcare, Horten, Norway) and stored on a dedicate workstation for off-line analysis (EchoPAC, version 201, GE Healthcare). For each echocardiographic measurement, at least two cardiac cycles were averaged. Conventional echocardiographic measurements were performed in accordance with the guidelines.[13–15] Valvulo-arterial impedance (Zva) was calculated as the sum of systolic blood pressure and mean transaortic gradient, divided by indexed LV stroke volume. Strain analysis was based on speckle-tracking approach, measured by an experienced cardiologist and expressed as an absolute value. The acquisitions were performed in apical long-axis, four-, and two-chamber views (frame rate 70–90 frames/s).[16,17] LV was divided into six myocardial segments in each view, and GLS calculated as the average LS at end-systole. For measuring layer-specific strain, attention was taken to cover the entire myocardial wall thickness by the region of interest (ROI) of each segment. Calculation of transmural variation of LS across the entire myocardium was based on the assumption of a linear distribution. Endocardial and epicardial LS were measured on the endocardial and epicardial ROI border, respectively, whereas the mid (centre line) of the ROI represented the average values of the transmural wall thickness (GLS). LS gradient was calculated as the difference between endocardial and epicardial LS.[18] Right ventricle (RV) LS was calculated as the average of regional strain from RV free wall segments and interventricular septum.

Clinical follow-up

Patients were routinely followed-up and managed according to available guidelines, and clinical information was obtained from direct patient interview, telephone calls with physicians, patients, or next of kin, or review of autopsy records and death certificates. Cardiovascular-related mortality was the endpoint.

Statistical analysis

Data are reported as mean ± standard deviation for continuous variables or percentages of individuals for categorical variables. The χ^2 test or Fisher's exact test was used to compare qualitative variables. One-way analysis of variance test was used to compare the three groups. When a significant difference was found, *post hoc* testing with Bonferroni comparisons for identified specific group differences was used. Variables with a *P*-value <0.05 on univariable analysis were incorporated into the multivariable logistic regression model for the prediction of symptoms and cardiovascular mortality. Receiver operator characteristics (ROC) curves were generated to determine the cut-off value that best predicted the occurrence of symptoms and cardiovascular mortality. The Kaplan–Meier method was used for cumulative survival analysis with the log-rank test for assessing statistical differences between the curves. Statistical analyses were performed using IBM-SPSS, version 23 (SPSS Inc., Chicago, IL, USA). Reproducibility analyses were previously published by our group.[17,19]

Results

Baseline patients' characteristics

Of the 211 patients, 114 (54%) were classified as symptomatic baseline (syncope = 4, dyspnoea = 98, angina = 7, and acute pulmonary oedema = 5) (*Table 1*). Compared with the 97 (56%) asymptomatic patients, they did not differ in age, gender, LV ejection fraction, and presence of risk factors but had higher body mass index, systolic blood pressure, aortic pressure gradients, brain natriuretic peptide (BNP) levels, and smaller aortic valve area. Symptomatic patients also had more pronounced cardiac chambers remodelling, diastolic dysfunction, and impaired RV function. Despite similar LV ejection fraction between groups, multilayer strain values (GLS, endocardial, epicardial, and gradient LS) were significantly lower in symptomatic patients (*Figure 1*). Asymptomatic patients also had lower strain values when compared with controls. In all groups, endocardial systolic strain was higher than epicardial strain.

Symptomatic vs. asymptomatic AS

The impact of specific layer strains on symptoms was evaluated in two multivariable models, where GLS was taken as the reference (GLS vs. endocardial LS or epicardial LS). In the first model, concentric remodelling [$P = 0.044$, odds ratio (OR) = 2.294], indexed left atrial volume ($P = 0.006$, OR = 1.035), and endocardial LS ($P = 0.012$, OR = 1.150) emerged as independent cofactors associated with symptoms after adjustment for body mass index, BNP level, types of remodelling, and severity of AS (*Table 2*). In the second model, concentric remodelling ($P = 0.04$, OR = 2.429), indexed left atrial volume ($P = 0.006$, OR = 1.036), and GLS ($P = 0.015$, OR = 1.17) emerged as independent cofactors associated with symptoms after adjustment for body mass index, BNP level, LV mass, types of remodelling, and severity of AS (*Table 3*). At ROC curve analysis (*Figure 2*), a

Table 1 Baseline clinical and echocardiographic characteristics

Variables	Controls ($n = 50$)	Asymptomatic AS group ($n = 97$)	Symptomatic AS group ($n = 114$)	*P*-value
Clinical variables				
Age (years)	71.1 ± 4.7	71.9 ± 12.2	74.9 ± 11.0	0.071
Male gender, *n* (%)	25 (50)	55 (57)	64 (56)	0.713
Body mass index (kg/m^2)	25.5 ± 3.4	26.1 ± 4.0	27.8 ± 5.8[a,b]	0.007
Body surface area (m^2)	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	0.472
Systolic arterial pressure (mmHg)	128 ± 11	137 ± 18[a]	135 ± 20	0.028
Diastolic arterial pressure (mmHg)	77 ± 8	73 ± 10	73 ± 11	0.052
BNP (log)		4.4 ± 1.1	4.9 ± 1.1[b]	0.007
Diabetes mellitus, *n* (%)		20 (21)	30 (26)	0.353
Hypertension, *n* (%)		69 (73)	89 (78)	0.362
Hypercholesterolaemia, *n* (%)		64 (67)	74 (65)	0.790
Current smoking, *n* (%)		15 (16)	16 (14)	0.746
Coronary artery disease, *n* (%)		10 (10)	21 (19)	0.092
LV dimensions and geometry				
Interventricular septum (mm)	9.6 ± 1.2	12.2 ± 2.0[a]	13.7 ± 2.4[a,b]	<0.001
LV posterior wall (mm)	9.8 ± 1.9	10.6 ± 1.6[a]	11.5 ± 1.7[a,b]	<0.001
LV end-diastolic diameter (mm)	42.7 ± 5.3	44.9 ± 5.9	45.5 ± 6.2[a]	0.023
LV end-systolic diameter (mm)	29.3 ± 5.1	30.1 ± 6.0	29.8 ± 5.7	0.732
LV mass index (g/m^2)	76.8 ± 20.2	103.7 ± 27.2[a]	120.0 ± 27.5[a,b]	<0.001
Relative wall thickness	0.46 ± 0.1	0.48 ± 0.10	0.51 ± 0.11[a]	0.019
Normal geometry, *n* (%)	19 (38)	19 (23)[a]	10 (10)[a,b]	<0.001
Concentric remodelling, *n* (%)	25 (50)	30 (36)[a]	19 (18)[a,b]	<0.001
Concentric hypertrophy, *n* (%)	5 (10)	26 (31)[a]	62 (60)[a,b]	<0.001
Eccentric hypertrophy, *n* (%)	1 (2)	9 (11)	13 (12)	0.109
Aortic valve severity				
Mean pressure gradient (mmHg)		43.8 ± 12.9	47.7 ± 14.4[b]	0.044
Peak aortic velocity (m/s)		4.2 ± 0.6	4.3 ± 0.6	0.143
Aortic valve area (cm^2)		0.81 ± 0.15	0.78 ± 0.20	0.153
Indexed aortic valve area (cm^2/m^2)		0.45 ±0.08	0.42 ± 0.09[b]	0.017
Indexed stroke volume (mL/m^2)		45.5 ± 10.0	44.7 ± 9.2	0.554
Zva (mmHg/mL/m^2)		4.2 ± 1.0	4.3 ± 1.0	0.530
Low flow–low gradient, *n* (%)		11 (11)	7 (6)	0.184
low flow–high gradient, *n* (%)		4 (4)	8 (7)	0.358
Normal flow–low gradient, *n* (%)		22 (23)	17 (15)	0.156
Normal flow–high gradient, *n* (%)		60 (62)	79 (70)	0.219
LV-RV dimension function				
LV end-diastolic volume (mL)	83.0 ± 24.3	89.8 ± 31.8	94.7 ± 34.8	0.138
LV end-systolic volume (mL)	30.2 ± 10.4	33.9 ± 14.9	35.9 ± 15.0	0.095
LVEF (%)	64 ± 5	63 ± 7	62 ± 16	0.325
Indexed left atrial volume (mL/m^2)	26.5 ± 8.6	35.3± 12.5[a]	44.9 ± 19.6[a,b]	<0.001
Mitral *E*/*A* ratio	0.9 ± 0.3	0.9 ± 0.3	1.0 ± 0.9	0.363
Average *E*/e'	7.4 ± 1.8	12.9 ± 5.6[a]	13.5 ± 5.2[a]	<0.001
TTPG (mmHg)	17 ± 8	29 ± 11[a]	31 ± 12[a]	<0.001
TAPSE (mm)	22 ± 3	23 ± 4	23 ± 3	0.747
RV s' (cm/s)	13 ± 3	13 ± 3	12 ± 3[a]	0.014
Right atrial volume (mL)	32.7 ± 10.8	40.3 ± 20.0	44.3 ± 28.7[a]	0.015
LV-RV longitudinal strain				
RV GLS (%)	20.3 ± 4.5	19.7 ± 3.6	20.2 ± 4.0	0.285
LV GLS (%)	20.7 ± 2.1	18.5 ± 2.8[a]	17.4 ± 2.8[a,b]	<0.001

Continued

Table 1 *Continued*

Variables	Controls ($n = 50$)	Asymptomatic AS group ($n = 97$)	Symptomatic AS group ($n = 114$)	*P*-value
Endocardial LS (%)	23.4 ± 2.5	21.1 ± 3.2[a]	19.6 ± 3.4[a,b]	<0.001
Epicardial LS (%)	18.3 ± 1.8	16.2 ± 2.5[a]	15.4 ± 2.6[a,b]	<0.001
Gradient endocardial-epicardial LS	5.1 ± 1.1	4.8 ± 1.1	4.3 ± 1.6[a,b]	0.001

Values are expressed as *n* (%) or mean ± SD.
AS, aortic stenosis; BMI, body mass index; BNP, brain natriuretic peptide; BSA, body surface area; EF, ejection fraction; GLS, global longitudinal strain; LV, left ventricle; RV, right ventricle; TTPG, trans-tricuspid pressure gradient; Zva, valvulo-arterial impedance.
[a]$P < 0.05$ vs. controls.
[b]$P < 0.05$ vs. asymptomatic group.

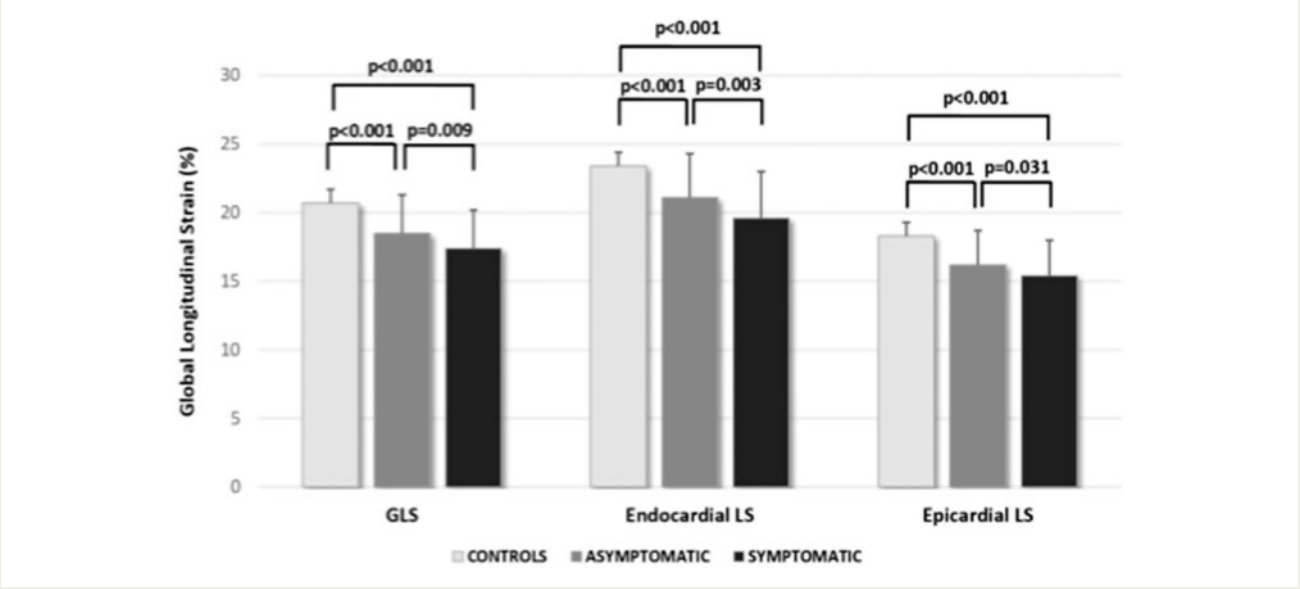

Figure 1 GLS (mid-myocardial), endocardial, and epicardial longitudinal strain in controls, asymptomatic and symptomatic severe AS patients.

Table 2 Univariable and multivariable logistic regression analyses of clinical and echocardiographic parameters associated with symptoms (Model 1)

Parameters	Univariate			Multivariate		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Body mass index	1.070	1.011–1.132	0.020			
BNP	1.425	1.097–1.851	0.008			
LV mass index	1.023	1.011–1.036	<0.001			
Normal geometry	2.748	1.200–6.291	0.017			
Concentric remodelling	2.485	1.274–4.849	0.008	2.294	1.021–5.150	0.044
Concentric hypertrophy	3.293	1.796–6.037	<0.001			
Mean pressure gradient	1.021	1.000–1.046	0.047			
Indexed aortic valve area	0.024	0.001–0.538	0.019			
Indexed left atrial volume	1.044	1.021–1.067	<0.001	1.035	1.010–1.061	0.006
GLS	1.124	1.032–1.223	0.007			
Endocardial LS	1.118	0.039–1.204	0.003	1.150	1.032–1.282	0.012

BNP, brain natriuretic peptide; CI, confidence interval; GLS, global longitudinal strain; LS, longitudinal strain; LV, left ventricle; OR, odds ratio.

Table 3 Univariable and multivariable logistic regression analysis of clinical and echocardiographic parameters associated with symptoms (Model 2)

Parameters	Univariate			Multivariate		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Body mass index	1.070	1.011–1.132	0.020			
BNP	1.425	1.097–1.851	0.008			
LV mass index	1.023	1.011–1.036	<0.001			
Normal geometry	2.748	1.200–6.291	0.017			
Concentric remodelling	2.485	1.274–4.849	0.008	2.429	1.084–5.445	0.040
Concentric hypertrophy	3.293	1.796–6.037	<0.001			
Mean pressure gradient	1.021	1.000–1.046	0.047			
Indexed aortic valve area	0.024	0.001–0.538	0.019			
Indexed left atrial volume	1.044	1.021–1.067	<0.001	1.172	1.032–1.332	0.015
GLS	1.124	1.032–1.223	0.007	1.036	1.010–1.032	0.006
Epicardial LS	1.118	0.039–1.204	0.003			

BNP, brain natriuretic peptide; CI, confidence interval; GLS, global longitudinal strain; LS, longitudinal strain; LV, left ventricle; OR, odds ratio.

subendocardial LS of 21% in patients with severe AS was associated with symptoms with a sensitivity of 70% and a specificity of 65% [area under the curve (AUC) = 0.692, $P < 0.001$].

Predictors of clinical outcome

After a median follow-up period of 22 months (interquartile range: 7–51 months), 145 patients with severe aortic stenosis underwent AVR (transcatheter AVR = 38, surgical replacement = 107), and 33 patients died of a cardiovascular event (after AVR = 20, heart failure = 4, sudden death = 7, cardiac tamponade = 1, stroke = 1). In the univariable Cox-regression analysis, patients who died were older ($P = 0.002$), had higher values of BNP and LV mass ($P < 0.001$), greater LV end-diastolic diameter ($P = 0.004$), and LV volumes ($P = 0.044$ for end-diastolic, $P = 0.015$ for end-systolic), right and left atrial volumes ($P < 0.001$), diastolic dysfunction and pulmonary hypertension ($P < 0.001$). In addition, significant correlations between GLS ($P = 0.006$), endocardial LS ($P = 0.003$), epicardial LS ($P = 0.045$) and mortality were observed. For the other parameters, including severity of AS, no significant correlations with the outcome were found ($P > 0.1$ for all) (*Table 4*). On multivariable Cox-regression analysis, age ($P = 0.029$), BNP values ($P = 0.003$), LV mass index ($P = 0.0065$), LV end-systolic volume ($P = 0.012$), and endocardial LS ($P = 0.0057$) emerged as independently associated with cardiovascular death. The best endocardial LS values associated with outcome was 20.6% (sensitivity 70%, specificity 52%, AUC = 0.626, $P = 0.022$) (*Figure 3A*). The cumulative event rate for cardiovascular death was significant higher in AS patients with more impaired endocardial LS (<20.6%) compared to those with preserved endocardial LS (≥20.6%) (21.6% vs. 11.3% at 5-year follow-up, respectively; log-rank $P = 0.035$) (*Figure 3B*).

During a median period of 30 months (interquartile range: 14–36 months), 9 (9%) out of the 97 asymptomatic patients died from cardiovascular deaths (most of them after symptoms development). These patients had higher values of BNP, more pronounced cardiac chambers remodelling, diastolic dysfunction, and pulmonary hypertension. Both GLS (16.9 ± 2.9 vs. 18.2 ± 2.8, $P = 0.031$) and endocardial LS (19.1 ± 3.3 vs. 20.7 ± 3.3, $P = 0.02$) but not epicardial LS (15.2 ± 2.8 vs. 15.9 ± 2.5, $P = 0.10$) were reduced in patients who died.

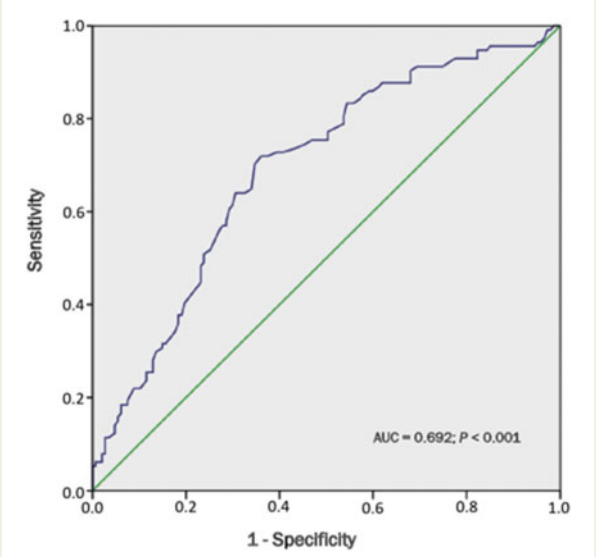

Figure 2 ROC curve of endocardial longitudinal strain associated with symptoms in patients with severe AS.

Discussion

In patients with severe AS and preserved LVEF, the present study demonstrates that: (i) GLS (mid-myocardial), as well as endocardial and epicardial LS values are lower in patients with severe AS as

Table 4 Univariable and multivariable predictors of cardiovascular mortality

Parameters	Univariable			Multivariable		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.067	1.025–1.110	0.002	1.12	1.017–1.32	0.029
Body mass index	1.012	0.939–1.090	0.762			
Body surface area	1.972	0.357–10.905	0.436			
Systolic arterial pressure	0.986	0.967–1.005	0.142			
Diastolic arterial pressure	0.968	0.934–1.004	0.084			
LogBNP	2.160	1.457–3.201	<0.001	2.12	1.03–4.45	0.003
Diabetes mellitus	0.606	0.294–1.250	0.175			
Hypertension	0.719	0.296–1.747	0.466			
Hypercholesterolaemia	1.336	0.655–2.726	0.426			
Current smoking	1.172	0.452–3.042	0.744			
Coronary artery disease	0.334	0.155–0.722	0.005			
LV mass index	1.022	1.009–1.035	0.001	1.06	1.017–1.12	0.0065
Interventricular septum	1.092	0.917–1.300	0.325			
LV posterior wall	1.173	0.949–1.451	0.141			
LV end-diastolic diameter	1.087	1.027–1.151	0.004			
LV end-systolic diameter	1.060	0.998–1.125	0.059			
Relative wall thickness	0.343	0.009–13.159	0.565			
Normal geometry	2.534	0.602–10.660	0.205			
Concentric remodelling	1.187	0.507–2.782	0.693			
Concentric hypertrophy	0.735	0.354–1.523	0.407			
Eccentric hypertrophy	0.701	0.267–1.839	0.471			
Mean pressure gradient	0.989	0.963–1.016	0.425			
Peak aortic velocity	0.691	0.404–1.182	0.177			
Aortic valve area	4.607	0.567–37.437	0.153			
Indexed aortic valve area	22.554	0.325–1565	0.150			
Indexed stroke volume	1.009	0.973–1.046	0.646			
Zva	0.864	0.578–1.292	0.476			
LV end-diastolic volume	1.009	1.000–1.018	0.044			
LV end-systolic volume	1.025	1.005–1.045	0.015	1.107	1.02–1.20	0.012
LV EF	0.951	0.900–1.005	0.073			
Indexed left atrial volume	1.034	1.020–1.049	<0.001			
Average *E*/e'	1.096	1.045–1.149	<0.001			
TTPG (mmHg)	1.052	1.026–1.079	<0.001			
TAPSE (mm)	0.934	0.848–1.028	0.163			
RV s'	0.857	0.716–1.025	0.092			
Right atrial volume	1.019	1.009–1.029	<0.001			
RV GLS	1.008	0.963–1.054	0.744			
LV GLS	1.212	1.057.1.390	0.006			
Endocardial LS	1.190	10.061–1.334	0.003	2.75	1.33–5.69	0.0057
Epicardial LS	1.164	1.003–1.351	0.045			
Gradient endocardial-epicardial LS	1.308	1.101–1.552	0.002			

AS, aortic stenosis; BNP, brain natriuretic peptide; CI, confidence interval; EF, ejection fraction; GLS, global longitudinal strain; HR, hazard ratio; LS, longitudinal strain; LV, left ventricle; TTPG, trans-tricuspid pressure gradient; Zva, valvulo-arterial impedance.

compared to controls; (ii) symptomatic patients with severe AS have decreased values of all layers of LV strain compared to asymptomatic patients with similar LVEF; (iii) endocardial LS is more sensitive than GLS and epicardial LS to characterize the symptomatic status of AS patients; (iv) endocardial LS is an independent predictor of cardiovascular outcome.

Multilayer strains and symptoms

Symptom development and a LVEF <50% are the main triggers for AVR in patients with severe AS. However, symptoms are subjective, patients may be unable to perform an exercise test to characterize them, and a LVEF <50% already demonstrates advanced myocardial involvement (i.e. extensive myocardial fibrosis) with limited

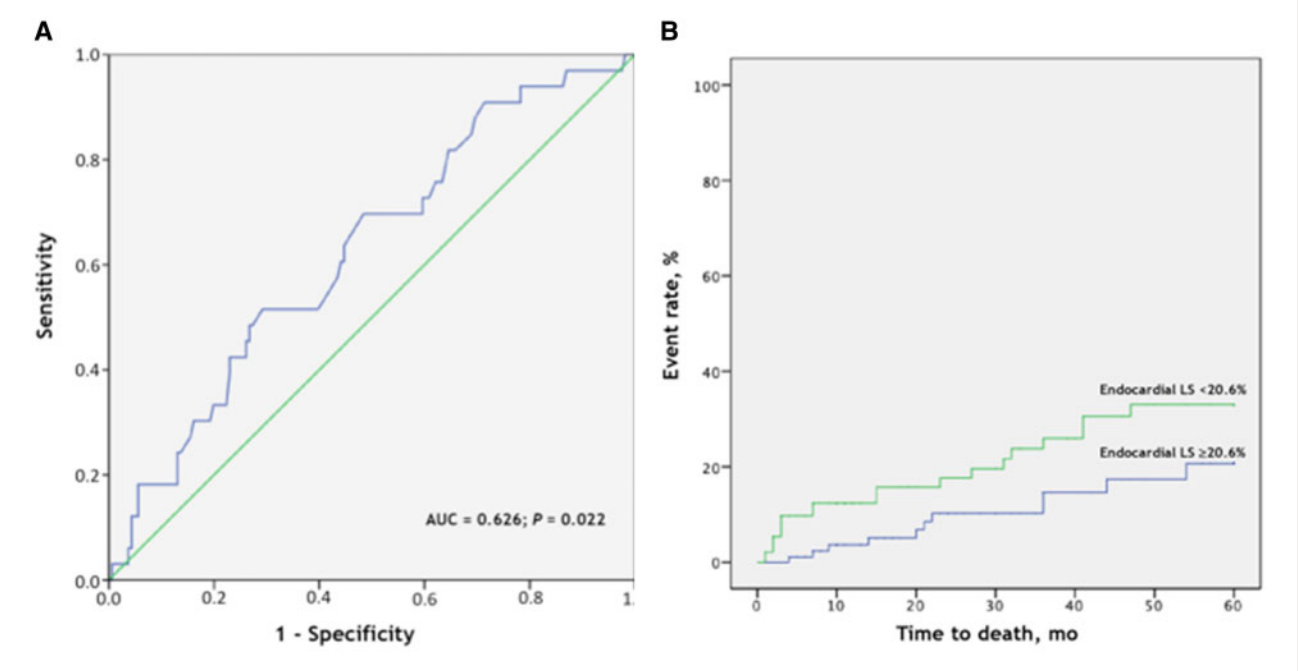

Figure 3 ROC curve of endocardial longitudinal strain associated with cardiovascular death in patients with severe AS (*A*). Kaplan–Meier estimates for cardiovascular death during follow-up in patients with severe AS divided into two groups according to baseline endocardial longitudinal strain: more impaired (<20.6%, green line) vs. more preserved (≥20.6%, blue line) (*B*).

reversibility after AVR.[20,21] In the HAVEC registry, patients with LVEF between 50% and 59% had less favourable outcomes and experienced more heart failure-related deaths than those with LVEF >60%, even after AVR.[4] Reduced LV GLS is an early marker of impaired contractile function when LVEF is still preserved and is also associated with the presence of myocardial fibrosis.[22] Recent series in patients with AS have also linked GLS with subsequent cardiac events and worsening of strain abnormalities as AS progresses despite the lack of a simultaneous fall in LVEF.[23–29] Spatial configurations of ventricular myocardial fibres in the subendocardial and subepicardial layers provide sequential contractile activity of the ventricle and contribute to LV GLS. The endocardium undergoes greater dimensional changes (both thickening and shortening) during systole than does the epicardium in healthy myocardium. In AS, as the subendocardial fibres are more sensitive to microvascular ischaemia (subendocardial blood flow maldistribution related to LV hypertrophy and increased wall stress) and fibrosis, the longitudinal function is likely the first to be altered.[30–32] However, as the AS progresses, all myocardial layers are gradually affected but to a different extent. Cho *et al.*[33] reported lower epicardial, mid-wall, and endocardial LS in 45 patients with severe AS compared to 18 healthy controls, and correlated LS with LV mass index, LVEF, left atrial volume, and N-terminal pro-B-type natriuretic peptide.[33] In 36 AS patients, Ozawa *et al.*[12] correlated the impairment of multilayer LS, particularly of endocardial LS, with the severity of AS. The present study confirms and extends these findings in a larger population and provides new insights into the relationship between regional strain impairment and symptoms in AS. As observed, all layer-specific strains were decreased in patients with AS as compared to controls. However, the reduction in regional strains, particularly of endocardial LS, was more pronounced in symptomatic patients. Hence, the assessment of multilayer strains appears to be promising and may complement conventional echocardiographic parameters (e.g. LV remodelling, left atrial volume) to discriminate the symptomatic status in AS.

Multilayer strains and outcomes

Comorbidities are frequent in patients with AS (e.g. age, coronary artery disease) and increase the overall cardiovascular risk profile of patients. Biomarkers have consistently shown to be associated with patient outcome. Higher BNP values are associated with increased mortality risk. Echocardiography also plays a major predictive role in AS.[32,34] As reported, the severity of AS, the degree of LV hypertrophy and remodelling, the diastolic burden (e.g. increased in LV filling pressure, left atrial enlargement), the augmented pulmonary pressures and dilated right atrium, and the extent of regional LV systolic dysfunction as estimate by GLS are all potential predictors of poor outcome. These data are also confirmed in our study in which we also show a prognostic value of layer-specific strains. Alteration of endocardial LS was strongly and independently associated with higher cardiovascular mortality rate in patients with AS and preserved LVEF. Reduced endocardial strain was observed in patients who died regardless of the symptomatic status at the entry point. Consequently, LVEF, which only takes into account the LV chamber or wall thickness as a whole, is insufficient to estimate the degree of dysfunction within the different layers of the myocardial wall, which represents a more sensitive marker of myocardial involvement and outcome. An endocardial LS below 20.6% yielded the strongest predictive

accuracy for cardiovascular death, even if with moderate accuracy, likely due to low hard event rates. Further prospective studies with larger number of patients could confirm the data and determine the exact role of endocardial LS in predicting cardiovascular events.

Limitations

This study has some limitations. We included in the study only patients with severe AS based on aortic valve area and preserved LV ejection fraction. The sub-categorization of AS according to flow-gradient pattern was not performed. The presence of patients with coronary artery disease could affect our data. However, coronary artery disease incidence was similar in both groups with and without symptoms, and patients with wall motion abnormalities were preventively excluded from the analysis. The gradient of strain across the myocardium is a nonlinear phenomenon, and the definition of the layers is arbitrary and is based on simple division into three parts. Because the spatial resolution of ultrasound is limited, there will always be a certain degree of overlap. Despite interobserver and intraobserver reproducibility of LV GLS have demonstrated to be comparable with conventional echocardiography parameters, the variability of LS measurement related to ultrasound system and the software for the off-line analysis could represent a limitation. The decision to perform surgery was made by individual cardiologists in charge of the patients. Serial echocardiographic assessment over time was not performed.

Conclusions

In severe AS, LS impairment involves all myocardial layers and is more prominent in the endocardial layer. This impairment becomes even more evident in the advanced phases of the disease when the symptoms occur. Regardless of the symptomatic status, reduced LS conveys a worse outcome. Further studies are needed to better determine the role of endocardial LS in predicting the progression of aortic valve disease and the occurrence of cardiovascular events.

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Impact of global left ventricular afterload and transaortic gradient on myocardial work in patients with aortic stenosis and preserved ejection fraction

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Background: Myocardial work (MW) is a new parameter that derives from myocardial strain and provides an incremental value to myocardial function, incorporating measurement of deformation and load. To date, little is known about the changes in MW related to AS severity and arterial compliance.

Purpose: We investigated the effect of severity of AS, valvulo-arterial impedance (Zva) and stroke volume in patients with AS and preserved LV ejection fraction (EF).

Methods: 283 patients (60% males, mean age 71±12 years old) with varying grades of AS and LVEF≥50% were enrolled. Exclusion criteria were more than mild associated cardiac valve lesion, left bundle branch block, and suboptimal quality of speckle-tracking image analysis. The control group included 50 patients matched for age and sex. Clinical, demographic and resting echocardiographic data were recorded, including quantification of 2D global longitudinal strain (GLS), global work index (GWI), global constructive work (GCW), global wasted work (GWW) and global work efficiency (GWE).

Results: Patients with AS had higher systolic (p=0.017) and diastolic arterial pressure (p=0.007), increased LV wall thickness, mass index (p<0.001) and volumes (p=0.045) compared to controls. Greater indexed left atrial volume, E/e' and trans-tricuspid gradient were also observed in the AS group (p<0.001). As expected, speckle tracking analysis revealed significant lower GLS in AS than

in control group (18.7±3.2 vs 20.7±2.1%, p<0.001). Conversely, increased values of GCW and GWI (respectively 2965±647 vs 2360±353 mmHg%, and 2535±559 vs 2005±302 mmHg%, p<0.001) were observed in patients with AS. Besides, GWW was significantly increased in AS vs controls (147±108 vs 90±49 mmHg%, p=0.001), with no changes in terms of GWE (95±4 vs 96±2%, p=0.110). When patients were stratified according to the AS severity, the analysis of variance revealed that GCW, GWI and GWW significantly increased with higher transaortic mean gradient and lower aortic valve area (p<0.001). Also Zva demonstrated to impact on CGW (p=0.040) and GWW (p<0.001), with increased values in presence of increased global LV afterload (Zva>4.5 mmHg/ml/m2). Conversely, patien.ts with low-flow AS (stroke volume index < 35 ml/m2) showed lowers values of GCW (p=0.014) and GWI (p=0.001) compared to normal flow AS, but increased GWW (p=0.041) and reduced GWE (93±7 vs 95±4%, p=0.010). At multivariable analysis, mean gradient (p<0.001), Zva (p=0.038), systolic blood pressure (p<0.001) and GLS (p<0.001) were independently associated with GWI and GCW, while only GLS was associated with GWW.

Conclusion: In patients with AS and preserved LVEF, GLS reduction is accompanied by an increase of GCW, GWI and GWW, without affecting the GWE. These MW modifications seem to be mainly correlated to the severity of AS and increased global LV afterload.

Prognostic role of global work index in asymptomatic patients with aortic stenosis

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Background: in asymptomatic patients with aortic stenosis (AS), the optimal timing for intervention is still challenging. Previous studies demonstrated that advanced stages of cardiac damage are associated with excess mortality. The role of myocardial work to identify cardiac dysfunction in AS and to predict prognosis has not been investigated.

Purpose: We aimed to evaluate the modification of myocardial work indices related to AS stages and their prognostic value.

Methods: This study retrospectively analysed the clinical, demographic, echocardiographic,and outcome data of 170 patients with asymptomatic AS (aortic valve area ≤1.5 cm^2) and preserved ejection fraction (LVEF≥50%). Exclusion criteria were: more than mild associated cardiac valve lesion, left bundle branch block and suboptimal quality of speckle-tracking image analysis. The control group included 50 patients matched for age and sex. Global work index (GWI), global constructive work (GCW), global wasted work (GWW) and global work efficiency (GWE) were estimated by LV pressure-strain loops. In AS group, LV pressure was evaluated by adding trans-aortic mean gradient to systolic blood pressure. Patients were classified according to the following staging classification: no cardiac damage associated with the valve stenosis (Stage 0), left ventricular damage (Stage 1), left atrial or mitral valve damage (Stage 2), pulmonary hypertension or tricuspid valve damage (Stage 3), or right ventricular damage or subclinical heart failure (Stage 4).

Results: While global longitudinal strain was significantly lower in AS than in control group (18.7±2.8 vs 20.7±2.1%, p<0.001), increased values of GCW and GWI (respectively 2948±598 vs 2360±353 mmHg%, and 2528±521 vs 2005±302 mmHg%, p<0.001) were observed in patients with AS. Besides, GWW was significantly increased in AS vs controls (139±90 vs 90±49 mmHg%, p=0.001), with no changes in terms of GWE (95±4 vs 96±2%, p=0.110). When patients were stratified according the stages of cardiac damage, MW indices didn't different significantly between the stages, except for the GWI, which was significantly lower in Stage 3 to 4 compared to Stage 0 and Stage 2 (2268±469 vs 2623±503 vs 2610±503 mmHg% respectively, p=0.025). During a mean follow up of 27 months (IQ range 12-48 mo), 18 patients had a CV death. The best GWI value associated with outcome was 1866 mmHg% (sensitivity 45%, specificity 96%, AUC= 0.701, p=0.01). The presence of a GWI at baseline lower than 1866 mmHg% was associated with a higher rate of CV events at 4-year follow-up (57% vs 7%, log-rank p<0.001). On multivariable Cox-regression analysis, BNP values (P=0.014) and GWI <1866 mmHg% (P=0.033) emerged as independently associated with CV death.

Conclusion: in asymptomatic patients with AS, advanced stages of cardiac damage are characterized by reduced values of GWI, that are associated with increased mortality. Thus, the evaluation of MW indices may allow a better phenotyping of asymptomatic patients at higher risk of developing cardiovascular events during follow-up.

I.F.C. Italian Federation of Cardiology

Global and regional myocardial function and outcomes after transcatheter aortic valve implantation for aortic stenosis and preserved ejection fraction

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Aim **To investigate the effects of transcatheter aortic valve implantation (TAVI) on early recovery of global and segmental myocardial function in patients with severe symptomatic aortic stenosis and preserved left ventricular ejection fraction (LVEF) and to determine if parameters of deformation correlate with outcomes.**

Methods **The echocardiographic (strain analysis) and outcome (hospitalizations because of heart failure and mortality) data of 62 consecutive patients with preserved LVEF (64.54 ± 7.97%) who underwent CoreValve prosthesis implantation were examined.**

Results **Early after TAVI (5 ± 3.9 days), no significant changes in LVEF or diastolic function were found, while a significant drop of systolic pulmonary artery pressure (PAP) occurred (42.3 ± 14.9 vs. 38.1 ± 13.9 mmHg, *P* = 0.028). After TAVI global longitudinal strain (GLS) did not change significantly, whereas significant improvement in global mid-level left ventricular (LV) radial strain (GRS) was found (−16.71 ± 2.42 vs. −17.32 ± 3.25%; *P* = 0.33; 16.57 ± 6.6 vs. 19.48 ± 5.97%, *P* = 0.018, respectively). Early significant recovery of longitudinal strain was found in basal lateral and anteroseptal segments (*P* = 0.038 and 0.048). Regional radial strain at the level of papillary muscles [*P* = 0.038 mid-lateral, *P* < 0.001 mid-anteroseptum (RSAS)] also improved. There was a significant LV mass index reduction in the late follow-up (152.42 ± 53.21 vs. 136.24 ± 56.67 g/m^2, *P* = 0.04). Mean follow-up period was 3.5 ± 1.9 years. Parameters associated with worse outcomes in univariable analysis were RSAS pre-TAVI, LV end-diastolic diameter after TAVI, relative wall thickness, and mitral *E* and *E/A* after TAVI.**

Conclusion **Global and regional indices of myocardial function improved early after TAVI, suggesting the potential of myocardium to recover with a reduced risk for clinical deterioration.**

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Keywords: aortic stenosis, echocardiography, speckle tracking, strain, transcatheter aortic valve implantation

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Introduction

Transcatheter aortic valve implantation (TAVI) has been established as a promising procedure for patients with severe symptomatic aortic stenosis who are not suitable candidates for surgery.[1–6] The procedure has been shown to be well tolerated and feasible in the short- and mid-term follow-up period.[7,8] In patients with severe aortic stenosis, because of chronic pressure overload, compensatory mechanisms lead to geometry and functional changes: left ventricular (LV) hypertrophy, diastolic dysfunction, fibrosis and global systolic dysfunction. Myocardial strain, especially longitudinal strain, is a sensitive tool for detecting subtle intrinsic myocardial function damage, even when standard indices of myocardial performance, that is, ejection fraction, are still preserved.[9,10] Acute changes in myocardial function may be seen immediately after TAVI partly because of pressure unloading.[11] In the mid-term follow-up, occurrence of geometrical changes known as reverse remodelling, that is, LV mass and volume regression,[12,13] can be detected with conventional transthoracic echocardiography (TTE). Global longitudinal strain (GLS) improvement after TAVI correlates with symptomatic improvement after intervention.[9] Patients with reduced LV ejection fraction benefit the most in terms of longitudinal reverse remodelling,[14–16] although impaired LVEF itself is associated with adverse outcomes.[15] However, the impact of elevated afterload is not the same in all LV segments according to the Laplace's law. Whether regional LV deformation is associated with outcomes remains unknown. We sought to investigate the effects of TAVI on early recovery of global and segmental myocardial function and mechanics in

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patients with severe aortic stenosis and preserved LVEF, and to determine if parameters of deformation correlate with prognosis. We hypothesized that after TAVI, regional longitudinal strain in basal LV segments and also regional radial strain measured at the level of the papillary muscles improve because of acute pressure unloading and that those changes are associated with prognosis.

Methods

Study population

A single-centre retrospective longitudinal study was performed using hospital charts and digitally stored standard TTE protocols. Consecutive patients with severe symptomatic aortic stenosis who underwent CoreValve (Medtronic, Minneapolis, Minnesota, USA) implantation at the University Hospital of Liège, Belgium in the period 2008–2014 and met inclusion criteria were enrolled. Inclusion criteria were: severe aortic stenosis with preserved LVEF (>50%), high calculated operative risk or contraindication for surgery, available comprehensive TTE protocol before and early after TAVI. Exclusion criteria were: reduced LVEF, unresolved coronary artery disease, signs of scared myocardium and poor quality of echocardiographic images.

Echocardiography

TTE was performed using Vivid 9 echocardiographic ultrasound system (GE Vingmed Ultrasound, Horton, Norway). All TTE studies conducted prior to TAVI, in the early postprocedural period (mean 5 ± 3.9 days), and, if available, after 1-year follow-up were analysed using offline software (EchoPAC-PC, GE Vingmed Ultrasound). LVEF and LV volumes were calculated according to Simpson's biplane method from apical two-chamber and four-chamber views. LV dimension and mass were measured in the long parasternal view. LV mass was then indexed to body surface area. Data concerning diastolic function were collected from apical four-chamber view using pulse wave Doppler on tips of mitral leaflets and tissue Doppler pulse wave Doppler on septal and lateral part of the mitral annulus – simplified approach for patients with preserved ejection fraction was used according to recommendations.[17] Right ventricular function was estimated from apical four-chamber view using tricuspid annulus systolic excursion, fractional area change and tissue Doppler velocity of the tricuspid annulus. Left and right atrial volumes were measured from apical four-chamber and two-chamber views at end systole. Maximum and mean aortic valve gradients were measured from continuous wave Doppler and the aortic valve area was calculated using the continuity equation.

Two-dimensional speckle-tracking strain analysis

Speckle-tracking strain analysis was performed offline using EchoPAC-PC software according to recommendations.[18,19] Two-dimensional views were obtained from the apical (four-chamber, two-chamber and three-chamber views) and parasternal papillary muscle view. Three consecutive cardiac cycles of each view were acquired during a breath hold. Special care was taken to avoid foreshortening in apical views. Peak systolic longitudinal strain was analysed from apical three-chamber, two-chamber and four-chamber views. GLS was measured automatically using an 18-segment model. Segmental longitudinal strain and strain rate (Sr) were measured from six LV basal segments. Radial strain and Sr and circumferential strain and Sr were measured at the mid-ventricular level in short-axis view (papillary muscle level) from six segments because radial strain in basal LV segments is an inaccurate measure because of reduced spatial resolution in this tracking direction. Global radial (GRS) and global circumferential strain (GCS) were calculated manually as an average value from these mid-ventricular six segments.[18] Global right ventricular longitudinal strain (RVLS) was measured from apical four-chamber view. After manual careful tracing of the endocardial borders, epicardial borders were automatically traced by the software. Tracing quality and derived curves were visually checked and manual adjustment was performed when necessary. Timing of aortic valve closure was set manually. The region of interest width was adjusted in order to get optimal tracing. Only segments with satisfactory tracking were used for further analysis. Image quality was checked for each examination and only cine-loops with 2D frame rate greater than 50 fps were used. Global and regional deformation indices were recorded. Intraobserver variability was within 5% measured on 10 randomly chosen patients, whereas interobserver variability was less than 10%, which is in concordance with previously reported data.

Outcomes

Data concerning functional status and outcomes (hospitalizations because of heart failure and death) were collected by direct patients' interview, telephone interview with patients, their physicians or next of kin, or review of hospital or autopsy records and death certificates. Functional status was estimated according to New York Heart Association (NYHA) class. Mean time of follow-up after TAVI to telephone interview was 3.5 ± 1.9 years.

Statistical analysis

Data are reported as mean ± standard deviation for continuous variables or percentages of individuals for categorical variables. Comparisons among patient groups before and after TAVI were performed using appropriate statistical tests, depending on data types and distributions (paired *t*-test, Wilcoxon signed-rank test, or McNemar's test). Paired sample correlations among different variables before and after TAVI were also measured. Survival and outcome analysis was performed using Kaplan–Meier curves, log-rank test and Cox regression. The results were

Table 1 Baseline patients' characteristics and comorbidities

	Number of patients	Percent
Sex (men)	23	37
Arterial hypertension	44	71
Percutaneous coronary intervention	16	26
Coronary arterial bypass grafting	12	19
Hyperlipidaemia	35	56
Pulmonary hypertension	20	32
Atrial fibrillation	17	27
Diabetes	16	26
Chronic obstructive pulmonary disease	14	23
Pacemaker	13	21
Peripheral arterial disease	10	16
Porcelain aorta	10	16
Renal failure	13	21
Stroke/transitory ischemic attack	10	16
Valve surgery	4	6
Carotid disease	3	5

considered statistically significant with a P value of 0.05 or less. Statistical analysis was done using IBM SPSS version 21-software.

Results

Patients' characteristics

Out of all 156 TAVI procedures performed during the period 2008–2014, a total of 62 patients who underwent successful TAVI and met inclusion criteria were enrolled in the study (39 women, mean age 84.5 ± 6.6 years). Patients' baseline characteristics and comorbidities are shown in Table 1. Twenty-eight patients had undergone coronary artery revascularization prior to TAVI (percutaneous in 16, surgical in 12), but had no signs of myocardial scar on TTE.

Procedural data

Mean dimension of the CoreValve implanted was 28.2 ± 2.2 mm. Transfemoral approach was used in the majority of patients (88.7%), subclavian in six patients (9.7%) and carotid in one patient (1.6%). Complications after the procedure included: eight pacemaker implantations early after the procedure, four lesions of the femoral artery, one tamponade requiring surgical drainage, three valves re-sheathed and three recaptured, two strokes, two transitory ischemic attacks and blood transfusion because of bleeding in two patients.

Conventional echocardiographic measurements

Echocardiographic data acquired prior and within 1 week after TAVI revealed no changes in either the LVEF, LV diastolic function, or in the cavity dimensions and volumes (Table 2). As expected, a significant drop in aortic pressure gradients and maximal jet velocity as well as an increase in the aortic valve area was observed (Table 2). Postprocedural aortic regurgitation was found in two-thirds of the patients [trace aortic regurgitation in 32 (52%); mild in 7 (11%); and moderate aortic regurgitation in 2 patients (3%)]. At baseline, transmitral Doppler parameters revealed severely impaired myocardial relaxation, elevation of filling pressures (E/e' 17.7 ± 7.5), left atrial dilatation and concomitant pulmonary artery hypertension. Early after the procedure, no change in either the diastolic parameters, left atrial dimensions or in the right ventricular function was observed, whereas a significant drop in the pulmonary artery pressure occurred ($P = 0.028$) (Table 2).

Table 2 Changes in echocardiographic parameters in the early follow-up

	Before TAVI	Early after TAVI	P value
LVEF (%)	64.54 ± 7.97	66.18 ± 8.42	0.124
LVIDd index (cm)	39.96 ± 15.79	38.56 ± 18.15	0.657
RWT	0.49 ± 0.15	0.45 ± 0.10	*0.023**
LV mass index (g/m^2)	152.4 ± 53.2	136.2 ± 56.7	*0.046**
LVEDV (ml)	73.3 ± 29.5	71.7 ± 26.2	0.435
LVESV (ml)	26.7 ± 13.3	25.3 ± 12.6	0.190
E wave (m/s)	1.02 ± 0.37	1.09 ± 0.34	0.101
E/A	1.08 ± 0.71	0.97 ± 0.57	0.228
e' septal	0.05 ± 0.01	0.05 ± 0.01	0.234
e' lateral	0.06 ± 0.02	0.02 ± 0.07	0.186
E/e'	17.76 ± 7.44	18.64 ± 6.09	0.367
Left atrial volume (ml)	88.9 ± 40.9	87.6 ± 35.7	0.734
PAP (mmHg)	42.3 ± 14.9	38.1 ± 13.9	*0.028**
Maximum aortic PG (mmHg)	75.7 ± 28.9	15.25 ± 9.6	*<0.001**
Mean PG (mmHg)	46.8 ± 17.3	7.8 ± 4.7	*<0.001**
AVA (cm^2)	0.77 ± 0.21	1.93 ± 0.69	*<0.001**
AVA/BSA (cm^2/m^2)	0.43 ± 0.13	1.12 ± 0.42	*<0.001**
TAPSE (mm)	19.8 ± 4.3	19.2 ± 3.8	0.265
FAC (%)	63.1 ± 11.6	60.7 ± 10.4	0.343
RV s' (m/s)	0.10 ± 0.02	0.11 ± 0.02	0.185

AVA, aortic valve area; BSA, body surface area; E', early myocardial tissue velocity at septal mitral annulus; E, early transmitral velocity; FAC, fractional area change; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volume; LVIDd, left ventricular internal diameter diastole; PAP, pulmonary artery pressure; PG, pressure gradient; RV, right ventricle; RWT, relative wall thickness; TAPSE, tricuspid annular plane systolic excursion. * $P < 0.05$.

Two-dimensional speckle-tracking strain analysis

Changes in the global and regional longitudinal strain and strain rate early after TAVI are shown in details in Table 3. Before TAVI, the global indices of LV deformation (GLS, GRS) were significantly reduced, with a mild insignificant change in GLS and significant improvement in GRS early after TAVI. Significant regional improvement in longitudinal strain was found in the basal lateral and basal anteroseptum segments, whereas a nonsignificant improvement in the regional basal longitudinal strain was observed in all the other segments. Interestingly, LSr increased significantly after TAVI in almost all basal LV segments. Radial strain and RSr at the mid-ventricular segments also showed a trend of improvement. However, significant changes in the mid-anteroseptum and mid-lateral radial strain were observed. Right ventricular longitudinal deformation did not change significantly after TAVI.

Outcome clinical data

Survival rate 30 days after TAVI was 100%, with a significant improvement in the functional status at the early follow-up period: 20 were in NYHA I (41%), 15 in NHYA II (31%), 7 in NYHA III (14%) and 7 in NYHA IV (14%), whereas before TAVI there were 23 in NYHA II

Table 3 Global and regional strains before and early after transcatheter aortic valve implantation

	Before TAVI	Early after TAVI	*P* value
Global strain			
GLS (%)	−16.71 ± 2.42	−17.32 ± 3.25	0.333
GRS (%)	16.57 ± 6.96	19.48 ± 5.97	*0.018**
GCS (%)	−25.59 ± 5.29	−24.75 ± 6.13	0.508
RVLS (%)	−19.30 ± 5.50	−18.60 ± 5.20	0.558
Regional basal longitudinal strain and strain rate			
LS basal septum (%)	−9.90 ± 4.84	−11.09 ± 4.11	0.105
LSr basal septum (s^{-1})	−0.61 ± 0.28	−0.74 ± 0.29	*0.007**
LS basal lateral (%)	−12.09 ± 4.57	−14.64 ± 7.41	*0.038**
LSr basal lateral (s^{-1})	−0.82 ± 0.29	−1.13 ± 0.46	*<0.001**
LS basal inferior (%)	−12.60 ± 5.75	−13.22 ± 5.05	0.471
LSr basal inferior (s^{-1})	−0.81 ± 0.05	−1.01 ± 0.06	*0.008**
LS basal anterior (%)	−12.85 ± 3.77	−14.02 ± 6.63	0.245
LSr basal anterior (s^{-1})	−0.80 ± 0.31	−0.97 ± 0.43	*0.017**
LS basal posterior (%)	−13.85 ± 4.79	−15.20 ± 5.39	0.237
LSr basal posterior (s^{-1})	−1.03 ± 0.32	−1.24 ± 0.56	0.064
LS basal anteroseptum (%)	−14.07 ± 4.48	−16.43 ± 5.39	*0.048**
LSr basal anteroseptum (s^{-1})	−0.92 ± 0.35	−1.05 ± 0.38	0.129
Regional mid-ventricular radial strain and strain rate			
RS septum (%)	21.07 ± 11.16	19.53 ± 10.49	0.634
RSr septum (s^{-1})	1.48 ± 0.57	1.81 ± 1.52	0.341
RS lateral (%)	16.05 ± 6.75	21.24 ± 11.38	*0.038**
RSr lateral (s^{-1})	1.51 ± 0.52	1.91 ± 0.62	*0.041**
RS inferior (%)	18.10 ± 9.52	18.27 ± 10.33	0.937
RSr inferior (s^{-1})	1.53 ± 0.58	1.60 ± 0.62	0.796
RS anterior (%)	10.51 ± 5.40	15.85 ± 8.53	0.068
RSr anterior (s^{-1})	1.24 ± 0.45	1.70 ± 0.91	0.075
RS posterior (%)	20.08 ± 16.17	23.03 ± 15.45	0.121
RSr posterior (s^{-1})	1.47 ± 0.49	1.69 ± 0.74	0.181
RS anteroseptum (%)	11.08 ± 5.73	17.25 ± 8.15	*<0.001**
RSr anteroseptum (s^{-1})	1.09 ± 0.51	1.46 ± 0.57	*0.025**

GCS, global circumferential strain; GLS, global longitudinal strain; GRS, global radial strain; LS, longitudinal strain; LSr, longitudinal strain rate; RS, radial strain; RSr, radial strain rate; RVLS, right ventricular longitudinal strain. * $P < 0.05$.

(37%), 35 in NYHA III (56%) and 4 patients in NYHA IV (7%) functional status. In the late follow-up period of 3.5 years, 28 patients (45%) died and 6 were lost from the final following-up. Among patients who died, 21% ($N = 6$) suffered from cardiac-related death. Concerning hospitalizations, there were altogether 10 hospitalizations (16%) because of heart failure. Echocardiographic measurements before and early after TAVI (5 ± 3.9 days) were analysed in correlation with mortality and composite outcomes (mortality and hospitalizations because of heart failure) (significant correlations are shown in Table 4). Diastolic function was found to correlate with mortality. Abnormal relaxation ($E < A$) early after TAVI was found to have better prognosis compared with patients with more severe diastolic dysfunction (*E*/*A* 0.78 vs. *E*/*A* 1.23, $P = 0.038$; $\rho = 0.300$, $P = 0.041$). Concerning composite outcomes, higher *E* wave velocity before (i.e. worse diastolic function, abnormal relaxation and increase in diastolic filling pressures) correlated with worse composite outcomes (0.93 ± 0.34 vs. 1.14 ± 0.38; $P = 0.041$, $\rho = 0.29$, $P = 0.033$). Also, early after TAVI, E wave velocity and *E*/*A* ratio were found to correlate with composite outcomes: higher *E* wave velocity (0.99 ± 0.31 vs. 1.20 ± 0.36; $P = 0.028$; $\rho = 0.36$, $P = 0.008$) and higher *E*/*A* ratio (0.77 ± 0.20 vs. 1.20 ± 0.81, $P = 0.045$; $\rho = 0.370$, $P = 0.022$) was associated with worse prognosis. Kaplan–Meier survival curves showed worse long-term outcomes (both mortality and composite outcomes) in patients with *E*/*A* ratio higher than 1.5 (Fig. 1). Noncompromised RV function and significant postprocedural PAP reduction did not show an effect on survival. Concerning deformation indices, lower segmental radial strain values in the mid-LV anteroseptal region (RSAS) before TAVI significantly correlated with mortality (17.53 ± 7.90 vs. 12.26 ± 6.11%; $P = 0.031$; $\rho = -0.351$, $P = 0.036$); it also showed a trend towards worse composite outcomes (17.50 ± 8.15 vs. 12.56 ± 6.08%, $P = 0.045$; $\rho = -0.312$, $P = 0.064$). Kaplan–Meier curves (Fig. 2) show that patients with RSAS less than 18% had higher morbidity and mortality than patients with better radial strain in this segment

Follow-up echocardiographic data

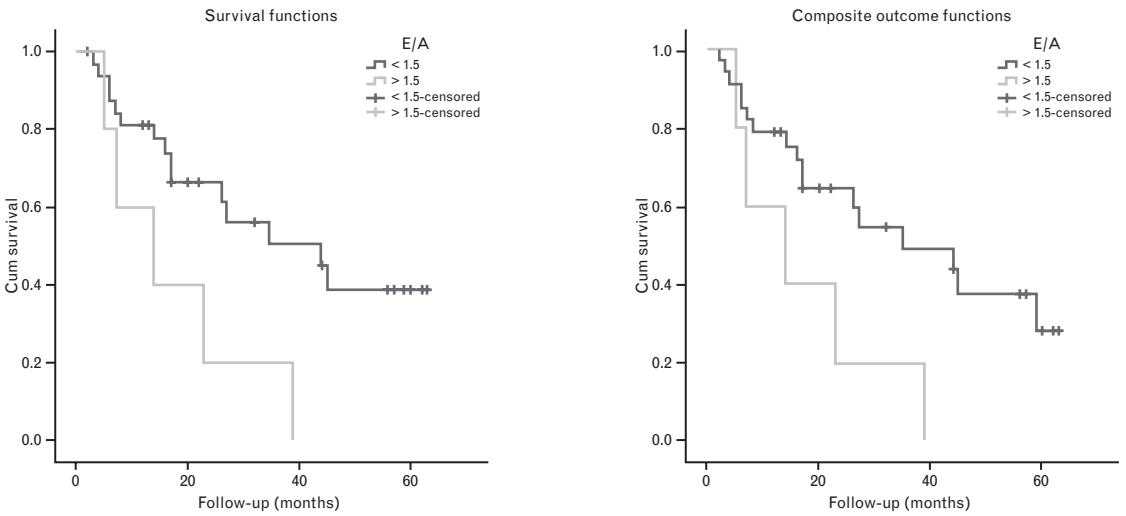
After 1-year follow-up, 21 patients had a complete TTE. Only a few significant differences were found when compared with preprocedural and early postprocedural TTE data. A significant reduction in LV mass index was found (152.4 ± 53.2 pre-TAVI vs. 136.2 ± 56.7 g/m^2 post-TAVI, $P = 0.04$), whereas relative wall thickness, LV volumes and dimensions showed no significant changes. Function of the valve prosthesis was well preserved after 1 year, with no significant changes in maximal ($P = 0.75$) and mean pressure gradients ($P = 0.85$) or in the valve area ($P = 0.86$). There was a nonsignificant improvement in GLS and mid-LV GRS. Improvement in NYHA status was found to significantly correlate only with regional improvement in longitudinal strain of basal interventricular septum ($r = 0.446$, $P = 0.015$).

Table 4 Univariable predictor analysis of mortality and composite outcomes

Outcome	*E* Pre-TAVI	RSAS Pre-TAVI	LVIDd After TAVI	RWT After TAVI	*E* After TAVI	*E*/*A* After TAVI
Mortality	$\rho = 0.260$	$\rho = -0.351$	$\rho = -0.348$	$\rho = 0.376$	$\rho = 0.252$	$\rho = 0.365$
	$P = 0.060$	$P = 0.036$*	$P = 0.009$*	$P = 0.009$*	$P = 0.069$	$P = 0.024$*
	Exp (*B*) = 3.821	Exp (*B*) = 0.892	Exp (*B*) = 0.970	Exp (*B*) = 742.965	Exp (*B*) = 3.978	Exp (*B*) = 12.915
	$P = 0.096$	$P = 0.038$*	$P = 0.073$	$P = 0.038$*	$P = 0.131$	$P = 0.042$*
Composite outcome	$\rho = 0.294$	$\rho = -0.312$	$\rho = -0.344$	$\rho = 0.344$	$\rho = 0.360$	$\rho = 0.370$
	$P = 0.033$*	$P = 0.064$	$P = 0.009$*	$P = 0.018$*	$P = 0.008$*	$P = 0.022$*
	Exp (*B*) = 5.489	Exp (*B*) = 0.902	Exp (*B*) = 0.962	Exp (*B*) = 322.015	Exp (*B*) = 9.556	Exp (*B*) = 16.721
	$P = 0.051$	$P = 0.058$	$P = 0.045$*	$P = 0.066$	$P = 0.036$*	$P = 0.042$*

E, E wave on transmitral flow; *E*/*A*, *E* wave/*A* wave ratio on transmitral flow; LVIDd, left ventricular internal diastolic diameter; RSAS, radial strain of anteroseptum; RWT, relative wall thickness; TAVI, transcatheter aortic valve implantation.

Fig. 1



Kaplan–Meier analysis according to *E/A* ratio early after transcatheter aortic valve implantation: poorer survival and composite outcomes in patients with *E/A* greater than 1.5 (green line) than in patients with *E/A* less than 1.5 (blue line) ($P = 0.022$ and $P = 0.033$).

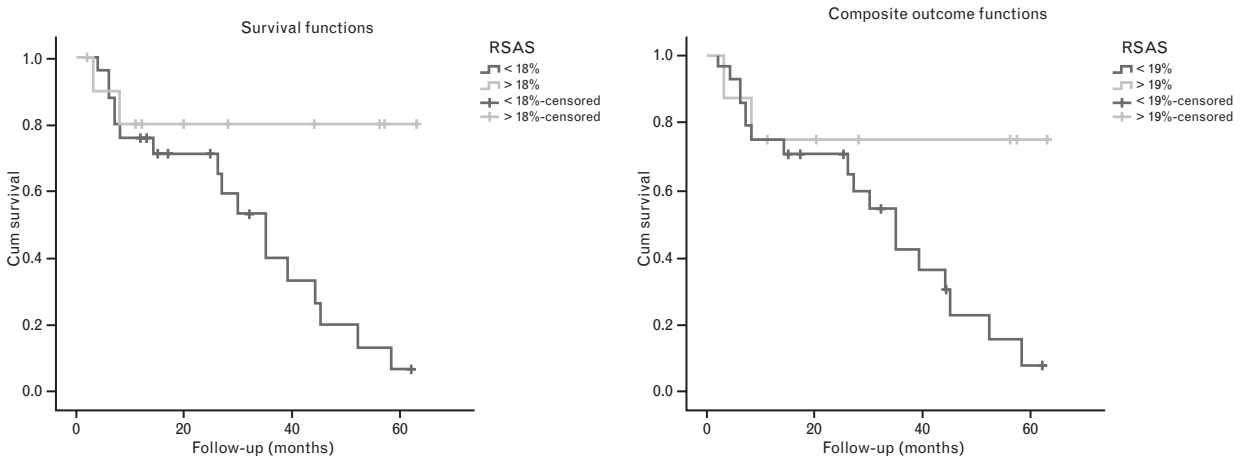
Discussion

We hypothesized that after TAVI, regional longitudinal and radial strain improve because of acute pressure unloading and that those changes are associated with prognosis. The main findings of our study are as follows: LV systolic function as assessed by radial mid-LV GRS and regional longitudinal basal strain of LV anteroseptum and lateral segments improved early after TAVI procedure; radial strain of mid-LV anteroseptum and diastolic dysfunction were associated with a worse prognosis; neither right ventricular function nor the changes in pulmonary pressures after TAVI predicted individual outcome. Those results support given hypothesis.

Left ventricular remodelling in aortic stenosis

In aortic stenosis, the chronically increased afterload results in progressive LV remodelling.[20,21] Early in this compensatory phase, afterload mismatch implies that myocardial contractility is not irreversibly depressed, and that after pressure unloading, recovery of the LV function will be allowed.[22] LVEF measured by conventional means of echocardiography will be preserved even

Fig. 2



Kaplan–Meier analysis according to mid-anteroseptum radial strain before transcatheter aortic valve implantation: RSAS less than 18% implicates worse survival rates ($P = 0.035$) and RSAS less than 19% is associated with worse composite outcomes ($P = 0.084$) (green lines).

at this point, although progressive stepwise impairment of longitudinal and radial deformation can be detected by 2D strain.[23] In our study, LVEF was preserved, whereas GLS and GRS were reduced. So, early signs of subnormal LV function can be reliably detected by 2D strain imaging,[24] as a potential surrogate marker of the presence and severity of myocardial fibrosis, and of likelihood for progression to heart failure. Strain imaging is superior to LVEF in the assessment of latent LV dysfunction.[22] Global indices of longitudinal strain were shown to be a predictor of worse outcomes in both asymptomatic and symptomatic aortic stenosis with preserved LVEF.[25–27] Although the increase in LV wall thickness is a compensatory mechanism that reduces systolic wall stress, it can result in impaired LV relaxation, reduced LV compliance and increased metabolic demands. These early hemodynamic changes can be detected by quantification of LV diastolic function, which is known to have an important role in the progression of symptoms and development of heart failure.[22] In the present study, we showed that prior to TAVI, myocardial relaxation was severely impaired, left atrium was significantly enlarged, and pulmonary artery pressures markedly increased.

Pressure unloading, left ventricular function and outcomes

After TAVI, the so-called 'reverse remodelling' often occurs.[11] We found no change in LVEF measured by conventional echocardiography in the early postoperative period, which is in concordance with previous studies.[28] LV mass and relative wall thickness deceased in the early and late stages after TAVI and predicted individual outcome, suggesting that the vulnerable myocardium can still recover.[10,21]

Diastolic dysfunction may persist in various degrees for a longer period of time[22,24,29] after TAVI. In our study, although diastolic parameters did not change significantly in the early postimplantation period, they were associated with the outcome. Diastolic function both prior to and after TAVI showed positive correlations with mortality and composite end-points. Hypertrophied LV with reduced compliance, impaired relaxation and consequently elevated filling pressures implies pulmonary arterial hypertension even with normal right ventricular function. Such a normal functioning right ventricle may worsen after surgical aortic valve replacement because of unsatisfactory cardioprotection.[30] After TAVI, as we have shown, RV function remained normal, suggesting that the drop in pulmonary pressure was mainly because of LV filling pressure reduction.

Strain is known to be a more sensitive measure of LV function and mechanics.[23] In previous studies, longitudinal strain was mostly analysed. In our study, we also extended the analysis of deformation parameters to regional radial strain and strain rate. Călin *et al.*[22] have demonstrated that longitudinal fibres are most prone to damage during pressure overload, whereas radial and circumferential functions determined by mid-LV fibres are capable of compensating and preserving LVEF for a longer period of time. Radial deformation is normally expressed more, so changes could be easier to detect. Early after TAVI, there was a slight immediate improvement in GLS and in GRS (Table 3). Moreover, segmental strain also showed significant improvement in some segments. Due to acute pressure unloading, significant improvement in regional longitudinal strain was recorded in basal LV anteroseptal and lateral segments. Intriguingly, we did not observe early improvement in longitudinal strain in the region of basal interventricular septum, known to be the most prone to pressure overload.[31] Absolute longitudinal strain values were the worst in this segment before TAVI, not related to outcomes. This could be because of patient's age and longstanding hypertension in more than two-thirds of our patient population (71%), both factors known to influence the function of this segment, leading to more permanent damage (fibrosis).[32–34] Also the low implantation of the prosthesis in some patients might have played a role. On the other hand, early significant improvement in global radial strain and regional radial strain was also detected in mid-LV anteroseptal and lateral segments. Before TAVI, anteroseptum radial strain was found to be associated with outcomes in our population. The better the anteroseptum radial strain prior to the procedure, the more extended was the recovery after TAVI and better was the outcome. Altogether, these changes in strain advocate the presence of an early 'reverse remodelling' phenomenon[10] or 'reverse function'.

Clinical implications

The assessment of LV global and regional function in patients with aortic stenosis has shown to be associated with individual patient outcome. After surgical aortic valve replacement, the extent of improvement in the myocardial deformation parameters reflects the magnitude of LV reverse remodelling and of functional reserve recruitment. In the present study, we extended these observations to TAVI patients showing additionally that LV segmental strain improvement was not uniform. Moreover, we also found that some specific segments were not only improving more than others after TAVI but also they are associated with outcomes. Interestingly, only segments that improved most significantly in longitudinal and radial directions early after intervention showed correlations with prognosis. According to our results, the assessment of anteroseptal LV segment function was of most interest. Impairment in its radial strain identified patients with a worse prognosis. Remarkably, this segment had the highest potential to improve its function early after TAVI. As already described, the use of strain rate was also more sensitive to these changes than strain parameters in our study. So the longitudinal strain rate may thus be a more specific tool to measure the

impact on the LV of pressure unloading in the damaged myocardium. Furthermore, regular assessment of radial strain in the anteroseptal mid-LV segment might be used as a signal to select patients for earlier intervention.

Limitations

The main limitation of our study is its relatively small size with the inclusion of a heterogeneous group of patients concerning comorbidities. As age and comorbidities per se are one of the indications for TAVI, it is hard to obtain a homogenous population with, for example, no coronary artery disease or hypertension. It would be interesting to follow up this population for a longer period of time and to compare our results with magnetic resonance estimation of fibrosis. Also, the results of this hypothesis-generating only study should be cautiously interpreted and confirmed by further investigations on larger populations.

Conclusion

The improvement in LV regional (longitudinal and radial) function early after TAVI, suggesting the potential of the myocardium to recover, is associated with a reduced risk for clinical deterioration. So, not only global but also indices of segmental LV function before and early after TAVI may affect patient prognosis. Diastolic dysfunction before TAVI represents a major outcome determinant after intervention. Altogether, predictors of worse outcomes for composite end-points according to our study are: more hypertrophied, smaller and more stiffened LV with worse diastolic function and decreased radial deformation in the mid-LV anteroseptum segment.

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Conflicts of interest

There are no conflicts of interest.

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LETTER TO THE EDITOR

Myocardial Function in Patients With Radiation-Associated Aortic Stenosis Undergoing Transcatheter Aortic Valve Replacement

A Layer-Specific Strain Analysis Study

Patients with aortic stenosis (AS) and prior mediastinal radiotherapy (XRT) represent a challenging group (1-3). The detrimental effects due to the pressure overload associated with AS may be compounded by the presence of radiation heart disease. Echocardiographic speckle-tracking strain analysis can reliably estimate left ventricular (LV) systolic function and the subtle changes in LV performance after transcatheter aortic valve replacement (TAVR) (4). Because of the different vulnerability to pressure overload of the 3 myocardial layers, multilayer strain analysis can better characterize the extent of damage in AS (3). No studies have assessed the effect of XRT on myocardial function in patients with AS and whether elimination of AS may lead to improvement of LV systolic function. The aim of the present retrospective study was to investigate the impact of prior XRT on layer-specific strains and on post-TAVR early outcomes and recovery of myocardial function in patients with AS.

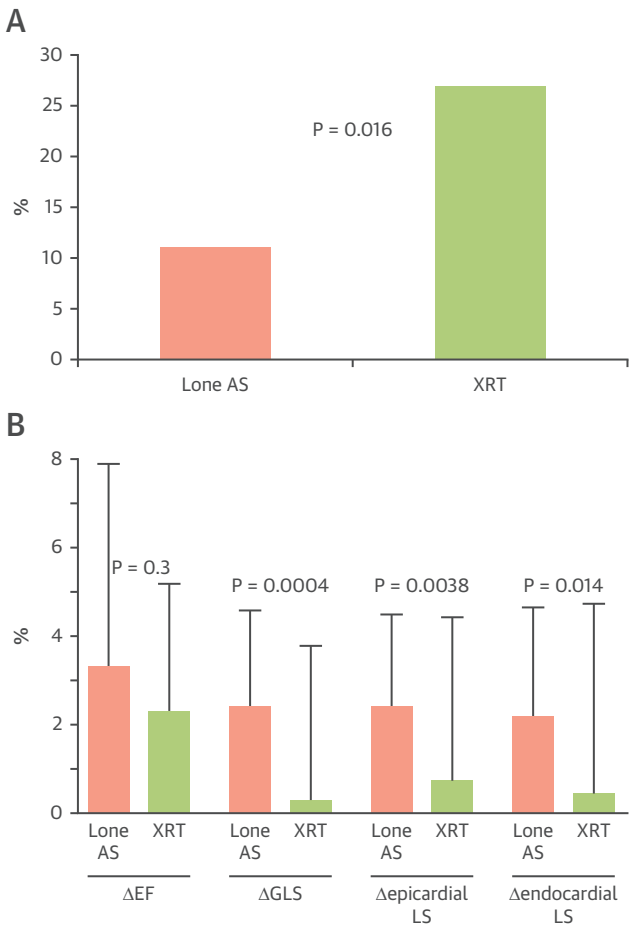
Of 227 patients with severe AS (aortic valve area $\leq$0.6 cm^2/m^2) who underwent TAVR between January 2013 and February 2018, 58 patients were excluded because of suboptimal quality of speckle-tracking image analysis, unclear radiation status, nonsinus rhythm, or valve-in-valve TAVR. The study was approved by the university hospital local ethics committee of Liège, Belgium.

Of the remaining 169 patients (Society of Thoracic Surgeons risk score 6.9 ± 4.1%), 33 (20%) had histories of XRT. All TAVR procedures were performed with CoreValve self-expandable biologic prostheses (Medtronic, Minneapolis, Minnesota) via the transfemoral (n = 146) or transaxillary (n = 23) approach. Clinical endpoints were independently adjudicated according to the Valve Academic Research Consortium-2 criteria. Post-TAVR echocardiographic assessment was performed immediately before discharge and at 6 ± 1.5 month follow-up.

Patients with XRT did not differ from those with lone AS in terms of clinical data (age, sex, cardiovascular risk factors, presence of cardiovascular disease, baseline brain natriuretic peptide level) and baseline conventional echocardiography data (aortic valve area and pressure gradients) except for mitral calcifications (69% vs. 27%; $p < 0.001$) and stenosis (greater than mild, 15% vs. 3.6%; $p < 0.035$). Five patients (15%) in the XRT group presented with porcelain aorta ($p < 0.05$). ST2 level was significantly higher in patients with XRT ($p = 0.026$). Conversely to LV ejection fraction (LVEF) (53 ± 11% vs. 56 ± 11%; $p = 0.26$), longitudinal strains (LS) (epicardial LS 13.8 ± 4.2% vs. 12.2 ± 4.04% [$p = 0.04$], endocardial LS 18.04 ± 5.5% vs. 15.8 ± 4.9% [$p = 0.036$], and global LS 15.6 ± 4.7% vs. 14.1 ± 4.2% [$p = 0.07$]) were significantly decreased in patients with XRT. The rates of stroke (1 [0.7%] vs. 3 [9%]; $p = 0.024$) and delirium (5 [3.7%] vs. 7 [21%]; $p = 0.0023$) after TAVR were higher in patients with XRT, whereas in-hospital death (11 [8.1%] vs. 2 [6.1%]; $p = 0.51$) and major vascular complications (12 [8.9%] vs. 6 [18%]; $p = 0.11$) were similarly distributed between groups. The rate of paravalvular aortic regurgitation was also higher in patients with histories of XRT after TAVR (15 [11%] vs. 9 [27%] for greater than moderate; $p = 0.016$) (**Figure 1A**). Follow-up echocardiography was performed in 103 patients (30 of 33 [91%] of the XRT group). At follow-up, post-TAVR LV systolic function had improved significantly, with increases in LVEF, transmural global LS, and epicardial and endocardial LS ($p < 0.05$ for all). However, except for LVEF, the rate of change was significant only in patients without histories of XRT (ΔLVEF 3.3 ± 4.6% vs. 2.3 ± 2.9% [$p = 0.30$], Δglobal LS 2.4 ± 2.19% vs. 0.29 ± 3.5% [$p = 0.0004$], Δepicardial LS 2.4 ± 2.1% vs. 0.73 ± 3.7% [$p = 0.0038$], and Δendocardial LS 2.16 ± 2.5% vs. 0.44 ± 4.3% [$p = 0.014$]) (**Figure 1B**).

Patients with histories of chest radiation for cancer and severe symptomatic AS have more marked impairment of LV systolic function than those with lone AS. Such alteration mainly concerns a decrease in longitudinal function as assessed by layer-specific strains and is underestimated by the study of LVEF. After TAVR, recovery of heart function is better in patients with lone AS. Conversely, in the presence of radiation cardiomyopathy, myocardial recovery is significantly impaired, with no post-procedural improvement in LS. History of chest radiation is associated with more paravalvular aortic regurgitation and may increase the risk for neurologic events

FIGURE 1 Rate of Paravalvular Regurgitation and Evolution of Layer-Specific Strains After TAVR



(A) Rate of paravalvular regurgitation moderate or greater in patients with and those without histories of radiotherapy (XRT). **(B)** Pre- and post-transcatheter aortic valve replacement (TAVR) differences in layer-specific strains in patients with and those without histories of XRT. AS = aortic stenosis; EF = ejection fraction; GLS = global longitudinal strain; LS = longitudinal strain.

during the hospital stay. A larger cohort of patients, along with longer follow-up, would be necessary to evaluate the impact of our observations on long-term outcomes.

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Part III

Ischemia-driven coronary revascularization: how stress echocardiography can make difference

Complete Revascularization in Acute and Chronic Coronary Syndrome

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KEYWORDS

• Multivessel coronary artery disease (MVD) • Complete revascularization (CR)
• Incomplete revascularization (IR) • Culprit-only revascularization
• Percutaneous coronary intervention (PCI) • Coronary artery bypass graft (CABG)
• Acute coronary syndrome (ACS) • Chronic coronary syndrome (CCS)

KEY POINTS

- Multivessel coronary artery disease (MVD) is a common finding both in acute (ACS) and chronic coronary syndrome (CCS) and poses challenges to revascularization strategy.
- Complete revascularization (CR) has been based on anatomic or functional definitions, both in ACS and CCS.
- In ACS, mainly ST-segment elevation myocardial infarction, CR improves prognosis, but how define significant nonculprit lesions and when treating them still remain highly debated.
- In CCS, when myocardial revascularization (percutaneous coronary intervention or coronary artery bypass graft) is deemed beneficial, a functionally guided CR should be encouraged.
- Heart-team is essential to personalize strategies and reach balanced and optimized decision-making.

INTRODUCTION

Multivessel coronary artery disease (MVD) is a common finding both in acute (ACS) and chronic coronary syndrome (CCS) and poses challenges to revascularization strategy.

Despite the question of whether patients with MVD should undergo complete (CR) versus incomplete revascularization (IR) has been investigated in several studies, this issue still remains debated. This is attributable to conflicting results in clinical studies as well as to an evolved definition of coronary artery disease (CAD) over time, with a shift toward pursuing functional CR. Indeed, various definitions of CR exist and, to date, there is no consensus.[1,2] Although the anatomic-based definition has been the most widely used classification, in contemporary practice, a functional/physiological approach is encouraged.[3–5] Moreover, in the acute setting, the identification of nonculprit lesions (NCLs) poses relevant questions on their management. Finally, the optimal timing for

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reaching CR, in particular in acute presentations, and type of revascularization strategy also remain critical issues to be fully clarified. In this review, we provide an overview of recent evidence and current indication to perform a CR in patients with ACS or CCS and MVD.

COMPLETE REVASCULARIZATION IN ACUTE CORONARY SYNDROME

The identification of NCL is frequent in both ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation ACS (NSTE-ACS). Although in the latter setting there are no dedicated prospective studies on the revascularization strategy with MVD, there are relevant randomized trials for STEMI. In patients with STEMI, primary percutaneous coronary intervention (PCI) to treat the infarct related artery (IRA) or culprit lesion is essential to reduce myocardial damage and prevent reperfusion injury.[6] However, up to half of patients with STEMI show additional significant stenosis.[7,8] In this setting, the optimal management of NCL and whether to perform a CR has been a matter of discussion for years. Indeed, if on one hand most NCLs are asymptomatic or induce limited myocardial ischemia, conversely, it has been demonstrated that MVD following primary PCI associates with worse outcome than single-vessel disease.[7,8] This worse prognosis could be attributable to an increased disease burden, or a pan-coronary process of vulnerable plaque development, responsible for multiple plaque rupture even distant from the culprit lesion throughout the coronary tree.[9] One option could be a culprit-only revascularization with initial medical therapy followed by eventual further revascularization guided by recurrent symptoms. Alternatively, NCL revascularization (anatomically or functionally guided) may be performed immediately during the index procedure or as staged procedure, and the latter, in turn, could be performed during the index hospitalization or on a subsequent readmission. Thus, main open issues are as follows: (1) Is CR really beneficial? (2) If yes, how to optimally define NCLs needing revascularization? (3) Which is the optimal timing for NCL revascularization?

Clinical Evidence

Some randomized trials investigated the preferred strategy for patients with STEMI with MVD (CR vs IRA-only PCI), and also the optimal timing for CR (during index procedure or staged) (**Table 1**). In a small single-center trial, IRA-only PCI was associated with the highest risk of repeat unplanned revascularization, rehospitalization, and in-hospital death at 2.5-years compared with CR (at index PCI or staged).[10] The Preventive Angioplasty in Acute Myocardial Infarction (PRAMI) trial randomized 465 patients with STEMI with MVD to treatment of IRA lesion alone (n = 231) or revascularization of all obstructive (>50% angiographic stenosis) non-IRA lesions during the index procedure (n = 234).[11] Recruitment was stopped prematurely due to highly significant benefit of preventive PCI that at a mean of 23 months significantly reduced the composite of cardiac death or nonfatal myocardial infarction (MI) or refractory angina, as well as cardiac death and nonfatal MI, whereas cardiac death alone did not differ significantly. Interestingly, the benefit was evident within 6 months and maintained thereafter. Similarly, the Complete versus Lesion-only Primary PCI trial (CvLPRIT) (n = 269) showed that CR (>70% angiographic stenosis or 50% in 2 orthogonal views) during index hospitalization significantly reduced death, reinfarction, heart failure, or ischemia-driven revascularization, compared with IRA-only PCI.[12,13] There was a 40% reduction of primary endpoint after 5.6-year median follow-up, with most of the benefit occurring early. The composite of all-cause mortality and MI was also significantly lower in CR, whereas no significant difference was observed in individual components, although all were numerically lower in the CR group. Both the PRAMI and the CvLPRIT trial used the anatomic definition of significant stenosis to guide the CR, and did not evaluate the role of Fractional Flow Reserve (FFR) for MVD. Conversely, 2 randomized trials have proposed FFR to guide NCL revascularization.[14,15] The Third Danish Study of Optimal Acute Treatment of Patients with STEMI: Primary PCI in Multivessel Disease (DANAMI-3 PRIMULTI) trial (n = 627) showed a reduction in composite endpoint (all-cause mortality, reinfarction and ischemia-driven revascularization) with FFR-guided CR versus IRA-PCI only after a mean follow-up of 27 months, although this benefit was mainly driven by reduction of reintervention.[14] Notably, a recent cardiac magnetic resonance substudy on 280 patients showed that CR had no impact on left ventricle function and remodeling, nor on final infarct size, whereas a large but not significant increase of new nonculprit MI, related to periprocedural MI occurring during nonculprit intervention, was observed.[16] The Comparison Between FFR Guided Revascularization versus Conventional Strategy in Acute STEMI Patients with MVD (COMPARE-ACUTE) enrolled 885 patients who were assigned (2:1) to receive IRA-only PCI or FFR-guided CR.[15] Again, FFR-guided CR significantly reduced the composite of all-cause death, nonfatal MI, revascularization, or

Table 1
Overview of randomized clinical trials in patients with acute STEMI with MVD comparing complete revascularization with culprit-only PCI

Characteristics	Politi et al.	PRAMI	DANAMI-3-PRIMULTI	CvLPRIT	Compare-Acute	COMPLETE
Inclusion period	2003–2007	2008–2013	2011–2014	2011–2013	2011–2015	2013–2017
Trial registration	None	ISRCTN73028481	NCT01960933	ISRCTN70913605	NCT01399736	NCT01740479
Multicenter	No	Yes	Yes	Yes	Yes	Yes
Population	214	465	627	296	885	4041
Mean age	65	62	63	65	61	62
Lesion criteria	>70% stenosis	≥50% stenosis	>50%	≥70% or >50% in 2 orthogonal views	≥50% + FFR ≤0.80	≥70% or FFR ≤0.80
FFR measurement of NCL	No	No	Yes	No	Yes	Yes
Timing complete revascularization	2 CR groups randomized to index procedure vs staged revascularization	Index procedure	Index hospitalization	Index procedure or index hospitalization	Index procedure	Index hospitalization or after hospital discharge (no later than 45 d)
Median time from randomization to 2nd procedure (d)	0 (index procedure, n = 65) or 56.8 (staged, n = 65)	0 (index procedure)	2	<2	0 (index procedure)	1 (during admission, n = 1285) or 23 (after discharge, n = 596)
Primary endpoint	CV or all-death, in-hospital death, MI, rehospitalization for ACS, RR	CV death, MI, refractory angina	All-death, MI, RR or non-IRA	Death, reinfarction, HF, any RR	Death from any cause, MI, revascularization, cerebrovascular events	1) CV death or new MI; 2) CV death, new non-fatal MI or ischemia-driven revascularization
Blinded adjudication of clinical events	No	Yes	Yes	Yes	Yes	Yes
FUP, mo	30	23	27	12 (primary) and 66	12	36

(*continued on next page*)

Table 1
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Characteristics	Politi et al.	PRAMI	DANAMI-3-PRIMULTI	CvLPRIT	Compare-Acute	COMPLETE
Main results	20% in CR staged, 23.1% in CR index vs 50% in IR (staged HR: 0.37; 95% CI: 0.19–0.69; *P* = .002; index HR: 0.41; 95% CI: 0.22–0.74; *P* = .003)	9% in CR vs 23% in IR (HR: 0.35; 95% CI: 0.21–0.58; *P*<.001)	13% in CR vs 22% in IR (HR: 0.56, 95% CI: 0.38–0.83; *P* = .004)	At 1y: 10% in CR vs 21.2% in IR (HR: 0.45; 95% CI: 0.24–0.84; *P* = .009); At 5.6 y: 24% in CR vs 37.7% in IR (HR: 0.57; 95% CI: 0.37–0.87; *P* = .008)	7.8% in CR vs 20.5% in IR (HR: 0.35; 95% CI: 0.22–0.55; *P*<.001)	7.8% in CR vs 10.5% in IR (HR: 0.74; 95% CI: 0.60–0.91; *P* = .004)

Abbreviations: ACS, acute coronary syndrome; CI, confidence interval; CR, complete revascularization; CV, cardiovascular; FFR, fractional flow reserve; FUP, follow-up; HF, heart failure; HR, hazard ratio; IR, incomplete revascularization; IRA, infarct-related artery; MACE, major adverse cardiac event; MI, myocardial infarction; MVD, multivessel coronary artery disease; NCL, nonculprit lesion; PCI, percutaneous coronary intervention; RR, repeat revascularization; STEMI, ST-segment elevation myocardial infarction.

cerebrovascular events at 12 months, mainly driven by lower reinterventions. Importantly, FFR-guided revascularizations were performed in 83.6% of cases during the index procedure and elective revascularizations of non-IRA performed within 45 days after primary PCI for clinical evaluations were not counted, as events in the group receiving IRA-only PCI (occurred in 10% of this group).

The most recent Complete versus Culprit-Only Revascularization to Treat Multivessel Disease After Primary PCI for STEMI (COMPLETE) trial was the first powered for hard outcomes (composite of death or MI and the composite of cardiovascular death, MI, or revascularization).[17] A total of 4041 patients who had NCL with at least 70% stenosis or FFR $\leq$0.80 were randomly assigned (1:1) to CR or IRA-only PCI. At a median of 3 years, cardiovascular death or new MI was lower in CR, mainly driven by lower MI. The decision to perform preventive revascularization during the index hospitalization or after discharge (within 45 days after randomization) was specified by investigator before randomization. Interestingly, the benefit of CR was independent of timing of NCL-PCI (Pinteraction = 0.62) and a landmark analysis demonstrated that CR benefit of cardiovascular death or new MI emerged mostly over the long-term, with continued divergence of Kaplan–Meier curves for several years.[18] In an optical coherence tomography substudy, NCLs were in large proportion characterized by thin-cap fibroatheroma, thus, contributing to explain the benefit associated with multivessel revascularization.

Therefore, COMPLETE, the largest trial on the topic, confirmed that CR in patients with STEMI is associated with a significant reduction of the need for repeated revascularization and recurrence of MI. It remained, however, unclear if the lack of benefit in terms of cardiovascular and all-cause mortality was related to unpowered sample size, or to patient characteristics. Indeed, patients were relatively young and with a low mean SYNTAX (Synergy between PCI with Taxus and Cardiac Surgery) score, that could not reflect the clinical setting, often characterized by sicker patients with more diffuse and complex CAD.

All individual trials were underpowered for cardiovascular mortality. A recent meta-analysis included all of them with 6528 patients with STEMI with MVD (3139 CR vs 3389 culprit-only) demonstrating that CR significantly reduced cardiovascular mortality, as well as recurrent MI and repeated revascularization (**Fig. 1**).[19] Notably, CR was not associated with a significant increase of acute kidney injury (AKI), suggesting no

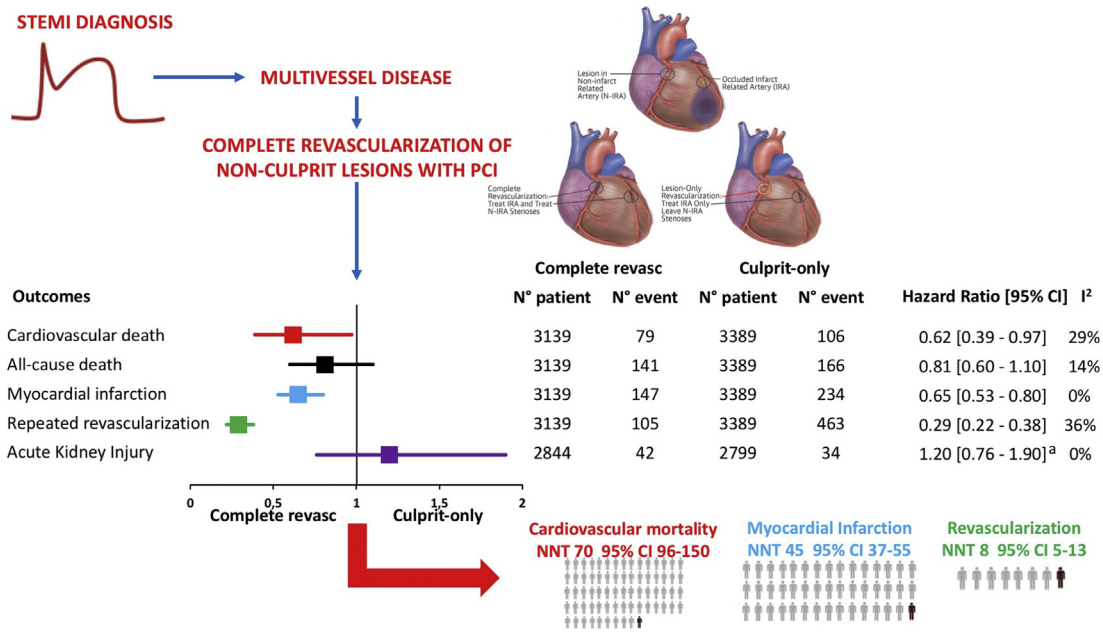

Fig. 1. Impact of complete revascularization on clinical outcomes in patients with STEMI. Summary results of a meta-analysis of 6 trials comparing CR versus culprit-only. CI, confidence interval; NNT, number needed to treat. [a] Risk ratio [95% CI]. (*Data from* Pavasini R, Biscaglia S, Barbato E et al. Complete revascularization reduces cardiovascular death in patients with ST-segment elevation myocardial infarction and multivessel disease: systematic review and meta-analysis of randomized clinical trials. Eur Heart J 2019.)

complications for this strategy; however, this outcome should be interpreted with caution considering that it was available in a limited number of patients and affected by heterogeneous definitions used. There was no benefit on all-cause mortality, likely related to low-risk population, or to length of follow-up. Conversely, a significant reduction in both all-cause mortality and MI was demonstrated in a previous meta-analysis in which the greater benefit was observed in CR performed during index PCI, suggesting that also timing of NCL treatment could affect prognosis.[20]

Data on patients with NSTE-ACS with MVD derive mainly form retrospective studies. In contrast with STEMI, in this setting to identify the culprit lesion is often difficult. Despite limited, some data indicate that CR might improve prognosis even in NSTE-ACS,[21–23] and a randomized trial showed that CR performed in a single procedure seems better than multistage PCI.[24] However, the long-term benefit of CR has to be balanced with the periprocedural risk of pursuing CR, mainly in those patients with complex coronary anatomy or chronic total occlusion (CTO).[25]

Practical Considerations and Future Perspectives

According to European Society of Cardiology (ESC) guidelines for STEMI, revascularization of NCL should be completed before hospital discharge (class IIa, level A).[6] Latest evidence will be incorporated in future recommendations and could change clinical practice. However, the decision whether to perform CR or not, and when/how, should take into account several factors, and data supporting benefits of CR should be interpreted with caution and in light of relevant considerations:

- COMPLETE showed small benefit in terms of cardiovascular mortality that instead was greater in older and smaller trials.
- There was huge variation in trial design, mainly on NCL evaluation (angio vs functional) and when CR was achieved (index vs staged PCI during same or subsequent hospitalization). In COMPLETE, treatment of NCL was mainly based on visual estimation, but nearly 60% of lesions had at least 80% stenosis, thus not requiring FFR. Therefore, beyond those lesions angiographically significant, FFR or instantaneous wave-free ratio (iwFR) may still be important in diagnosing intermediate lesions (50%–69%), and whether CR of such intermediate lesions further reduces the hard endpoints of death or MI at long-term remains unclear. Contrarily, some concerns on the value of functional assessment in the early phase of STEMI are related to concomitant microvascular dysfunction.[26]
- Different antiplatelet regimens may have influenced the findings described among studies conducted in different time periods. In a recent subanalysis of the TRITON-TIMI38 trial a more potent therapy with prasugrel reduced nonculprit MI compared with clopidogrel.[27] This supports that CR in patients with ACS should be attempted to prevent future events, but it could also be speculated that CR might influence the decision-making on the intensity of the antiplatelet therapy (ie, a more potent P2Y12 inhibitor should be always prioritized in patients with ACS, and deescalation to clopidogrel should not be considered in patients not receiving CR).
- Risks related to CR (including AKI and periprocedural MI) may have been underestimated and should never be forgotten because they can negatively impact on prognosis. Some concerns are related to perform CR during the index PCI; indeed, not rarely, an initial thrombotic burden, a nonoptimal IRA reperfusion result, or a significant coronary spasm that would cause inaccurate stent size, can occur and represent potential challenges to CR. Also the potential risk of AKI that may occur in some patients during primary PCI should be taken into account and could induce to decide for a staged approach. On the other hand, an advantage of CR during the index hospitalization is to avoid that patients after discharge do not return to complete procedure.
- Clinical factors always should be considered (patient's age and comorbidities, like chronic kidney disease), to avoid futile complex procedures in frail and old patients. Overall, patients participating in trials are generally less sick than those in the real world, and extending the results to patients with a greater risk of complications may not be safe. Yet, trials had specific exclusions criteria and were not designed to address the specific setting of cardiogenic shock in which MVD is frequent and associated with higher mortality. Guidelines recommended CR of all angiographic significant lesions during the index procedure (class IIa, level C),[6] but the recent CULPRIT shock trial, the largest randomized controlled trial in cardiogenic shock complicating MI (62% STEMI), showed that IRA-only PCI significantly reduced death or renal-replacement therapy at 30 days, and the difference was mainly driven by significantly

lower all-cause mortality. At 1-year, however, mortality did not differ significantly, suggesting that the benefit of culprit-only PCI was confined to the early period during which death in patients with cardiogenic shock mainly occurs.[28] Therefore, a subsequent document stated that in patients with cardiogenic shock complicating MI, primary PCI should be restricted to the IRA, whereas multivessel PCI should be limited to cases in which IRA is difficult to identify or incorrectly defined initially or when multiple culprit lesions are identified.[29]

In patients with NSTE-ACS, given the paucity of data, guidelines suggest to tailor CR to age, general patient condition and comorbidities, and to select a CR during a single procedure or with staged procedures based on clinical presentation, comorbidities, complexity of coronary anatomy, ventricular function, and revascularization modality.[30,31]

Available evidence supports NCL revascularization in patients with STEMI; however, the optimal tool(s) to guide NCL revascularization (which NCL to revascularize?) and the optimal timing for this (NCL assessment and revascularization) remain unsolved. Ongoing randomized trials will provide important insights in the future and are summarized in **Table 2**.

COMPLETE REVASCULARIZATION IN CHRONIC CORONARY SYNDROME

CAD is a chronic and frequently progressive disease that can present long, stable periods but can also become unstable at any time. Because of its dynamic nature, CAD can have different clinical presentations, including ACS or CCS. The latter group includes several clinical scenarios sharing the risk, although variable, of future cardiovascular events (mortality or MI).[32] Together with appropriate lifestyle modifications and optimal medical therapy (OMT), successful myocardial revascularization is crucial to reduce such risk.[30,32] OMT is essential to reduce symptoms, limit atherosclerosis progression, and prevent atherothrombotic events in patients with CCS, but on top of it (without supplanting it), myocardial revascularization (PCI or CABG) is fundamental for 2 main reasons: symptom relief and/or prognosis improvement. Huge evidence has shown that when compared with OMT alone, revascularization is effective in relieving angina, reducing the need for antianginal drugs, and improving exercise capacity and quality of life, as well as reducing the risk of major acute cardiovascular events, including MI and cardiovascular death.[30,32] A practical approach to the indication to revascularization in patients with CCS according to ESC guidelines is summarized in **Fig. 2**.

Selecting PCI or CABG remains a matter of ongoing discussion, but this is beyond our scope and is detailed elsewhere.[30,32–36] However, CR is key for both strategies; indeed, the benefit of CABG versus PCI has been attributed, in part, to greater degree of CR, and relevant evidence has demonstrated worse prognosis with IR compared with CR, either with PCI or CABG.[37–41]

Clinical Evidence

Most data evaluating the impact of CR is based on anatomic definition derived from studies comparing long-term outcomes of PCI versus CABG in MVD patients. In 2 pivotal trials, CR was more frequently reached with CABG, and the benefit of CR over IR was significant in patients with PCI but not in those with CABG.[42–44] Notably, they included PCI using bare-metal stents (BMS) or first-generation drug-eluting stents (DES, paclitaxel-eluting stent). More contemporary data on PCI with new-generation DES, specifically everolimus-eluting stents, showed that among 15,046 patients with MVD, CR was obtained in 30% and significantly reduced cardiovascular events including death compared with IR, and most relevant predictors of IR were the number of vessels diseased and the presence of a CTO.[39] Yet, data from 6539 patients demonstrated that surgical IR had negative impact on long-term survival, and this was strongly associated with age (higher mortality in <60 years but not in older patients).[45] A large meta-analysis on 89,883 patients comparing CR versus IR in MVD confirmed that CR was more often achieved with CABG than PCI and was associated with significantly better long-term mortality, MI, and repeat revascularization.[37] Remarkably, CR benefit was present in both PCI and CABG, and was independent of study design and definition. Similarly, in a pooled analysis of 3 trials including 3212 patients, CR rate was 61.7% (57.2% with PCI and 66.8% with CABG) and CR-PCI was associated with similar survival to CR-CABG at a median of 4.9 years.[46] Moreover, PCI resulting in IR had a higher risk of all-cause death and the composite of death/MI/stroke than CR-CABG. Importantly, these findings were consistent in subgroup analysis of MVD, high SYNTAX score (>32), and diabetes.

Overall, much evidence supports that CR improves outcomes, irrespective of whether achieved through PCI or CABG.

Table 2
Overview of ongoing randomized clinical trials on NCL management in STEMI and/or ACS as reported on clinicaltrials.gov

	FULL REVASC	iMODERN	FLOWER-MI	Safe STEMI for Seniors	FRAME-AMI	MULTISTARS AMI	BIOVASC	FIRE
Trial registration	NCT02862119	NCT03298659	NCT02943954	NCT02939976	NCT02715518	NCT03135275	NCT03621501	NCT03772743
Official title	Ffr-gUidance for compLete Non-cuLprit REVASCularization - a Registry-based Randomized Clinical Trial	Instantaneous Wave-free Ratio Guided Multi-vessel revascularizatiOn During Percutaneous Coronary intervEntion for Acute myocaRdial iNfarction	FLOW Evaluation to Guide Revascularization in Multi-vessel ST-elevation Myocardial Infarction	Study of Access Site for Enhancing PCI in STEMI for Seniors	Comparison of Clinical Outcomes Between FFR-guided Strategy and Angiography-guided Strategy in Treatment of Non-Infarction Related Artery Stenosis in Patients With Acute MI	MULTivessel Immediate vs STAged RevaScularization in Acute MI	Percutaneous Complete Revascularization Strategies Using Sirolimus Eluting Biodegradable Polymer Coated Stents in Patients Presenting With ACS and MVD	Functional vs Culprit-only Revascularization in Elderly Patients With MI and MVD
Estimated N	4052	1146	1170	875	1292	700	1525	1385
Type of patients	STEMI	STEMI	STEMI	STEMI	STEMI	STEMI	STEMI and NSTE-ACS	Elderly (>74y) STEMI and NSTEMI
Study start date	Aug 2016	Dec 2017	Dec 2016	Aug 2017	Aug 2016	Jan 2017	Jun 2018	Jul 2019
Estimated primary completion date	Jun 2021	Jan 2021	Dec 2019	Oct 2022	June 2020	Jun 2020	Dec 2020	Dec 2021

Intervention	FFR-guided PCI of NCL(s) during index hospital admission	iFR-guided revascularization of NCL with >50% diameter stenosis and iFR $\leq$0.89 during index procedure or index hospitalization	Angiography-guided PCI of NCL	iFR-guided revascularization of NCL	Angiography-guided PCI of NCL during index procedure or index hospitalization	Staged CR PCI (new hospitalization after 19–45 d, to complete the coronary revascularization)	Staged CR PCI (within 6 wk after index procedure)	Functionally-guided CR PCI
Alternative intervention	Initial conservative management of NCL	Adenosine stress perfusion CMR scan within 6 wk after STEMI, with revascularization of NCL associated with perfusion defects	FFR-guided PCI of NCL	Initial conservative management of NCL	FFR-guided PCI of NCL during index procedure or index hospitalization	Immediate CR PCI	Immediate CR PCI	Initial conservative management of NCL
Primary endpoint	all-cause mortality and MI during follow-up of minimum 1y	All-cause death, recurrent MI and hospitalization for heart failure at 1y	death, MI and unplanned hospitalization leading to urgent revascularization at 1y	cardiac death, infarct artery target-vessel MI, or ischemia-driven index IRA revascularization at 1y	Any death and any MI at 2 y	all-cause death, non-fatal MI, unplanned ischemia-driven revascularization, hospitalization for heart failure, and stroke at 1y	all-cause mortality, nonfatal type 1 MI, any unplanned revascularization, and cerebrovascular events at 1y	all-cause death, any MI, any stroke, any coronary revascularization at 1y

Abbreviations: ACS, acute coronary syndrome; CMR, cardiac magnetic resonance; CR, complete revascularization; FFR, fractional flow reserve; FUP, follow-up; HF, heart failure; iFR, instantaneous-wave free ratio; IRA, infarct-related artery; MI, myocardial infarction; NCL, nonculprit lesion; NSTE-ACS, non–ST-segment elevation ACS; NSTEMI, non–ST-segment elevation MI; PCI, percutaneous coronary intervention; STEMI, ST-segment elevation MI.

Data from NIH. National Library of Medicine. Clinicaltrials.gov.

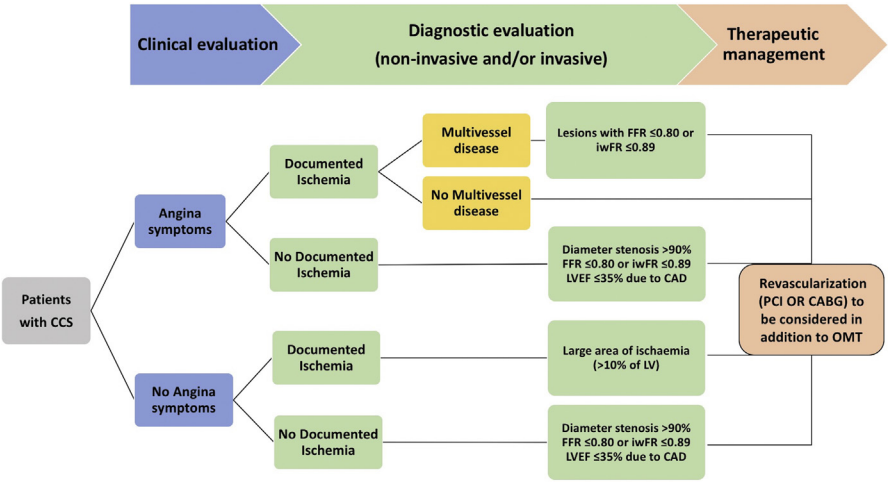

Fig. 2. Algorithm for patients undergoing invasive coronary angiography. CAD, coronary artery disease; FFR, fractional flow reserve; iwFR, instantaneous wave-free ratio; LV, left ventricle; LVEF, left ventricular ejection fraction; OMT, optimal medical therapy. (*Data from* Knuuti J, Wijns W, Saraste A et al. 2019 ESC Guidelines for the diagnosis and management of chronic coronary syndromes. Eur Heart J 2020;41:407-477.)

Since IR has been considered a surrogate marker of greater burden on anatomic coronary complexity and associated with worse outcome,[21] the residual SYNTAX score after PCI has been proposed as an objective measure of residual stenosis and indicator of clinical outcome.[47] In the PCI group of SYNTAX, a residual SYNTAX score greater than 8 was associated with increased long-term mortality and death/MI/stroke, whereas a residual SYNTAX $\leq$8 was associated with long-term mortality comparable with CR-PCI. This finding introduced the concept of "reasonable IR," which implies that an acceptable burden of obstructive CAD postrevascularization is associated with similar outcomes than CR.

Although the anatomy-based definition of CR has been the most widely used in previous studies and practice, optimized decision-making on myocardial revascularization should also account for vessel size, angiographic and functional/physiologic severity of lesions, and myocardial viability. In the past decade, functional-based definition of CR has reached great clinical relevance and attention. Functional CR is accomplished when all lesions causing resting or stress-induced ischemia are treated by either PCI or CABG.

A pivotal trial investigating the impact of functionally guided decision in CCS was the Clinical Outcomes Utilizing Revascularization and AGgressive drug Evaluation (COURAGE) study in which PCI (with BMS) plus OMT had apparently similar all-cause death and MI than OMT alone in 2287 patients with significant coronary lesions and evidence of myocardial ischemia, after a median of 4.6 years. This inevitably led to the conclusion that OMT is as effective as PCI in CCS.[48] However, a nuclear imaging substudy, despite underpowered for prognosis, provided insights into the importance of functional evaluation, indeed, reduction of $\geq$5% of myocardial ischemia was associated with significantly lower rates of death and MI, and this level of ischemia reduction was achieved more frequently with PCI, suggesting that CR might have developed a larger proportion of patients reaching a significant reduction of residual ischemia.[49] As an alternative to noninvasive stress-imaging, FFR provides a validated and recommended method for ischemia detection. In the Fractional Flow Reserve versus Angiography for Multivessel Evaluation (FAME) study, FFR-guided PCI in patients with MVD (cutoff FFR 0.80) was associated with a significant reduction of death, nonfatal MI, or repeat revascularization at 1 year,[50] and mortality plus MI at 2 years.[51] Furthermore, it was cost-saving and cost-effective, being associated with lower use of stents and contrast medium, compared with angiographically guided PCI.[52] In FAME-2, FFR-guided PCI of functional relevant lesions was superior to OMT in preventing urgent revascularization.[5] These results were confirmed at 3 and 5 years with a significant reduction of major adverse cardiac events (MACE), including death, MI, and urgent revascularization.[53,54] These important findings led to propose an FFR-guided SYNTAX score (so-called, "functional SYNTAX score") in patients with PCI with MVD. It showed a better predictive accuracy for MACE than classic

SYNTAX score and also led to decrease by 32% the number of higher-risk patients.[55] Further evidence supporting the functional CR concept rather than angiographic CR alone derived by FAME analysis showed that residual angiographic lesions not functionally significant did not predict poorer outcomes.[56]

A special setting of patients with CCS with MVD is characterized by those with CTO. CTO influences CR and can have an impact on the decision between PCI or CABG. Despite limited evidence from large trials, data from registries and small trials show encouraging results in favor of CTO revascularization (probably due to optimal CR), that improves angina symptoms, quality of life, exercise capacity, and left ventricular function; reduces the risk of ventricular arrhythmias; and improves clinical outcomes.[57]

Recent studies have questioned revascularization value in CCS and generated huge debate. The Objective Randomized Blinded Investigation with optimal medical Therapy of Angioplasty in stable angina (ORBITA) was the first trial to investigate the influence of PCI in a sham-controlled fashion on angina symptoms and exercise time.[58] Despite all included patients had anatomically and/or functionally significant stenosis, PCI failed to improve exercise times or chest pain frequency. However, this was a small study (n = 200) with relevant limitations that should be interpreted with caution when considering daily practice.[59] The recent International Study of Comparative Health Effectiveness With Medical and Invasive Approaches (ISCHEMIA) trial represents an important additional piece of evidence (**Fig. 3**, **Box 1**). ISCHEMIA questioned whether in stable patients with at least moderate ischemia on a stress test, there is a benefit to adding cardiac catheterization and, if feasible, revascularization to OMT.[60] The primary endpoint did not differ at 4 years between conservative and invasive strategy.

Practical Considerations and Future Perspectives

Large evidence and practice guidelines support the role of the heart team to consider myocardial revascularization, whether with PCI or CABG, in patients with CCS with symptoms and/or documented ischemia and MVD, based on a functional/physiologic approach (see **Fig. 2**). Therefore, reflecting contemporary practice of ischemia-based revascularization, a physiologic/functional approach (FFR or iwFR) is considered

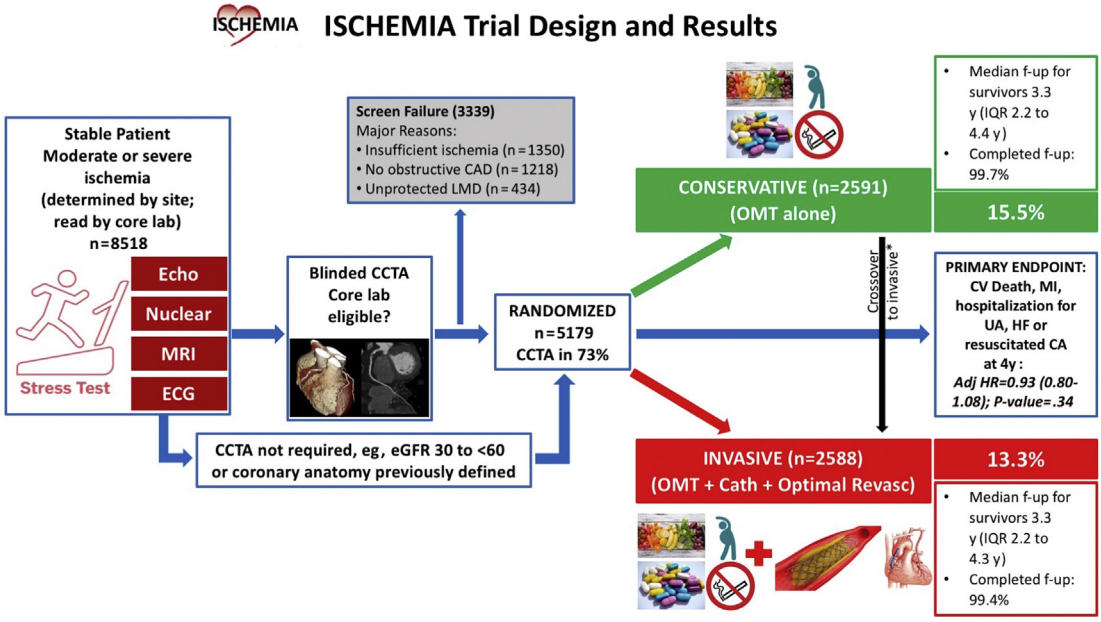

Fig. 3. Design and main results of the ISCHEMIA trial. CCTA, coronary computed tomography angiography; ECG, electrocardiogram; eGFR, estimated glomerular filtration rate; f-up, follow-up; HF, heart failure; HR, hazard ratio; IQR, interquartile range. *At 4 years, indications for cath in CON: 25.8% cumulative incidence 28%): suspected/confirmed event 13.8%; OMT failure 3.9%; nonadherence 8.1%, and revascularization in CON: 16% (cumulative incidence 23%). CA, cardiac arrest; CAD, coronary artery disease; Cath, cathererization; CV, cardiovascular; LMD, left main disease; MI, myocardial infarction; MRI, magnetic resonance imaging; OMT, optimal medical therapy; UA, unstable angina.

Box 1
Eligibility criteria of the ISCHEMIA (International Study of Comparative Health Effectiveness With Medical and Invasive Approaches) trial

Clinical and Stress Test Eligibility Criteria

Inclusion criteria

Age ≥21 years

Moderate or severe ischemia[a]:

- Nuclear ≥10% left ventricular ischemia (summed difference score ≥7)
- Echo ≥3 segments stress-induced moderate or severe hypokinesis, or akinesis
- Cardiac Magnetic Resonance:
  - Perfusion: ≥12% myocardium ischemic, and/or
  - Wall motion: ≥3/16 segments with stress-induced severe hypokinesis or akinesis

Exercise Tolerance Testing (ETT) >1.5 mm ST depression in greater than 2 leads or >2 mm ST depression in single lead at less than 7 METS, with angina

Major exclusion criteria

New York Heart Association Class III-IV heart failure

Unacceptable angina despite medical therapy

Left ventricular ejection fraction <35%

Acute coronary syndrome within 2 months

Percutaneous coronary intervention or coronary artery bypass grafting within 1 year

Estimated glomerular filtration rate less than 30 mL/min or on dialysis (ISCHEMIA chronic kidney disease study)

Coronary Computed Tomography Angiography Eligibility Criteria

Inclusion criteria

≥50% stenosis in a major epicardial vessel (stress imaging participants)

≥70% stenosis in a proximal or mid vessel (ETT participants)

Major exclusion criteria

≥50% stenosis in unprotected left main

[a] Ischemia eligibility determined by sites. All stress tests interpreted at core laboratories.

more reasonable and should be encouraged for appropriate CR.

In past years the so-called "hybrid" revascularization approach in patients with MVD has emerged as alternative to PCI or CABG alone with the aim to achieve CR by reducing the risks of a conventional CABG. Hybrid CR is characterized by the graft of internal mammary artery to the left anterior descending coronary artery through a small thoracotomy and then PCI with DES to other diseased vessels. Promising data support this approach, although potential limitations are also present (technically demanding, bleeding risks related to dual antiplatelet therapy in the immediate postoperative setting). Current ESC guidelines state that hybrid procedures may be considered in specific patient subsets at experienced centers (class IIb, level B).[30] Future studies will offer new insights (NCT03089398).

Despite small and inconclusive, ORBITA highlights that patients with CCS should be carefully evaluated before PCI. Yet, ISCHEMIA results overcome the previous COURAGE limitations (eg, PCI with new-generation DES, revascularization including both PCI and CABG) and reinforce the concept that probably not all patients with CCS with demonstrated ischemia/lesions should undergo revascularization. While waiting for its results be digested by the scientific community and incorporated into guidelines, some considerations can be made:

- Coronary computed tomography angiography reinforced its role in screening patients with suspected CAD, confirming the extent of disease and excluding left main disease.
- OMT and lifestyle changes are essential to all patients.
- Results cannot be extended to all patients with CCS (main exclusion criteria were ACS within 2 months, highly symptomatic patients, left main stenosis, and heart failure or left ventricular ejection fraction <35%).
- In people with chest pain symptoms, revascularization improved symptoms better than conservative strategy and the more symptomatic the patient was, the more symptoms improved after revascularization.
- Procedural MI was increased with an invasive strategy, but spontaneous MI was reduced.
- There were very low rates of procedure-related stroke and death, and all-cause death was low in both groups.
- During follow-up, a not negligible proportion of conservative patients required invasive management.
- Data on CR are not yet available.

SUMMARY

In patients with MVD, CR is the most biologically plausible approach irrespective of definition (anatomic or functional) or type (PCI or CABG) or clinical setting (ACS or CCS). It aims at minimizing residual ischemia, relieving symptoms and reducing the risk of future cardiovascular events. Large evidence supports CR benefits in ACS, predominantly STEMI, except cardiogenic shock, although the optimal tool to evaluate NCL and timing for achieving it remain to be clarified. In CCS, when revascularization is deemed appropriate, a functional CR should be attempted. Therefore, the heart-team plays a crucial role in the individualization of therapies aimed at selecting the ideal strategy for each patient to optimize decision-making. Ongoing studies will further inform our current knowledge.

DISCLOSURE

The authors have nothing to disclose.

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ORIGINAL PAPER

Accuracy of global and regional longitudinal strain at peak of dobutamine stress echocardiography to detect significant coronary artery disease

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Abstract
Dobutamine stress echocardiography (DSE) is sensitive but subjective diagnostic tool to detect inducible ischemia. Nowadays, speckle tracking allows an objective quantification of regional wall function. We aimed to investigate the feasibility and accuracy of global (GLS) and regional longitudinal strain (RLS) during DSE to detect significant coronary stenosis (SCS). We conducted a prospective observational multicenter study including patients undergoing DSE for suspected SCS. 50 patients with positive DSE underwent coronary angiography. Besides visual regional wall motion score index (WMSI), GLS and RLS were determined at rest and at peak stress by Automated Function Imaging. DSE GLS feasibility was 96%. Among 35 patients with SCS, 12 patients were affected by multivessel disease, 18 had stenosis of left anterior descending artery (LAD), 18 of left circumflex (LCX) and 15 of right coronary artery (RCA). At peak stress, both GLS reduction ($p=0.037$) and WMSI worsening ($p=0.04$) showed significant agreement with coronary angiography for detecting SCS. When single lesion was considered, peak stress GLS and LAD RLS were lower in the obstructed LAD regions than in normo-perfused territories (17.4 ± 5.5 vs. $20.5\pm4.4\%$, $p=0.03$; 17.1 ± 7.6 vs. $21.6\pm5.5\%$, $p<0.02$, respectively). Furthermore, the addition of RLS to regional WMSI was able to improve accuracy in LAD SCS prediction (AUC 0.68, $p=0.037$). Conversely, in presence of LCX or RCA SCS, LS was less accurate than WMSI at peak stress. In conclusion, DSE strain analysis is feasible and may improve prediction of LAD SCS, whereas regional WMSI assessment performs better in presence of SCS of LCX and RCA.

Keywords Dobutamine stress echocardiography · Speckle tracking · Regional longitudinal strain · Anterior myocardial ischemia

Introduction

Dobutamine stress echocardiography (DSE) is a recognized test to detect presence and location of coronary artery disease, thanks to its good diagnostic accuracy [1, 2]. However, it remains a subjective method, limited by operators experience on image acquisition and interpretation [3–5]. Moreover, the detection of myocardial ischemia during DSE seems to be even more challenging in the presence of pre-existing wall motion abnormalities [6, 7]. In the last years, speckle tracking echocardiography has emerged as an effective tool for the quantitative assessment of regional wall function [8–11]. Accordingly, the use of this advanced technology during DSE has been proposed as a more objective method to reveal inducible ischemia, being also validated against sonomicrometry in experimental studies [12–14]. To date,

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few reports have described the ability of speckle tracking to detect myocardial ischemia during DSE and mostly in patients without previous regional wall motion abnormalities [15–17]. Considering the exclusion of patients with previous history of coronary artery disease from these studies, their results cannot be extended to this subset of patients. In the present study, we sought to assess the feasibility and accuracy of global (GLS) and regional longitudinal strain (RLS) during DSE to detect significant coronary artery disease in both patients with and without previous wall motion abnormalities.

Methods

This is a prospective observational multicentre study, which included 88 consecutive patients referred for DSE to our cardiac imaging laboratory from October 2015 to December 2018. DSE was prescribed on the suspicion of obstructive coronary artery disease based on symptoms and/or the results of exercise ECG. Patients affected by acute coronary syndrome, significant valvular and congenital heart disease, atrioventricular block, persistent atrial fibrillation, complex ventricular arrhythmias, idiopathic cardiomyopathy, poor acoustic window which prevented a correct evaluation of all myocardial segments, were excluded from the study. The study protocol also included invasive coronary angiography, which was performed on an average of 12 ± 11 days following DSE. The study was conducted in accordance with the amended Declaration of Helsinki. All patients gave their written informed consent at enrollment. Patients data were collected in an anonymous way.

DSE was performed according to standardized staged protocol [18, 19] through an intravenous peripheral infusion with a mechanical pump, starting at dose of 5 µg/Kg/min and increasing at 3-min intervals to 10, 20, 30 and 40 µg/Kg/min. Intravenous atropine up to 1 mg was given at the end of the final stage if needed to increase the heart rate to the target response (85% of age-predicted maximal heart rate). ECG was monitored continuously, and blood pressure was measured at each stage. The test was considered positive in case of development of angina pectoris, new wall-motion abnormalities in at least two contiguous regional segments at any stage of dobutamine infusion, left ventricular end-systolic dilation or severe ischemic ECG changes [19].

Transthoracic echocardiography was performed with subjects in the left lateral decubitus position by experienced echocardiographers (RD, RE), using Vivid E95 and M4S transducer (General Electric Healthcare, Horten, Norway) and stored on a dedicate workstation for off-line analysis (EchoPAC, GE Healthcare). Standard 2D grey scale images of three apical views (four- and two-chamber view, and apical long-axis), parasternal long-axis and parasternal short-axis at the level of papillary muscles were acquired at rest, at low dobutamine dose (20 µg/Kg/min), at peak stress and at early recovery (within 2 min after stress). Left ventricular end-diastolic volume, end-systolic volumes and ejection fraction were measured at rest and at peak stress using Simpson's biplane method of discs [20].

Wall motion was assessed by at least 2 experienced independent observers (FI, CS) using a 17 myocardial segment model, as recommended by guidelines [20]. A semiquantitative scoring system was used to analyse each segment (1 = normal or hyperkinetic; 2 = hypokinetic; 3 = akinetic; 4 = dyskinetic). Global wall-motion score index (WMSI) was calculated as the average of the scores assessed for each segment at rest and at each DSE stage. Figure 1 shows the 17 segments model divided according to perfusion territories of the three major coronary arteries, based on a standardized perfusion model [21]. According to this model, basal, midventricular and apical segments of anteroseptal and anterior walls, and the apex were attributed to left anterior descending artery (LAD) territory. Basal and midventricular segments of posterior wall, and all lateral wall segments (basal, midventricular and apical) were attributed to left circumflex (LCX) coronary artery territory. All inferior wall segments and basal and midventricular segments of posterior septum were attributed to right coronary artery (RCA) territory (Fig. 1). Regional WMSI for each coronary territory was calculated.

Strain analysis was based on speckle-tracking approach and measured by an experienced cardiologist blinded to the clinical history and coronary angiography results (FI, CS). In order to enable GLS analysis, two-dimensional

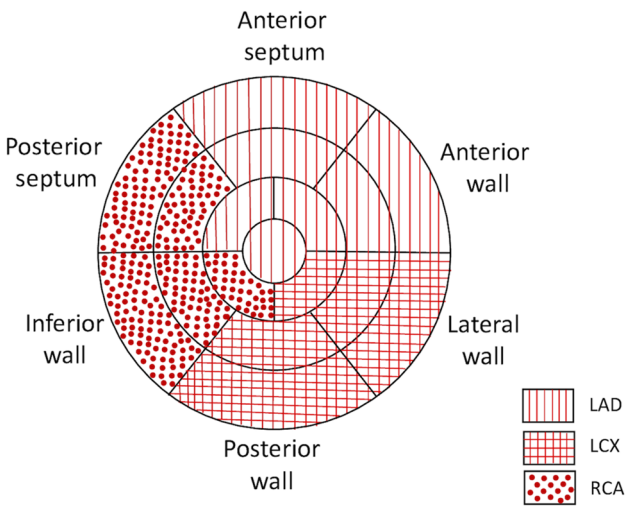

Fig. 1 Circumferential polar plot of the 17 myocardial segments derived by visual wall motion assessment and speckle tracking echocardiography, showing the definition of regions supplied by respective coronary arteries. *LAD* left anterior descending artery; *LCX* left circumflex coronary artery; *RCA* right coronary artery

grayscale images from the apical four-chamber, two-chamber, and long-axis views were acquired at frame rates between 50 and 80 frames/s (mean 63.1 ± 4.4 frames/s), in loops of three successive cardiac cycles. Speckle tracking was performed using the Automated Function Imaging (AFI) algorithm, that is incorporated in a quad-screen of the echo machine, and automatically analyses myocardial motion by tracking frame-to-frame speckle changes. When necessary, manual adjustments were performed to ensure correct 'anchorage' to the mitral annulus, to exclude papillary muscles and chordae from tracking, and to correctly include the left ventricular apex. The width of the region of interest was eventually adjusted to cover the entire myocardial wall thickness. The left ventricle was divided into six myocardial segments in each view, and GLS was calculated as the average of peak longitudinal strain of all segments at end systole [22]. RLS resulted from the sum of the territorial segmental strain divided by the number of segments visualized, applying the same perfusion model used for the regional WMSI analysis (Fig. 1). Inadequate tracked segments were automatically excluded from the analysis. When more than 2 segmental strain within the same coronary territory was not measurable, the strain analysis was considered unfeasible.

Selective coronary angiography was performed in multiple projection according to the Judkins technique, in all patients who underwent positive DSE for inducible ischemia, within 8 weeks of echocardiographic examination. All images were interpreted by experienced operators (GE, ES, PC), who based the estimation of the degree of coronary artery narrowing on visual assessment of angiograms. Significant coronary stenosis (SCS) were defined as ≥ 50% for left main coronary artery, ≥ 70% for the others epicardial arteries. When visual assessment was suggestive of intermediate coronary lesions (40–70% obstruction), fractional flow reserve was performed and SCS was defined with fractional flow reserve < 0.80.

Statistical analyses were performed using IBM-SPSS, version 23 (SPSS Inc, Chicago, IL, USA).

Continuous variables were expressed as mean ± SD and compared with unpaired Student t-test. Categorical data were expressed as percentage and comparisons were made by χ^2 test. A p value < 0.05 was considered statistically significant. Patients were divided in two groups according to the presence of obstructive coronary artery disease detected at coronary angiography. Inter-rater agreement Kappa (κ) was used to evaluate agreement between DSE or RLS analysis and coronary angiography considered as gold standard. When there is perfect agreement, κ is 1. Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic performance of RLS, regional WMSI analysis and their combination.

Results

A total of 88 patients performing DSE were screened for the study. 33 patients were excluded from further analysis because of absence of inducible ischemia at DSE; 3 patients refused the angiographic examination; 2 patients were rejected due to suboptimal RLS quality. The final study population consisted of 50 patients with positive DSE for inducible ischemia. In 8 cases, stress test was interrupted before reaching the target heart rate because of development of angina pectoris (n = 4), occurrence of complex ventricular ectopy (n = 2) or symptomatic hypotension (n = 2) associated with new/worsening wall-motion abnormalities. All patients underwent elective coronary angiography; in 35 (70%) of them SCS was detected. During coronary angiography, the estimation of coronary stenosis severity required fractional flow reserve evaluation in 10 cases. Table 1 reports demographic, clinical and angiographic results of the study population according to the presence or absence of SCS at coronary angiography. Previous percutaneous coronary interventions or coronary artery bypass grafting were more prevalent in patients with SCS (77.1 vs. 33.3% respectively, p = 0.003).

The feasibility of DSE GLS was 96% (n = 50/52). Of the 850 analyzed myocardial segments, 13 (1.5%) at peak stress were rejected due to poor tracking. Table 2 summarizes the main echo parameters at rest and during DSE. Of note, 31 (62%) patients had already wall motion abnormalities at rest, with no significant differences between the groups of patients with or without coronary artery disease (69% vs. 47% respectively, p = 0.144). Left ventricular ejection fraction and volumes, WMSI and GLS did not differ between the two groups at rest and at peak stress. Table 3 reports the concordance in detecting SCS between coronary angiography and both global and regional WMSI and LS at peak stress. The agreement at DSE peak was mild between both WMSI and GLS with coronary angiography (p = 0.041 and p = 0.037, respectively). The agreement between DSE and coronary angiography was higher for RLS for LAD SCS (p = 0.022) compared to regional WMSI (p = 0.031). More precisely, in 94.3% of cases RLS correctly identified significant LAD stenosis, compared to only 56% properly diagnosed by regional WMSI.

When considering SCS of LCX and RCA territories, a significant, despite mild, agreement with coronary angiography was observed only with regional WMSI (p = 0.05 and p = 0.005, respectively). Figure 2 depicts comparisons results of global and regional WMSI, GLS and RLS according to the perfusion territory supplied by the given SCS at peak stress. Regional WMSI appeared to be significantly higher when SCS occurred, independently from which coronary was affected (LAD p = 0.029, LCX

Table 1 Baseline demographic data, clinical and angiographic characteristics according to the presence or absence of significant coronary artery disease

Variables	Total (n = 50)	CAD (n = 35)	No CAD (n = 15)	*P* value
Clinical variables				
Age, years	66.3 ± 8.2	67.2 ± 6.7	63.9 ± 10.7	0.190
Male, gender	41 (82%)	30 (85.7%)	11 (73.3%)	0.296
Body surface area, m^2	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	0.800
Previous CABG/PCI	32 (64%)	27 (77.1%)	5 (33.3%)	**0.003**
Risk factors				
Hypertension	38 (76%)	25 (71.4%)	13 (86.7%)	0.248
Dyslipidemia	33 (66%)	23 (65.7%)	10 (66.7%)	0.948
Diabetes	18 (36%)	12 (36.4%)	6 (40%)	0.809
Current/previous smoker	19 (38%)	15 (48.4%)	4 (30.8%)	0.282
Family history of CAD	10 (20%)	6 (19.4%)	4 (30.8%)	0.410
Chronic kidney disease	10 (20%)	7 (22.6%)	3 (21.4%)	0.931
Systolic arterial pressure, mmHg				
Rest	137 ± 17	137 ± 17	137 ± 16	0.906
Stress	142 ± 30	143 ± 32	139 ± 25	0.656
Diastolic arterial pressure, mmHg				
Rest	77 ± 8	74 ± 10	75 ± 8	0.665
Stress	69 ± 13	70 ± 13	67 ± 14	0.460
Heart Rate, bpm				
Rest	68 ± 13	68 ± 13	68 ± 14	0.892
Stress	130 ± 14	130 ± 15	130 ± 13	0.997
Cardiovascular medications				
Aspirin	38 (76%)	28 (84.8%)	10 (66.7%)	0.15
P2Y12 inhibitors	18 (36%)	14 (42.4%)	4 (26.7%)	0.29
Beta-blocker	33 (66%)	23 (69.7%)	10 (66.7%)	0.83
Statin	40 (80%)	28 (84.8%)	12 (80%)	0.68
ACEi/ARB	37 (74%)	25 (75.8%)	12 (85.7%)	0.45
Calcium Channel Blocker	12 (24%)	9 (27.3%)	3 (20%)	0.59
Nitrate	1 (2%)	1 (2.9%)	0	0.51
Single-vessels disease		23 (65.7%)		
LAD		7 (22.6%)		
LCX		9 (25.7%)		
RCA		7 (22.6%)		
Two-vessels disease		9 (25.7%)		
LAD and LCX		3 (8.6%)		
LCX and RCA		2 (5.7%)		
LAD and RCA		4 (11.4%)		
Three-vessels disease		4 (11.4%)		

Values in bold indicate statistically significant results

Data are expressed as n (%) or mean ± SD

ACEi angiotensin converting enzyme inhibitors; *ARB* angiotensin receptor blocker; *CABG* Coronary artery bypass grafting; *CAD* coronary artery disease; *LAD* left anterior descending artery; *LCX* Left circumflex coronary artery; *PCI* percutaneous coronary intervention; *RCA* right coronary artery

p = 0.011, RCA p = < 0.02). GLS and RLS were lower in the myocardial regions supplied by obstructed LAD than in regions supplied by patent LAD (GLS p = 0.035; RLS p = 0.021). Conversely, no differences were shown in strain analysis when obstruction of the LCX and RCA was observed.

The ROC curves and areas under the curve (AUC) values for RLS, regional WMSI and their combination for the

Table 2 Echocardiographic parameters according to the presence or absence of significant coronary artery disease

Variables	Total (n = 50)	CAD (n = 35)	No CAD (n = 15)	*P* value
LV EF, %				
Rest	56 ± 9	55 ± 9	59 ± 7	0.14
Stress	57 ± 10	56 ± 11	59 ± 7	0.25
End Diastolic Volume, ml				
Rest	97 ± 29	97 ± 31	96 ± 25	0.89
Stress	71 ± 26	74 ± 28	63 ± 19	0.19
End Systolic Volume, ml				
Rest	45 ± 23	46 ± 25	40 ± 16	0.36
Stress	31 ± 18	34 ± 20	26 ± 9	0.16
WMSI				
Rest	1.24 ± 0.33	1.27 ± 0.36	1.17 ± 0.25	0.30
Stress	1.47 ± 33	1.53 ± 0.35	1.34 ± 0.27	0.07
GLS, %				
Rest	19.3 ± 4.5	19.0 ± 4.7	20.2 ± 3.9	0.39
Stress	19.4 ± 5.0	18.8 ± 5.2	20.6 ± 4.6	0.26

Data are expressed as mean ± SD

CAD coronary artery disease; *GLS* global longitudinal strain; *LV EF* left ventricular ejection fraction; *WMSI* wall motion score index

detection of SCS according to the perfusion territory of the three major coronary arteries are shown in Fig. 3. In territories supplied by obstructed LAD, only the combination of RLS at peak stress with regional WMSI abnormality is able to provide a statistically significant AUC value (p = 0.037, AUC = 0.68) (Fig. 3a). As expected, regional WMSI showed good accuracy in detecting ischemia in presence of LCX SCS (p = 0.021, AUC = 0.69), while the addiction of RLS to visual wall motion assessment wasn't able to improve accuracy (p = 0.033, AUC = 0.68) (Fig. 3b). In myocardial segments supplied by obstructed RCA, instead, the addition of RLS to regional WMSI provided slightly higher accuracy in detecting SCS (p = 0.003, AUC = 0.77) compared to regional WMSI alone (p = 0.005, AUC = 0.75). (Fig. 3c).

Interestingly, considering only those patients with resting wall motion abnormalities (n = 31), GLS and RLS – but not global or regional WMSI—were able to identify SCS of LAD coronary artery (p = 0.011 and p = 0.021, respectively) (Fig. 4). Also in this subgroup of patients, GLS and RLS could not distinguish LCX SCS. In myocardial regions supplied by obstructed RCA, instead, RLS—but not GLS—was significantly lower than in regions supplied by patent coronary artery (p = 0.042) (Fig. 4).

Table 3 Agreement between coronary angiography and echocardiographic parameters at peak of DSE in diagnosis of significant coronary artery disease

		Coronary angiography		Agreement	
		No significant stenosis N (%)	Significant stenosis N (%)	Kappa	P value
Global					
WMSI	Normal	3 (20%)	1 (2.9%)	0.217	**0.041**
	Diseased	12 (80%)	34 (97.1%)		
GLS	Normal	4 (26.7%)	2 (5.7%)	0.253	**0.037**
	Diseased	11 (73.3%)	33 (94.3%)		
LAD					
Regional WMSI	Normal	24 (75%)	8 (44.4%)	0.306	**0.031**
	Diseased	8 (25%)	10 (55.6%)		
RLS	Normal	4 (26.7%)	2 (5.7%)	0.322	**0.022**
	Diseased	11 (73.3%)	33 (94.3%)		
LCX					
Regional WMSI	Normal	18 (56.2%)	5 (27.7%)	0.257	**0.05**
	Diseased	14 (43.8%)	13 (72.3%)		
RLS	Normal	14 (43.7%)	7 (38.8%)	0.043	0.74
	Diseased	18 (56.2%)	11 (61.2%)		
RCA					
Regional WMSI	Normal	22 (62.8%)	3 (2.9%)	0.360	**0.005**
	Diseased	13 (37.2%)	12 (97.1%)		
RLS	Normal	20 (57.1%)	8 (53.3%)	0.034	0.80
	Diseased	15 (42.9%)	7 (46.7%)		

Values in bold indicate statistically significant results

GLS global longitudinal strain; *LAD* left anterior descending artery; *LCX* left circumflex coronary artery; *RCA* right coronary artery; *RLS* regional longitudinal strain; *WMSI* wall motion score index

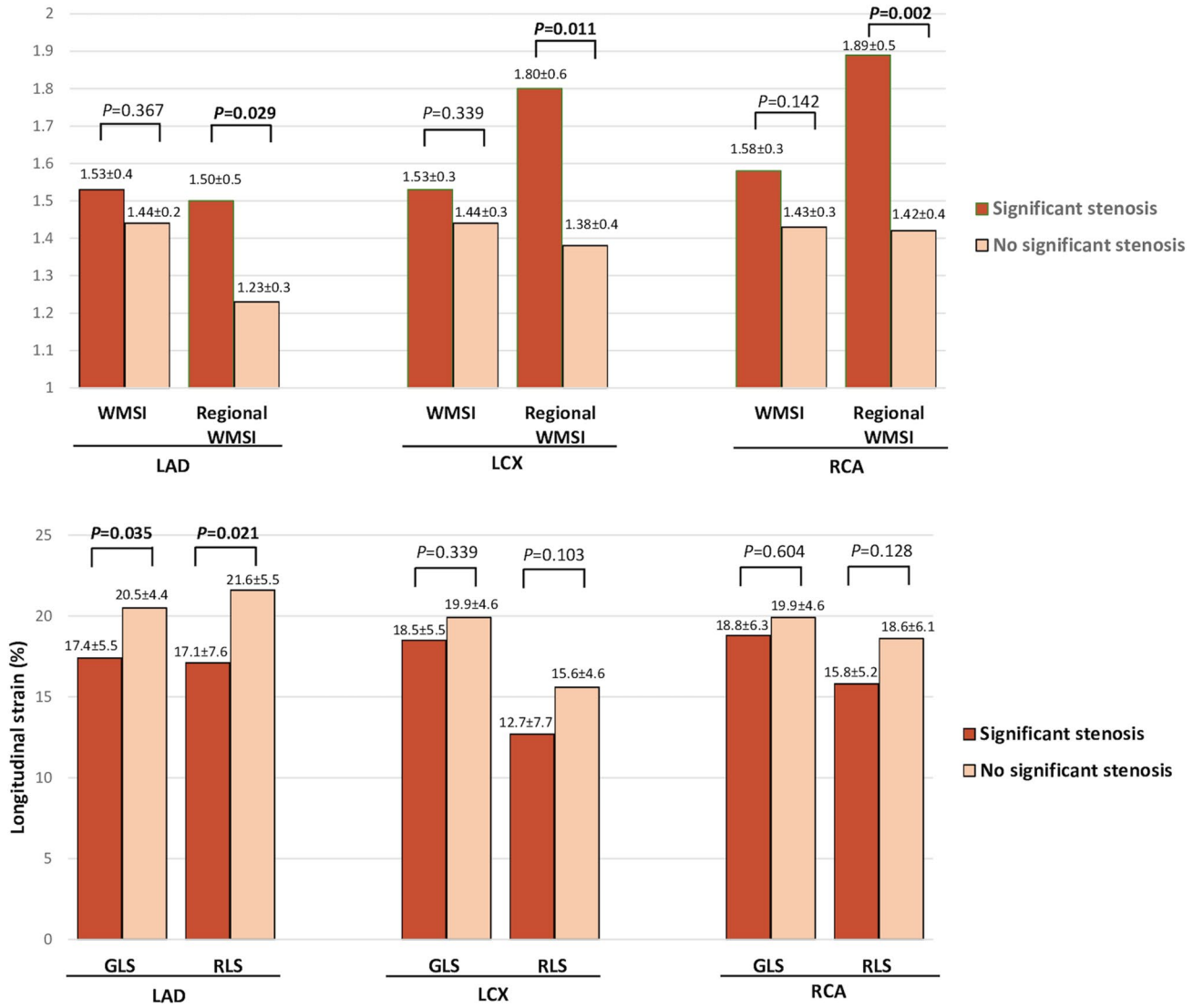

Fig. 2 Bar graph showing comparison between global and regional WMSI, GLS and RLS according to the perfusion territory supplied by the given SCS at peak stress

Discussion

This study demonstrated that: (i) the assessment of longitudinal strain during DSE is feasible even at the elevated heart rate reached during DSE peak; (ii) GLS and RLS at DSE peak are more sensitive than visual WMSI assessment to detect inducible ischemia in the myocardial territories supplied by LAD; (iii) these results are more evident when patients with resting wall motion abnormalities are considered; (iv) in territories supplied by LCX and RCA, GLS and RLS are less reliable to detect obstructive lesions.

Longitudinal strain analysis during DSE has been recently proposed as a quantitative method to overcome the limitation of the visual evaluation of regional wall motion. Despite an average accuracy of > 80% for detection of coronary artery stenosis [2], traditional stress echocardiography is characterized by a subjective interpretation of wall motion abnormalities, even among expert readers [1–3]. This limitation is still more evident in patients with history of coronary artery disease and previously known wall motion abnormalities, a setting in which the identification of residual and/or new areas of ischemia becomes very challenging [6, 7]. In fact, the growth of collateral circulation or the imperfect assignment of myocardial regions to coronary arteries may contribute to the underestimation of ischemia under these circumstances. In our previous experience, we have shown that AFI-derived GLS measured at DSE peak was more accurate than visual WMSI to detect inducible ischemia in patients with three vessel and left main disease [23]. The present study demonstrates that AFI technique can be performed

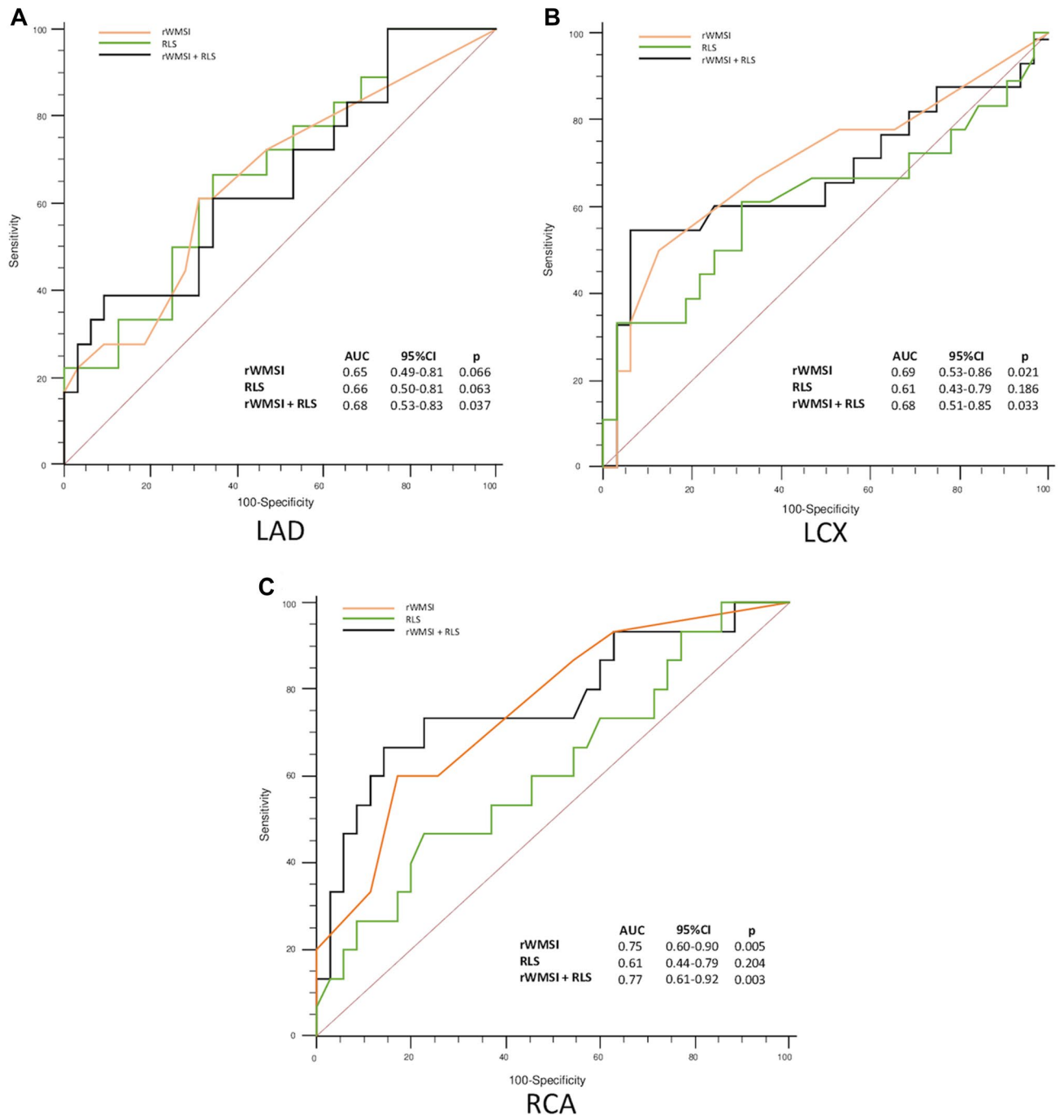

Fig. 3 Diagnostic performance of RLS, regional WMSI and their combination for the detection of regional myocardial ischemia in territories supplied by obstructed LAD (**a**), LCX (**b**) and RCA (**c**). *AUC* area under the curve; *CI* confidence intervals; *LAD* left anterior descending artery; *LCX* left circumflex coronary artery; *RCA* right coronary artery; *rWMSI* regional wall motion score index

during DSE, allowing a correct and almost complete analysis of myocardial deformation at every stages of stress protocol. AFI applied to DSE showed a feasibility of 96%, which is in line with previous literature, where feasibility ranged from 77 to 100% [19]. Non-angle dependent and better signal-to-noise ratio represent two main advantages for the use of strain analysis in the quantitative assessment of myocardial region supplied by obstructed coronary artery. Furthermore, the utilization of phased array probes and software advancement provide an enhanced visualization of structures at the sides of the sector in combination with higher frame rate. This allows to overcome the previously observed limitation

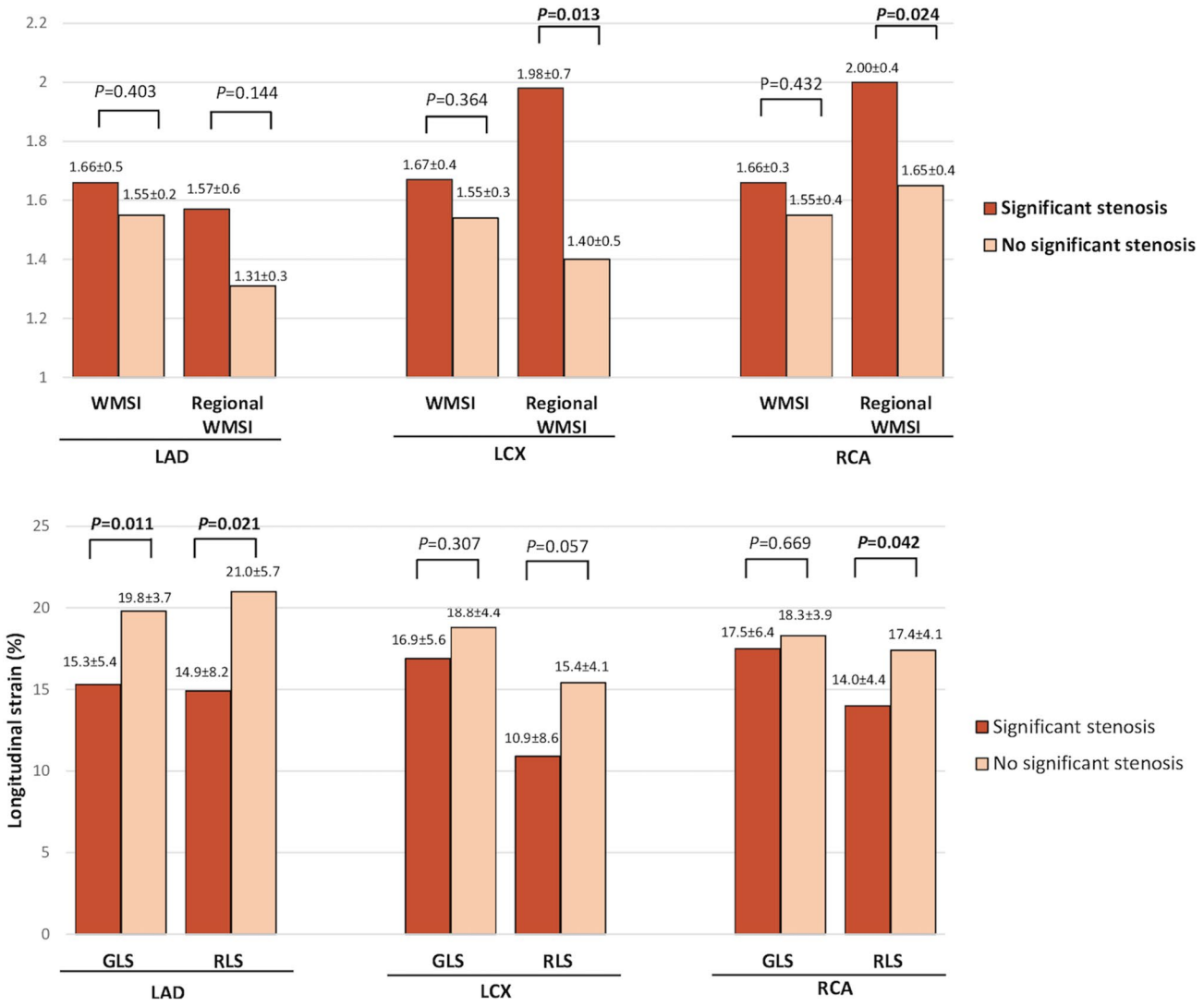

Fig. 4 Bar graph showing comparison between global and regional WMSI, GLS and RLS according to the perfusion territory supplied by the given SCS at peak stress in patients with previous wall motion abnormalities

of a poor speckles' identification occurring at DSE peak [23, 24]. Moreover, the automatic localization of endocardial borders enables a faster and objective quantitative analysis of myocardial longitudinal motion. The clear result presentation with a LV bullseye displaying segmental peak strain values and a corresponding polar map represents a handy tool to identify motion abnormalities even for less experienced operators (Fig. 5).

As a result of these technical improvements, in the present study, GLS at DSE peak stress showed a moderate diagnostic accuracy in detecting SCS, as compared with the results of coronary angiography. Interestingly, when the analysis was restricted to patients with significant LAD stenosis, GLS and RLS showed a better agreement compared to WMSI in detecting SCS. Moreover, the addiction of RLS to the visual wall motion assessment of myocardial ischemia has shown to significantly improve diagnostic accuracy in predicting LAD SCS, over visual wall motion alone. This incremental value of strain imaging at DSE peak could represent an additional tool in reducing false negative results obtained by visual assessment, especially in patients with suspected LAD disease. LAD is usually the largest of the 3 epicardial coronary artery and subtends about 50% of the LV myocardial mass [25]. The presence of significant LAD disease has been associated with worse prognosis than SCS involving other coronary arteries [26, 27]. Thus, given the extent and functional relevance of the myocardial territories supplied by LAD coronary artery, a properly and timely detection of LAD stenosis represents an appealing task. To notice, the incremental value of GLS and RLS in the detection of LAD SCS seems to be even more useful in patients with previous wall motion abnormalities. In this subset of

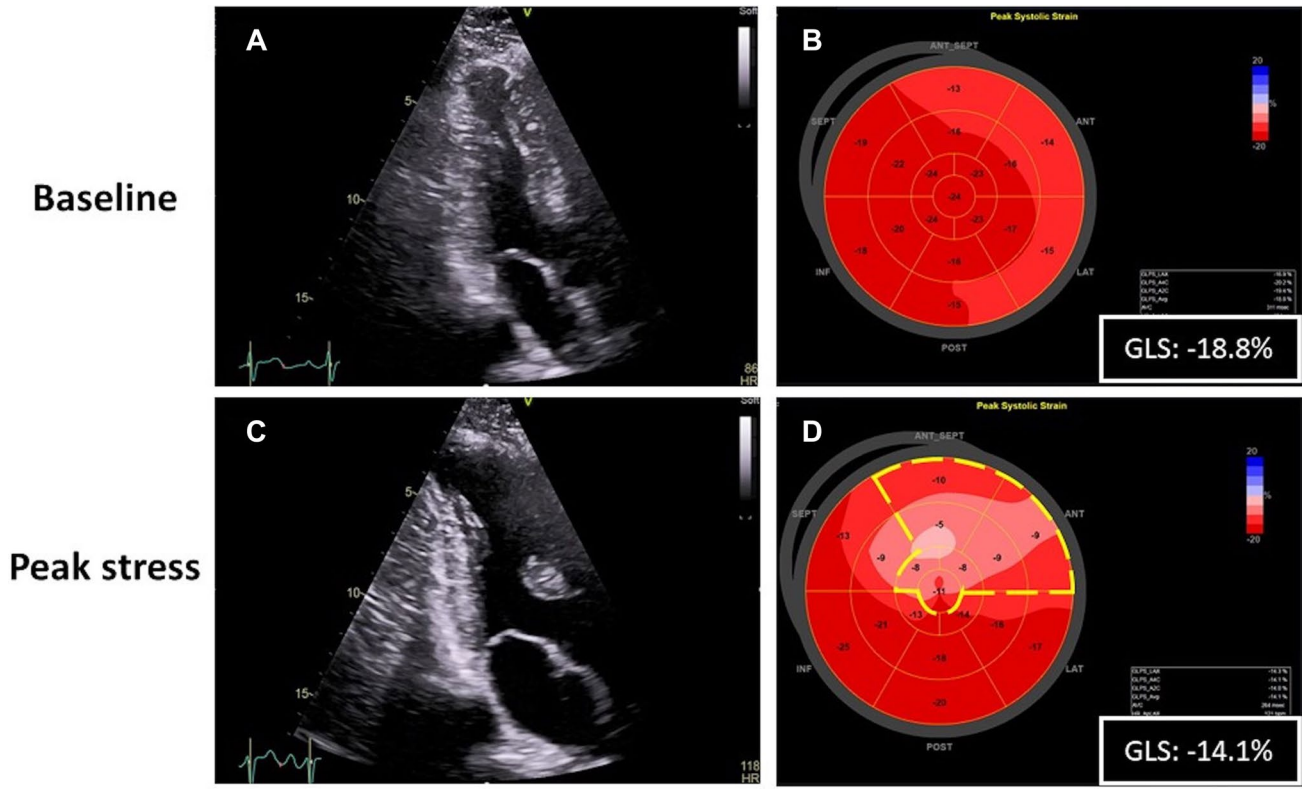

Fig. 5 Application of AFI analysis during dobutamine stress echo in patients with severe obstruction of the LAD. Upper panels showing resting apical long-axis view during end-systole (**a**), and strain analysis performed at the same time (**b**). Lower panels peak stress apical long-axis view depicting an abnormal response with increased left ventricular end-systolic diameter and hypokinetic contraction of the mid-apical segment of the anterior portion of the septum (**c**). Strain analysis performed at peak stress showing a clear concomitant RLS reduction in the LAD territory (dotted yellow lines) and GLS reduction compared to baseline values (**d**)

patients, indeed, strain analysis appears to be more sensitive than visual WMSI in identifying ischemia areas in myocardial segments supplied by LAD coronary artery.

The diagnostic power of both GLS and RLS was not accurate in detecting SCS of LCX and RCA territories where the visual analysis of regional wall motion showed a better agreement with coronary angiography in detecting SCS. It is commonly recognized that the ability to precisely identify a LAD obstruction during stress echocardiography exceeded that for the posterior circulation [19, 28]. Moreover, a discrepancy of the sensitivity between strain measurements in inferior and postero-lateral circulation than anterior coronary circulation had been described in previous studies [16, 17, 29]. In a study of 155 patients, Hanekom et al. reported the best sensitivity of 2D strain in LAD territories (sensitivity 77%, specificity 79%, accuracy 78%) [29]. Similarly, Aggeli et al. described a 2D-strain superior diagnostic efficiency for the evaluation of anterior coronary circulation, as compared to posterior circulation [17]. Roushdy et al. also demonstrated a superiority of 2D strain at DSE peak in detecting LAD and RCA lesions ($\kappa = 0.775$ and 0.415, respectively) [16]. A possible explanation of the reduced reliability of strain in inferior and posterior-lateral circulation could be attributable to the problematic visualization of the posterior endocardium, which compromises image quality, or to the overlap between right and circumflex artery territories, that makes a precise separation of these territories not feasible.

The main limitation of this study is the relatively limited sample size of the included population. Moreover, in the current study, we enrolled all patients with suspected ischemia, including those with previous coronary artery disease which could have represented a potential bias. The analysis of coronary artery disease localization was based on a standardized perfusion model, without considering the overlap in posterior circulation and anatomic variation regarding the coronary artery dominance of each patients. Load dependence of GLS need to be accounted considering dynamic changes in afterload during DSE [19]. However, even though it has not been considered in the design of this study, the effect of load changes throughout the different stages of DSE can be expected to be negligible when RLS is considered. Finally, the exclusion of patients with negative DSE from the study didn't allow the identification of true negative and false negative cases.

Conclusions

AFI-based strain imaging analysis appears to be feasible during DSE even at the highest heart rate achieved at peak stress. Its use during DSE provides a slightly better agreement with coronary angiography results in presence of SCS of LAD, particularly in presence of resting wall motion abnormalities. Conversely, strain analysis correlates poorly with identification of SCS of both right and circumflex arteries, possibly due to scarce visualization of myocardial segments perfused by these two arteries and/or to perfusion territory overlap. Future multicenter study on larger population sample size are needed to test the usefulness of strain imaging during DSE.

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Compliance with ethical standards

Conflict of interest The authors declares that they have no conflict of interest.

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Part IV

Platelet and microvascular function

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Different age-independent effects of nutraceutical combinations on endothelium-mediated coronary flow reserve

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Abstract

Background: Some components of Nutraceuticals (NUT) such as red yeast rice and *Morus alba* have demonstrated positive effects on the endothelial function in hypercholesterolemic subjects. Our aim was to compare the effects of two different NUT combinations on cold pressure test (CPT) derived coronary flow reserve (CFR) assessed by transthoracic echo-Doppler.

Results: In a randomized, single-blind study, 28 consecutive patients with a variety of cardiovascular risk factors received NUT A (LopiGLIK®: berberine, red yeast rice powder, and leaf extract of *Morus alba*) or B (Armolipid Plus®: policosanol, red yeast rice, berberine, astaxantine, folic acidandcoenzyme Q10). An echo-Doppler exam with evaluation of CFR was performed at baseline, 2 h (acute test) and 30 days after daily NUT assumption. Blood sampling for metabolic profile and platelet aggregometry was performed at baseline and after 30 days of daily NUT assumption. CFR was not significantly modified at the acute test. After 30 days, CFR improved with NUT A ($p < 0.0001$), because of the increase of hyperemic flow velocity ($p = 0.007$), but not with NUT B. CFR was comparable between the two groups at baseline but became significantly higher after 30 days in NUT A ($p < 0.02$), with a higher CFR percent variation versus baseline ($p = 0.008$). Total cholesterol and LDL-cholesterol were reduced with both NUT A ($p < 0.001$ and $p < 0.002$, respectively) and B (both $p < 0.02$), whereas platelet aggregation did not significantly change. In the pooled group of patients, after adjusting for age and percent changes of systolic blood pressure, heart rate, LDL-cholesterol and glycemia, NUT A – but not NUT B - was independently associated with CFR changes ($\beta = 0.599$, $p = 0.003$).

Conclusions: LopiGLIK® improved endothelial-derived CFR, independently of the beneficial effects exerted on the lipid profile. These findings can have clinical reflections on the prevention of age-related inflammatory diseases including coronary artery disease.

Trial registration: (NUTRENDO)"(ClinicalTrials.gov, NCT02969070).

Keywords: Cholesterol-lowering, Nutraceutical therapy, Coronary flow reserve, Cold pressure test, Endothelial function

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Introduction

Cardiac coronary system includes three different compartments, which are not well anatomically defined: a proximal compartment of epicardial coronary arteries, an intermediate compartment of pre-arterioles and a distal compartment of intramural arterioles, largely corresponding to coronary microcirculation [1]. Dysfunction of one of these compartments can take place even in the absence of alterations of the other compartments. Coronary system function can be tested by transthoracic Doppler echocardiography through the noninvasive assessment of coronary flow reserve (CFR), which is the maximal increase in coronary flow above its resting value for a given perfusion pressure [2]. It is well recognized that, in absence of significant stenosis of the epicardial coronary arteries, CFR represents an accurate expression of coronary microvascular function. Pharmacological agents used to induce maximal endothelium-independent hyperemia mainly include adenosine and dipyridamole. Hyperemia may even be provoked by a completely endothelium-dependent stimulus such as cold pressure test (CPT), which is performed by hand immersion in ice water for few minutes [2]. Endothelium-mediated regulation of coronary vascular tone acts through the production and release of several vasoactive mediators such as nitric oxide (NO). CPT-derived CFR is largely influenced by traditional cardiovascular risk factors and predicts future coronary events [3].

Nutraceuticals (NUT) are diet supplements that deliver concentrated forms of bioactive agents, isolated or purified from food, that are used in dosages exerting healing properties [4] and are well tolerated (hypoallergenic and digestible). NUT have shown clear beneficial effects on lipid profile [5–8]. According to recent guidelines [9–11], NUT can be used successfully either as alternatives or in addition to lipid-lowering drugs in patients with mild to moderate hypercholesterolemia.

Notably, some components of NUT such as red yeast rice (containing monacolins) and *Morus alba*, have demonstrated their positive effects on the endothelial function in hypercholesterolemic subjects [6, 12]. High cholesterol levels can reduce in fact NO's bioavailability, allowing, therefore, onset and development of atherosclerotic lesions [13, 14]. It is conceivable that this protective effect on endothelial function could be exerted even in patients with cardiovascular risk factors other than hypercholesterolemia. Accordingly, the aim of our study was to compare the acute and 30 days effects of two different NUT combinations on CPT-derived CFR, in a population of patients with different cardiovascular risk factors, wide-ranging hemodynamic profile and variable age.

Methods

Study protocol and population

This is an ancillary study of the clinical trial "Effects of Nutraceutical Therapies on Endothelial Function, Platelet Aggregation, and Coronary Flow Reserve (NUTRENDO)" (ClinicalTrials.gov, NCT02969070). In particular, it is a single center, randomized, single-blind study in which consecutive patients with cardiovascular risk factors received a NUT combination (combination A or combination B) for a 30 days period. The Combination NUT A contained: berberine (531.25 mg), red yeast rice powder (220 mg, 3.3 mg monacolin K) and leaf extract of *Morus alba* (200 mg) and has been approved in Italy (LopiGLIK®, Akademy Pharma). The Combination NUT B contained: policosanol (10 mg), red yeast rice (200 mg, 3 mg monacolin K), berberine (500 mg), astaxantine (0.5 mg), folic Acid (200 mcg) and coenzyme Q10 (2 mg) and is actually approved in Italy for the control of dyslipidemia (Armolipid Plus®, Rottapharm SpA).

The study population included consecutive adult patients (age > 18 years) with cardiovascular risk factors, recruited among the staff personnel of our Department during a screening period for cardiovascular prevention. Among the hypercholesterolemic patients, we enrolled those not requiring statins or statin intolerant patients. Exclusion criteria were intolerance to NUT compounds, pregnancy and high cardiovascular risk profile, overt coronary heart disease and/or heart failure, hemodynamically significant valvular heart disease, primary cardiomyopathies, permanent atrial fibrillation, and inadequate echocardiographic images. The study was carried out following the rules of the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of University of Naples Federico II (258/16). All participants gave their written informed consent.

Procedures

A targeted clinical and familial history was summarized for each patient. All blood samples were collected from the antecubital vein between 0800 and 0900 h after an overnight fast, for the assessment of metabolic profile, and adenosine diphosphate (ADP) and non-ADP platelet aggregometry. A complete echo-Doppler exam with CPT-derived CFR of left anterior descending coronary artery was performed at baseline (i.e., at the randomization time), and repeated after 2 h (acute test) and 4 weeks after the assumption of NUT combinations (chronic test).

Echocardiographic procedures

Echo-Doppler examinations were performed with a Vivid Seven Sound machine (GE) equipped with a 2.5 MHz phased array transducer with harmonic capability,

according to the procedures of our echo lab [15] and current recommendations [16, 17].

CPT and CFR

CPT was performed by placing the subject's hand and distal part of the forearm in an ice water slurry for 4 min [18, 19]. Coronary flow was visualized in the distal left anterior descending coronary artery by transthoracic Doppler echocardiography with a 5 MHz shallow-focus phased-array transducer in the low parasternal long-axis cross section under the guidance of color Doppler flow mapping, according to a standardized protocol of our echo laboratory [20–22]. Doppler sample volume was placed on the color signal of the left anterior descending artery, and the characteristic biphasic flow pattern with a larger diastolic and a smaller systolic component was recorded. Attention was taken to maintaining a constant incident angle (< 30°) between coronary flow and the Doppler beam during the overall exam duration. Coronary diastolic peak flow velocities (cm/s), heart rate, and blood pressure were measured at rest and soon after the CPT at maximal endothelial induced hyperemia. CFR was calculated as the ratio of hyperemic-to-resting diastolic peak velocities (the highest three spectral Doppler signals were averaged for each measurement). Blood pressure (BP) and heart rate were determined at the beginning and the end of CPT test. Reproducibility of CPT-derived CFR measurements of our echo laboratory has been previously reported (2.0% of intra-observer variability and 4.5% of inter-observer variability of 4.5%) [21]. All images were analyzed off-line by two operators who were blind to the patients' clinical characteristics and NUT assumption. The CFR technique operators were unaware of the NUT therapy prescribed to individual patients.

Blood sampling and platelet aggregation

Fasting blood samples for evaluating metabolic profile and platelet aggregation were performed at baseline and after30 days of daily NUT treatment.

The measurements of glucose, total cholesterol (TC) and triglycerides (TG) (all in milligrams per deciliter), were performed by enzymatic methods (Boehringer Mannheim). High-density lipoprotein cholesterol concentration (in milligrams per deciliter) was obtained after precipitation with dextransulfate/MgCl2. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald equation [23].

Platelet aggregation test was performed according to the standards of our laboratory [24]. In particular, pharmacodynamic testing of adenosine diphosphate (ADP) and non-ADP (collagen)-induced aggregation was performed using light transmittance aggregometry LTA (model 700; Chrono-Log, Havertown, PA). Venous blood was collected into sodium citrate tubes. Platelet-rich plasma was obtained after blood centrifugation at 900 rpm for 10 min and platelet-poor plasma obtained after centrifugation of the rest of the blood at 3000 rpm for 10 min at 24 C. All measurements were performed within 2 h of sample collection. Platelet aggregation was measured as the increase in light transmission for 6 min, with the addition of ADP (20 μM/L) and collagen (2 μg/ml). The results are reported as a percentage of maximum platelet aggregation. High platelet reactivity was defined as maximum platelet aggregation > 59% (LTA 20 μM/L) [25].

Results

We enrolled 28 consecutive patients (M/F: 18/10; age: 54.1 ± 9.4 years) with cardiovascular risk factors, randomly selected in two groups: Combination NUT A (n = 14) and Combination NUT B (n = 14). General characteristics of the pooled population and sub-analysis according to the type of NUT combination are presented in Table 1. The two groups were comparable for age, body mass index, diastolic BP and heart rate whereas systolic BP was significantly higher in NUT A (p = 0.034). The prevalence of cardiovascular risk factors did not differ significantly between the two groups. Fifteen of the 28 enrolled subjects (53.6%) presented elevated total cholesterol levels at baseline.

CPT-derived CFR

Acute test (Table 2)

At the CPT performed 2 h after NUT assumption, both NUT A and NUT B did not induce significant changes in patients' CFR or hemodynamic profile (BP and heart rate).

30- days test (Table 3).

In the NUT A group all the 14 patients repeated the CPT-CFR test after 30 days. In this group CFR substantially improved (p < 0.0001) in comparison with the baseline exam because of an increase of post-CPT coronary flow velocity (p = 0.007), whereas coronary flow velocity at rest did not significantly change. Figure 1 shows the improvement of CPT-CFR in a patient after 30 days NUT A therapy. In the NUT B group, one patient declined to repeat the CPT-CFR after 30 days because he declared an intolerance to the test (mainly, he suffered arm pain during the CPT at baseline and during the acute test). In the remaining 13 subjects, CFR was not significantly different in comparison with the baseline exam.

Notably, CPT-CFR was comparable between the two groups at baseline (p = 0.692) but became significantly different after 30 days (1.59 ± 0.14 in NUT A group versus 1.48 ± 0.16 in NUT B group, p < 0.02), with a higher CFR percent variation versus baseline in NUT A group

Table 1 Characteristics of the general population and sub-analysis according to NUT combination

Variable	Overall population ($n = 28$)	Combination A ($n = 14$)	Combination B ($n = 14$)	p value
Age (yrs)	54.1 ± 9.3	54.7 ± 9.8	52.9 ± 8.9	0.610
BMI (Kg/m^2)	27.2 ± 3.3	25.7 ± 2.6	1.9 ± 0.15	0.204
Systolic BP (mmHg)	127.4 ± 10.5	124.5 ± 10.6	133.7 ± 8.2	**0.034**
Diastolic BP (mmHg)	77.7 ± 8.4	77.2 ± 7.3	78.6 ± 10.5	0.670
Heart rate (bpm)	69.2 ± 12.1	68.9 ± 12.8	69.5 ± 11.3	0.899
Arterial hypertension (n, %)	13 (46.4%)	7 (50.0%)	6 (42.9%)	0.705
Diabetes mellitus (n, %)	5 (17.9%)	4 (28.6%)	1 (7.14%)	0.139
Hypercholesterolemia (n, %)	15 (53.6%)	8 (57.1%)	7 (50%)	0.705
Hypertriglyceridemia (n, %)	4 (14.3%)	2 (14.3)	2 (14.3%)	1.00
Smoke habit (n, %)	9 (32.0%)	4 (28,6%)	5 (35.7%)	0.686
CV Familiar history (n, %)	14 (50.0%)	7 (50%)	7 (50%)	1.00

BMI Body mass index, *BP* Blood pressure, *CV* Cardiovascular. Boldface= statistically significant p value

(16.0 ± 9.9) than in group B (3.4 ± 10.2) ($p = 0.008$) (Fig. 2).

Also, restricting the analysis to patients without baseline hypercholesterolemia ($n = 6$), CFR was significantly improved after 30 days in the NUT A group (1.61 ± 0.11 vs. 1.41 ± 0.22, $p < 0.02$) but not in the NUT B group ($p = 0.270$).

Table 2 CFR at time 0 and after 2 h NUT combination intake (acute test) (t-test for paired data)

	Time 0 ($n = 14$)	Acute Test ($n = 14$)	p value
Combination A			
Resting Systolic BP (mmHg)	124.6 ± 6.3	123.2 ± 13.2	0.675
Diastolic BP(mmHg)	79.3 ± 6.5	78.2 ± 8.2	0.487
Heart rate (bpm)	66.1 ± 13.1	68.2 ± 12.7	0.532
Coronary flow velocity (cm/s)	20.1 ± 4.4	19..2 ± 3.4	0.281
Post CPT Systolic BP (mmHg)	122.9 ± 9.9	123.2 ± 14.4	0.944
Diastolic BP (mmHg)	74.8 ± 7.5	78.6 ± 9.3	0.157
Heart rate (bpm)	71.6 ± 15.8	72.0 ± 13.7	0.934
Coronary flow velocity (cm/s)	28.6 ± 6.3	28.7 ± 4.6	0.957
CPT-derived CFR	1.44 ± 0.18	1.49 ± 0.16	0.160
Combination B			
Resting Systolic BP (mmHg)	133.9 ± 7.8	127.2 ± 14.8	0.236
Diastolic BP(mmHg)	80.6 ± 9.5	77.2 ± 9.0	0.169
Heart rate (bpm)	69.9 ± 10.8	71.9 ± 8.5	0.487
Coronary flow velocity (cm/s)	19.3 ± 4.5	19.6 ± 4.1	0.463
Post CPT Systolic BP (mmHg)	119.4 ± 14.7	123.9 ± 19.6	0.396
Diastolic BP (mmHg)	74.8 ± 8.6	78.3 ± 11.7	0.368
Heart rate (bpm)	71.1 ± 10.3	72.9 ± 13.8	0.567
Coronary flow velocity (cm/s)	27.5 ± 5.2	27.7 ± 4.8	0.658
CPT-derived CFR	1.44 ± 0.12	1.46 ± 0.13	0.213

BP Blood Pressure, *CPT* Cold Pressure Test, *CFR* Coronary Flow Reserve

Blood sampling and platelet aggregation

TC and LDL-Cl were significantly lower after 30 days in comparison with baseline values in both patients assuming combination NUT A ($p < 0.001$ and $p < 0.002$ respectively) and NUT B (both $p < 0.02$). No significant difference of HDL-cholesterol, TG, glycemia and platelet aggregation assays was found in both groups at this time (Table 4).

Independent associations of CPT-derived CFR percent changes

In the pooled group of patients, by a multiple linear regression analysis, which adjusted for potential confounders such as age and percent changes of systolic BP, heart rate, LDL-C and glycemia, NUT A – but not of NUT B - was the only variable to be independently associated with CPT-derived CFR changes (standardized β coefficient = 0.599, $p = 0.003$) (cumulative $R^2 = 0.17$, $p = 0.03$).

Discussion

The results of this interventional, single center, randomized, single-blind study demonstrate that in subjects with a variable amount of cardiovascular risk factors and without coronary artery disease (1) both the NUT combinations significantly reduce TC and LDL-cholesterol after 30 days treatment, (2) both NUT A and B are not able to exert a positive acute effect (2 h after the assumption) on CPT-derived CFR, (3) only NUT A combination significantly improves endothelium-mediated CFR after 30 days, and (4) the beneficial effect of NUT A on CFR is independent of LDL-cholesterol changes and other covariates including age, and is not mediated by significant changes in platelet aggregation.

Previous investigations showed that treatment with NUT combinations provide a beneficial effect on the control of dyslipidaemia in patients who have mild hypercholesterolemia and/or are intolerant to statins [4–8]. Current

Table 3 CFR at baseline (Time 0) and after 4 weeks of daily NUT combination intake (t-test for paired data)

Combination A	Time 0 ($n = 14$)	4 weeks CPT CFR ($n = 14$)	*p* value
Resting Systolic BP (mmHg)	124.6 ± 6.3	125.7 ± 8.9	0.865
Diastolic BP (mmHg)	79.3 ± 6.5	80.7 ± 8.0	0.391
Heart rate (bpm)	66.1 ± 13.1	71.2 ± 10.8	0.297
Coronary flow velocity (cm/s)	20.8 ± 4.0	20.4 ± 3.9	0.542
Post CPT Systolic BP (mmHg)	121.7 ± 15.8	125.7 ± 11.6	0.474
Diastolic BP (mmHg)	73.1 ± 9.1	75.5 ± 8.4	0.476
Heart rate (bpm)	69.5 ± 15.0	69.9 ± 7.7	0.521
Coronary flow velocity (cm/s)	28.9 ± 6.2	31.9 ± 6.2	**0.007**
CPT-derived CFR	1.39 ± 0.17	1.59 ± 0.14	**< 0.0001**
Combination B	Time 0 ($n = 13$)	4-weeks CPT CFR ($n = 13$)	*p* value
Resting Systolic BP (mmHg)	133.9 ± 7.8	131.2 ± 9.6	0.269
Diastolic BP (mmHg)	80.6 ± 9.5	79.4 ± 9.7	0.862
Heart rate (bpm)	69.9 ± 10.8	67.3 ± 6.1	0.351
Coronary flow velocity (cm/s)	19.3 ± 4.5	19.2 ± 3.7	0.820
Post CPT Systolic BP (mmHg)	120.8 ± 12.9	123.3 ± 14.9	0.600
Diastolic BP (mmHg)	75.7 ± 7.6	78.3 ± 10.9	0.417
Heart rate (bpm)	69.3 ± 10.1	70.3 ± 7.3	0.531
Coronary flow velocity (cm/s)	27.5 ± 5.2	28.3 ± 5.1	0.435
CPT-derived CFR	1.43 ± 0.12	1.48 ± 0.17	0.323

BP Blood pressure, *CFR* Coronary flow reserve, *CPT* Cold Pressure Test. Boldface= statistically significant *p* value

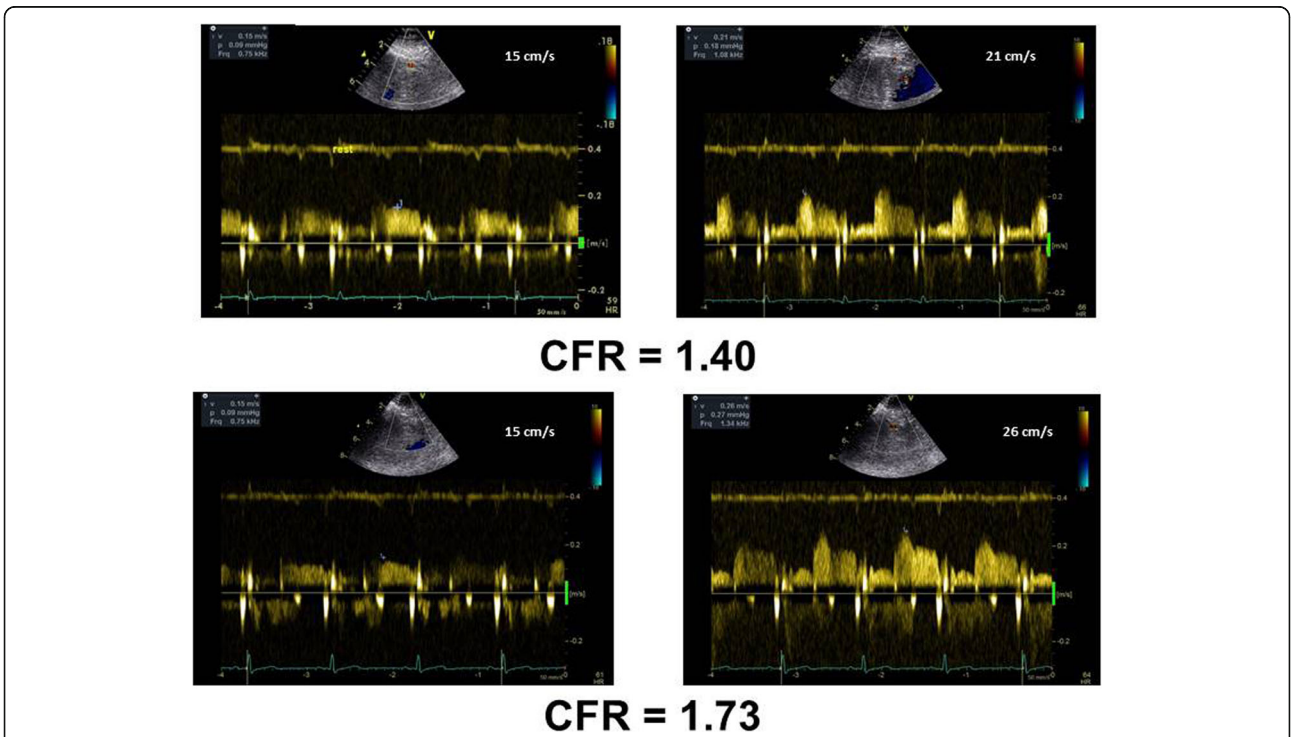

Fig. 1 Beneficial effect of NUT A combination on CPT-derived CFR after 30 days therapy. Time 0 CFR (upper panel): baseline (left) and post CPT (right) coronary flow velocities; Combination A Time 2 CFR (lower panel): baseline (left) and post CPT (right) coronary flow velocities. CFR = Coronary flow reserve. CPT = Cold pressure test

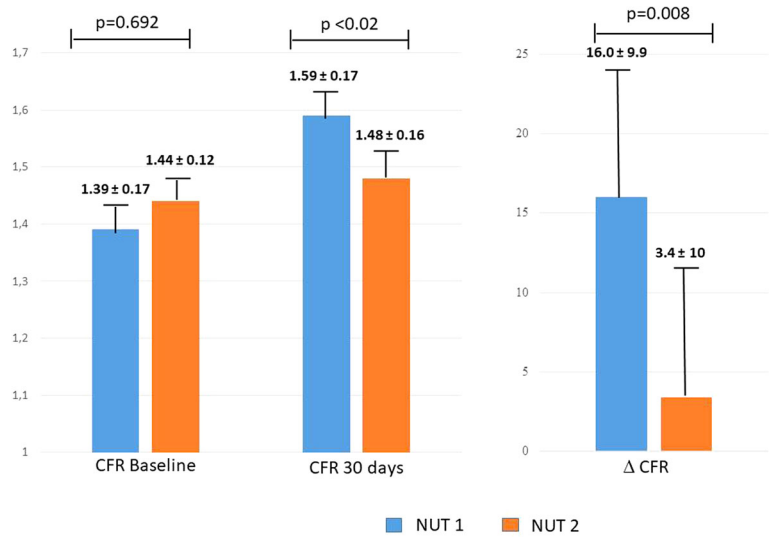

Fig. 2 Comparison of baseline and 30 days CFR, and CFR percent changes (30 days versus baseline) in NUT A and NUT B patients

international guidelines allow the use of NUT under these circumstances [9–11]. In a previous study, NUT A combination has already shown to be more effective than NUT B in reducing TC, LDL-C, TG and glycemia in patients with mild dyslipidaemia [6]. Our findings confirm these differences between the two NUT combinations and extend the beneficial effects of LopiGLIK® on TC and LDL-Cl also to subjects with other cardiovascular risk factors and normal TC levels (13 of the 28 enrolled subjects, 46.4%).

In the present study we tested the acute and 4 weeks effects of both NUT A and NUT B combinations on CPT-CFR. CPT is a well validated sympathetic stimulus able to induce a hyperemic vasodilation, which is totally dependent on NO endothelial release [18, 19]. In healthy subjects, *a*-adrenergically-induced CPT vascular smooth

Table 4 Blood assays at rest and after 4 weeks of daily NUT combination intake T-test for paired data)

Combination A	Time 0 (n = 14)	30 days CPT-CFR (n = 14)	p value
TC (mg/dL)	213.0 ± 39.4	197.1 ± 33.2	**< 0.001**
LDL-C(mg/dL)	138.2 ± 33.1	122.1 ± 25.9	**< 0.002**
Triglycerides (mg/dL)	130.1 ± 70.8	126.2 ± 52.7	0.828
Glycemia (mg/dL)	99.5 ± 24.3	98.2 ± 21.7	0.385
ADP(%)	66.1 ± 18.4	69.0 ± 20.1	0.700
Collagen(%)	60.1 ± 23.7	71.2 ± 25.9	0.124
ADP after insulin stimulation (%)	72.8 ± 12.1	74.5 ± 9.4	0.596
Collagen after insulin stimulation (%)	62.1 ± 26.2	67.7 ± 25.3	0.493
Combination B	Time 0 (n = 13)	30 days CPT-CFR (n = 13)	p value
TC (mg/dL)	209.6 ± 46.6	196.8 ± 35.6	**< 0.02**
LDL-C (mg/dL)	159.8 ± 42.9	148.5 ± 18.4	**< 0.02**
Triglycerides (mg/dL)	125.8 ± 17.7	103.6 ± 47.6	0.316
Glycemia (mg/dL)	91.6 ± 12.9	90.8 ± 7.4	0.861
ADP(%)	72.2 ± 25.4	77.3 ± 17.1	0.553
Collagen(%)	54.0 ± 39.4	69.8 ± 31.8	0.060
ADP after insulin stimulation (%)	71.5 ± 21.4	79.9 ± 11.0	0.167
Collagen after insulin stimulation (%)	55.4 ± 35.8	61.9 ± 38.6	0.156

ADP Adenosine diphosphate, *CPT* Cold Pressure Test, *LDL-C* Low density lipoprotein cholesterol, *TC* Total cholesterol. Boldface= statistically significant p value

muscle vasoconstriction is counterbalanced by a subsequent 'reactive' endothelium-dependent hyperemic vasodilation. In pathological conditions associated with reduced NO bioavailability, the vasoconstrictor effect becomes prominent and coronary blood flow does not increase or may even decrease despite the increase of cardiac work expressed by the rate-pressure product. This methodology has been applied to transthoracic Doppler echocardiography, which allows an easy visualization of coronary flow velocities in the left anterior descending coronary artery during the test. In previous studies, we successfully used this tool to evaluate coronary endothelial function in patients with Kawasaki disease [20] and in those with mild thyroid hormone deficiency [21], two diseases in which the endothelial damage is overt. Of interest, in a subsequent study we observed that recombinant human thyrotropin administration improves CPT derived CFR in differentiated thyroid cancer patients [22], highlighting therefore the ability of this test in evaluating of pharmacologic intervention.

In the present study, both the NUT combinations failed to show significant effects on CFR in the acute test, a result which could have been expected after only two hours from the NUT assumption. Conversely, after 4 weeks therapy, NUT A – but not NUT B combination – was associated with a significant, positive effect on hyperemic coronary flow velocities and thus on CFR. To the best of knowledge, the present study is the first to demonstrate a beneficial action of a NUT combination on the endothelium of coronary arteries. These findings extend to the coronary circulation our previous observation showing the improvement of peripheral flow-mediated dilation (assessed by digital pulse amplitude) produced by a NUT combination [6]. This effect could be mainly due to *Morus alba* (white mulberry), a component originally used in the traditional Chinese medicine, present in NUT A but not in NUT B. Through its effect on endothelial nitric oxide synthase (eNOS) signalling, *Morus alba* extract seems to act as a regulator of CV system, mainly in clinical conditions characterized by eNOS impairment [12, 26]. Of note, the beneficial effects of NUT A on CFR in the present study were not associated with any kind of action on ADP platelet aggregation, thus demonstrating to be independent of rheologic profile modifications. *Morus alba* has demonstrated to significantly inhibit arterial thrombosis [27] in vivo due to antiplatelet activity tested in experiments on rats [28, 29], mainly by impairing the glycoprotein VI pathway [29]. However, this action has never been confirmed in humans.

The novelty of our findings corresponds also to the fact that the positive effect of NUT A combination on endothelium derived CFR was exerted in a population with a variable amount of CV risk factors and a wide age range (33–78 years), even in absence of hypercholesterolemia. This effect was in fact observed even in patients with normal TC levels and remained independent of the percent reduction of both LDL-cholesterol and glycemia, i.e. of the variations of individual metabolic profile. Of interest, it was independent of BP and heart rate, i.e., of rate-pressure product, whose impact on NO endothelial release is well known [18, 19]. It was also independent of age, an important finding in relation with the recognized detrimental influence of aging on coronary endothelial function in patients with metabolic diseases [30].

Our results can be explained by multiple hypotheses. *Morus alba* seems to play a fundamental role in the inhibition of alpha-glucosidase, thereby promoting carbohydrate digestion and a better post-lunch glucose profile, as well as a better insulin sensitivity [31]. *Morus alba* extract reduces BP only in wild-type mice, while it fails to provoke any hemodynamic action in eNOS-deficient mice [10].A possible anti-inflammatory effect of NUT A combination, able to improve endothelial function, could also be considered. Among patients with low-grade systemic inflammation, an oral NUT combination has already shown to significantly improve the degree of systemic inflammation and the consequent endothelial injury [32].

Study limitations

The small sample size of the study population, mainly due to the complex protocol consisting in the repetition of three CPT tests in each patient, is the main limitation. CPT is not very well accepted since it can generate hand pain during the 4 min exposure to ice. One patient of the NUT B arm refused in fact to repeat the test after the 30 days period of daily NUT assumption. Another limitation could correspond to the absence of a definite cut-off point of normalcy for CPT-derived CFR and to the relatively small changes of the coronary flow induced by the hyperemic stimulus. However, our reproducibility of CPT-CFR has previously shown to be very good [21] with an intra-observer variability of only 2%, substantially lower than the percent increase of CFR provoked by both the NUT combinations (15 and 3% with NUT A and B, respectively).

Conclusions

The present study demonstrates a relevant effect of a novel NUT combination, LopiGLIK®, on CFR, in comparison with another combination which does not include *Morus alba*, an extract that has shown a recognized in vivo action on endothelial function [33]. The combination of NUT with dietary counseling has already shown the ability of improving lipid profile, glycemia, diastolic BP and risk scores, and of reducing the prevalence of metabolic syndrome in patients with moderate cardiovascular risk [34]. Our findings open additional new horizons on NUT therapy in blunting or

even preventing the endothelial damage and thus the atherosclerotic progression in patients with a variable amount of cardiovascular risk factors, independently of TC levels and of the effect of aging. These results could have interesting implications on the prevention of age-related inflammatory diseases including coronary artery disease.

Abbreviations
ADP: Adenosine diphosphate; BP: Blood pressure; CFR: Coronary flow reserve; CPT: Cold pressure test; eNOS: Endothelial nitric oxide synthase; LDL-C: Low density lipoprotein cholesterol; NO: Nitric oxide; NUT: Nutraceuticals; TC: Total cholesterol; TG: Triglycerides

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
Conceptualization: MG and RE; Methodology: RS; Software: GG and CS; Validation: RP, MA and FI. Avvedimento and FI; Formal Analysis: FR and GG; Investigation: MS, ME and FR; Data Curation: MA and RE; Writing – Original Draft Preparation: RE and RS; Writing – Review & Editing: RS and MG; Visualization: CS; Supervision:GE, VT and MG; Project Administration: GE and VT. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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ORIGINAL RESEARCH

Rac1 Modulates Endothelial Function and Platelet Aggregation in Diabetes Mellitus

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***Background*—**Vascular complications and abnormal platelet function contribute to morbidity and mortality in diabetes mellitus. We hypothesized that the Rho-related GTPase protein, Rac1, can influence both endothelial and platelet function and might represent a potential novel therapeutic target in diabetes mellitus.

***Methods and Results*—**We used both in vitro and ex vivo approaches to test the effects of pharmacological inhibition of Rac1 during hyperglycemic condition. We evaluated the effect of NSC23766, a pharmacological inhibitor of Rac1, on vascular function in diabetic mice and platelet aggregation in diabetic subjects. We demonstrated that the administration of NSC23766 protects from hyperglycemia-induced endothelial dysfunction, restoring NO levels, and reduces oxidative stress generated by nicotinamide adenine dinucleotide phosphate oxidase. Mechanistically, we identified Rho-associated coiled-coil serine/threonine kinase-1 as a downstream target of Rac1. Moreover, we reported that during hyperglycemic conditions, human platelets showed hyperactivation of Rac1 and impaired NO release, which were both partially restored after NSC23766 treatment. Finally, we characterized the antiplatelet effect of NSC23766 during hyperglycemic conditions, demonstrating the additional role of Rac1 inhibition in reducing platelet aggregation in diabetic patients treated with common antiplatelet drugs.

***Conclusions*—**Our data suggest that the pharmacological inhibition of Rac1 could represent a novel therapeutic strategy to reduce endothelial dysfunction and platelet hyperaggregation in diabetes mellitus. **(*J Am Heart Assoc*. 2018;7:e007322. DOI: 10.1161/JAHA.117.007322.)**

Key Words: endothelial dysfunction • NO • oxidative stress • cardiovascular disease • vascular reactivity

Macrovascular and microvascular complications contribute to morbidity and mortality in diabetes mellitus.[1–3] Endothelial dysfunction and abnormal platelet function represent the main determinants of the vascular accidents in diabetic patients, contributing to high incidence of thrombotic events.[4] Chronic hyperglycemia observed in type 2 diabetes mellitus induces platelet activation and increases reactive oxygen species (ROS) production in endothelium, playing an important role in the development of vascular damage.[5,6]

The small GTPase Rac1 is essential for the correct assembly of nicotinamide adenine dinucleotide phosphate oxidase (Nox) subunits.[7] Several pathways converge in the activation of Rac1, and some evidence suggests a role in different cellular mechanisms, such as cell adhesion, chemotaxis, and vascular permeability.[7] In addition to its role in ROS

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Accompanying Data S1, Tables S1, S2, and Figures S1 through S7 are available at http://jaha.ahajournals.org/content/7/8/e007322/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- The molecular mechanisms that govern endothelial dysfunction and enhanced platelet aggregation in diabetes mellitus are not completely elucidated.
- Herein, we show that Rac1 participates in diabetes mellitus–induced platelet alterations and endothelial dysfunction.

What Are the Clinical Implications?

- Rac1 inhibition reduces platelet hyperactivity and endothelial dysfunction in diabetes mellitus.
- Therefore, Rac-1 could represent a potential therapeutic target to ameliorate both pathophysiological alterations in diabetes mellitus.

generation in endothelium, Rac1 represents a key orchestrator of platelet actin cytoskeleton, modulating, in turn, platelet aggregation.[8] The role of Rac1 in hyperglycemia-induced platelet hyperaggregation is still poorly understood.

Antiplatelet drugs, such as aspirin, are prescribed to diabetic patients for prevention of ischemic cardiovascular diseases; however, many patients exhibit "aspirin resistance" with a high rate of cardiovascular events.[9–11] Furthermore, despite optimal antiplatelet therapy, many patients exhibit high residual platelet reactivity, which has been associated with higher risk of cardiovascular events as well.[12] Therefore, even a small variation in platelet activity might precipitate thrombotic events. This indicates the necessity of identifying new molecular targets to limit platelet aggregation in diabetes mellitus. In this regard, Rac1 could represent a good candidate able to modulate both endothelial function and platelet aggregation. Recently, a small molecule able to inhibit Rac1 activity, named NSC23766, has been developed.[13,14] NSC23766 has been shown to inhibit Rac1 activity by interfering with its binding domain involved in the determination of Rac1's specificity to a subset of guanine nucleotide exchange factors that catalyze the exchange of GDP to GTP to maintain Rac1 in its active, GTP-bound, form.[14,15] Recently, we have shown the beneficial effects of Rac1 inhibition through NSC23766 on endothelial dysfunction in human vessels.[16] However, the mechanisms by which NSC23766 exerts its protective role on endothelial function in hyperglycemia are still partially unknown.

Rho-associated coiled-coil serine/threonine kinase-1 (ROCK1) is the main downstream target of the small GTPase RhoA and has been involved in the regulation of multiple cellular functions involving cytoskeletal organization.[17] Given the important role of Rac1 in endothelial and platelet function, we hypothesized that ROCK1 might represent a potential downstream target of Rac1 activity.

To better understand the role of Rac1 in diabetes mellitus, we investigated the possible therapeutic role of NSC23766 on vascular and platelet alteration using both in vitro and ex vivo approaches in preclinical model of diabetes mellitus and human samples.

Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure on request to corresponding authors. All experiments involving animals were conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (publication 85-23, revised 2011) and were approved by the Istituto di Ricovero e Cura a Carattere Scientifico Istituto Neurologico Mediterraneo Neuromed review board. Human subjects were enrolled at the Cardiology Division of the University of Naples Federico II. The study protocol was conformed to the principles outlined in the Declaration of Helsinki and was approved by the institutional review board of the medical center, and each patient who accepted to participate provided written informed consent. All diabetic patients enrolled in our study fulfilled the criteria of the

Table. Baseline Characteristics of the Study Subjects

Characteristics	Control Subjects (n=11)	Diabetic Subjects (n=22)
Age, y	56.3±5.03	57.05±2.39
Women, %	36	30
Hypertension, %	40	35
Hyperlipidemia, %	22	25
BMI, kg/m^2	24.17±1.1	26.13±1.12
Smoking, %	46	45
HbA1c, %	5.5±0.31	8.3±1.32*
Drug therapy, n		
Statins	0	0
ACEIs	6	10
ARBs	6	10
CCBs	5	10
BBs	4	5
Anticoagulants	0	0
ASA	0	7
Other antiplatelet agents	0	0

Data are represented as mean±SD unless otherwise indicated. ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; BB, β blocker; BMI, body mass index; CCB, calcium channel blocker; and HbA1c, glycated hemoglobin.
*P=0.00012 vs control subjects.

National Diabetes Data Group for diabetes mellitus.[18] Characteristics of patients (demographics, concomitant medication therapy, and glycemic status) are summarized in the Table and Table S1. An expanded description of materials and methods used in this study is available in Data S1.

Statistical Analysis

Data are presented as bar graphs, box-and-whisker plots, or points and connecting line. Plots show mean, and the error bars represent SEM. Different data sets were generated to test differences in the experiments involving animals/cells or humans. In the animal/cells study, differences were analyzed by Mann-Whitney nonparametric test to compare 2 independent groups or by Kruskal-Wallis test in experiments including ≥3 groups. For vascular reactivity studies, differences were analyzed using nonparametric Friedman test, followed by Dunn's multiple comparison test, for the analysis of the effects of the pharmacological treatments on vascular reactivity function. Human data were presented as mean and SD and analyzed by 2-tailed Student *t* test (Table). No randomization was applied to allocate patients in the different groups because diabetic patients were chosen on the basis of glycated hemoglobin percentage (baseline difference). Therefore, no adjustment in the analysis was made because of the baseline differences. No repeated measurements on the same experimental unit over time were used. All experiments can be considered on different experimental units. A minimum value of $P<0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism software 7.0.

Results

Rac1 Inhibition Protects From Endothelial Dysfunction in a Mouse Model of Diabetes Mellitus

To evaluate the effects of NSC23766 on vascular function in diabetes mellitus, we used a previously described mouse model of streptozotocin-induced diabetes mellitus.[19] Mesenteric arteries were isolated from streptozotocin-treated mice and their control (vehicle-injected) littermates after IP injection of NSC23766 (5 mg/kg, as previously described),[20,21] at different time points (6, 12, 24, 36, 48, and 96 hours after injection) to perform vascular reactivity studies (Figure 1A through 1F). No effects on blood glucose levels and body weight were found after NSC23766 treatment in both control and streptozotocin-treated mice (Table S2).

As expected, diabetes mellitus caused impaired endothelial vasorelaxation, as demonstrated by reduced response to acetylcholine in mesenteric arteries of mice treated with streptozotocin (Figure 1). In contrast, smooth muscle relaxation induced by nitroglycerine was unaffected by diabetes mellitus (data not shown). Interestingly, in vivo administration of NSC23766 in streptozotocin-treated mice reduced endothelial dysfunction, ameliorating vasorelaxation starting after 6 hours from injection, with a sustained effect present up to 96 hours after administration (Figure 1A through 1F).

As expected, diabetic arteries also exhibited increased ROS production and Nox activity (Figure 2A). We also observed that, in streptozotocin-treated mice, both mRNA and protein levels of ROCK1 were increased (Figure 2B and 2C), coupled with significant downregulation of phosphoinositide 3-kinase/protein kinase B signaling pathway (Figure 2C). Interestingly, NSC23766 treatment abolished Rac1 activation in diabetic vessels, which, in turn, reduced RhoA and ROCK1 levels, restoring the phosphoinositide 3-kinase/protein kinase B signaling pathway and endothelial NO synthase (eNOS) phosphorylation (Figure 2B). These data support the role of Rac1 as an upstream modulator of ROCK1 involved in eNOS dysfunction and ROS production in diabetes mellitus.

NSC23766 Prevents High Glucose–Induced Endothelial Dysfunction by Restoring eNOS Phosphorylation and Reducing Oxidative Stress

To evaluate the in vitro effects of NSC23766 on glucose-induced endothelial dysfunction, mesenteric arteries from wild-type C57BL/6 mice were treated with 2 different glucose concentrations, mimicking normoglycemia (5 mmol/L) or hyperglycemia (25 mmol/L). Vessels exposed to 25 mmol/L of glucose for 30 minutes showed a significant reduction of acetylcholine-evoked vasorelaxation compared with vessels treated with 5 mmol/L of glucose (Figure 3A), whereas no differences between the 2 different doses of glucose were found in nitroglycerine-induced vasorelaxation (Figure S1). These data confirm the detrimental effects of high glucose levels on vascular function. Interestingly, pretreatment with Rac1 inhibitor, NSC23766 (30 μmol/L), was able to protect from endothelial dysfunction induced by high glucose (Figure 3A), restoring eNOS phosphorylation and reducing ROS production and Nox activity (Figure 3B and 3C).

ROCK1 Is Involved in Rac1-Dependent Effects on Vascular Function

It has been reported that Rac1 negatively modulates eNOS function.[22] This mechanism appears to be likely mediated by the reduction of ROCK1, a negative regulator of eNOS. In fact, ROCK1 inhibits eNOS gene expression and inhibits phosphoinositide 3-kinase/protein kinase B signaling, which phosphorylates and activates eNOS.[23,24] Accordingly, vessels treated with high glucose concentration in presence of

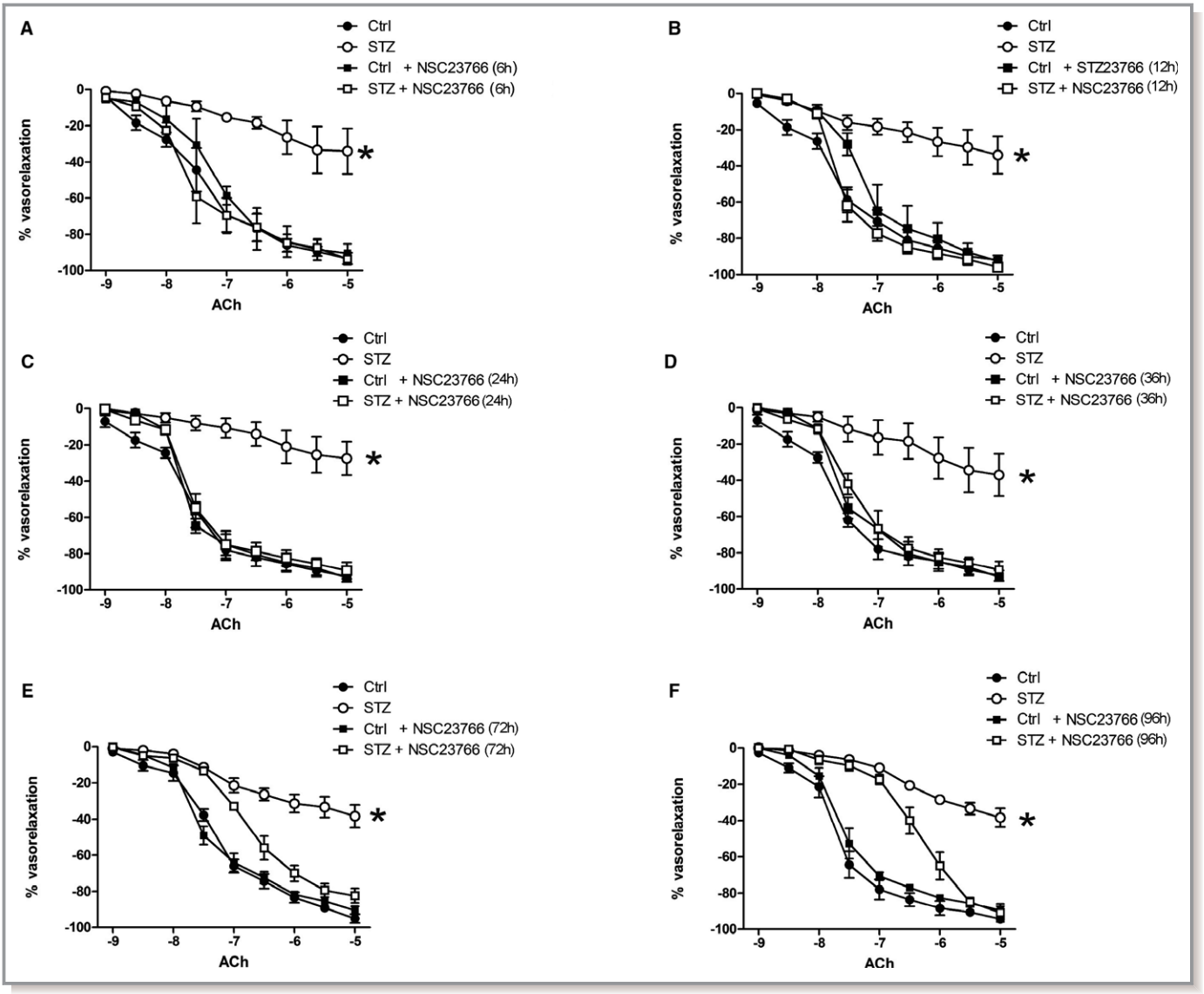

Figure 1. NSC23766 restores relaxation in diabetic vessels. Acetylcholine (ACh) vasorelaxation in preconstricted mesenteric arteries from vehicle-treated mice (control [Ctrl]; full circles), streptozotocin-treated mice (STZ; empty circles), from Ctrl mice treated with Rac1 inhibitor (Ctrl+NSC23766; full squares), and from STZ-treated mice plus Rac1 inhibitor (STZ+NSC23766; empty squares) at different time points from single injection of NSC23766: 6 hours (A), 12 hours (B), 24 hours (C), 36 hours (D), 48 hours (E), and 96 hours (F). n=4 for each group. **P*<0.05 vs all.

LY27632, an inhibitor of ROCK1 activity, showed increased eNOS phosphorylation and enhanced vasorelaxation compared with vessels treated with high glucose alone (Figure 3B and 3D). Notably, in LY27632-treated vessels, Rac1 was still activated, positioning it as an upstream modulator of ROCK1 (Figure 3B). Although the administration of Rac1 inhibitor in presence of LY27632 did not further enhance eNOS phosphorylation compared with LY27632 alone (Figure 3B), at functional level it was able to potentiate endothelial vasorelaxation, suggesting that additional mechanism(s) are recruited by Rac1 inhibitor to modulate endothelial function (Figure 3D). Interestingly, in the same model of hyperglycemia-induced vascular damage, inhibition of Rac1 but not inhibition of ROCK1 was able to protect the arteries from high glucose–induced ROS generation, as shown by a significant reduction in Nox levels after treatment with NSC23766 (Figure 3C).

To better evaluate the contribution of ROS in the differential response on vascular function observed with Rac1 and ROCK1 inhibitors, the antioxidant agent tiron (10^{-3} mol/L), a superoxide scavenger, was used in addition to ROCK1 inhibitor. Treatment with tiron+LY27632 restored endothelial vasorelaxation at similar level observed in presence of Rac1 inhibitor (Figure 3E). On the contrary, the addition of tiron to NSC23766 did not influence the vascular response evoked by Rac1 inhibition in presence of high glucose levels, whereas

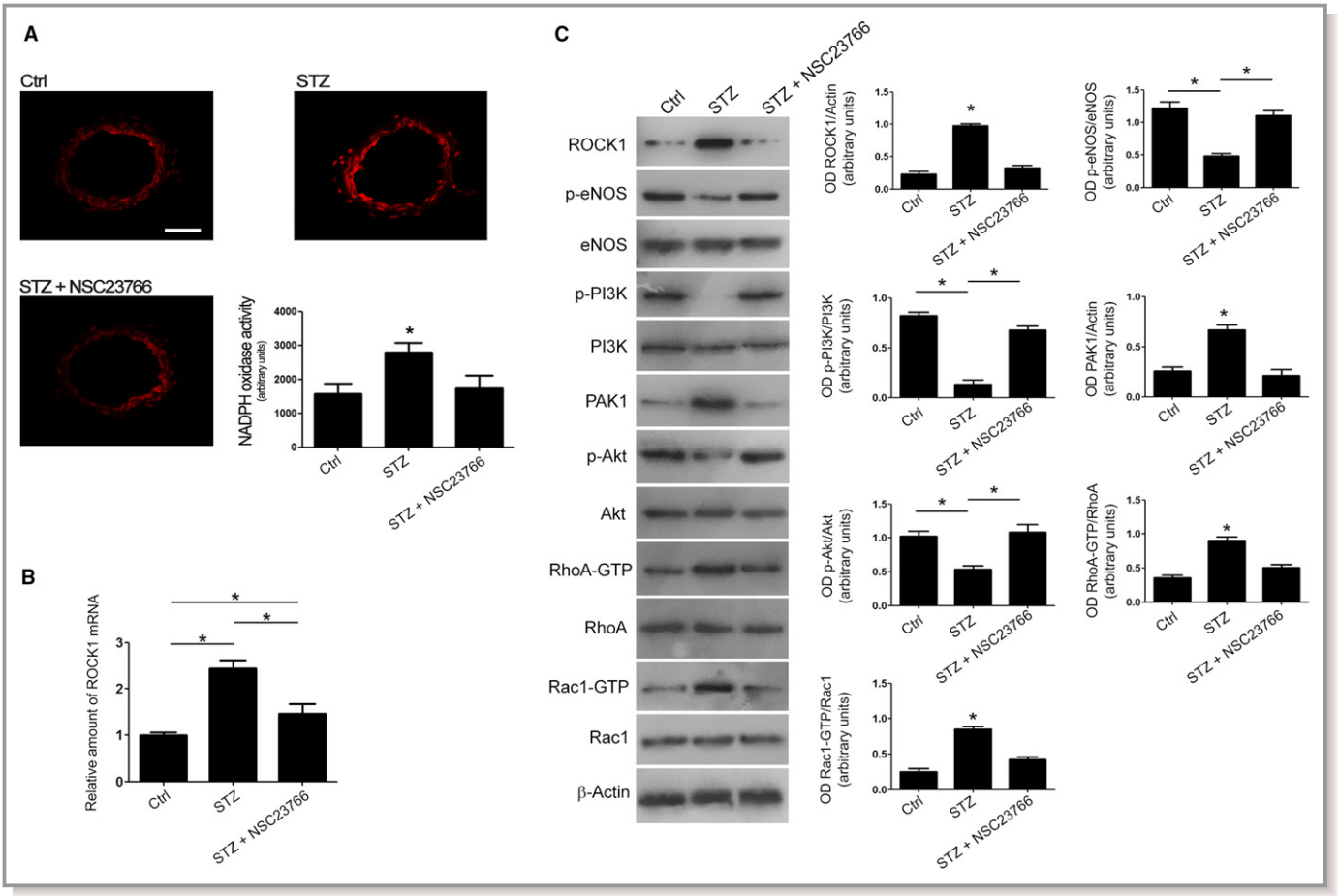

Figure 2. NSC23766 restores endothelial NO synthase (eNOS) function and reduces reactive oxygen species in diabetic vessels. A, Representative micrographs of Dihydroethidium staining to evaluate oxidative stress in mesenteric arteries from mice treated with vehicle (control [Ctrl]), with streptozotocin (STZ), or with streptozotocin plus NSC23766 (STZ+NSC23766; 48 hours). Representative images (n=3). Columns represent the effect of NSC23766 on nicotinamide adenine dinucleotide phosphate (NADPH)–induced lucigenin chemiluminescence in STZ mice mesenteric arteries. Data are expressed as increase of chemiluminescence per minute in arbitrary units. n=4 for each group. **P*<0.05 vs all. B, The mRNA levels of Rho-associated coiled-coil serine/threonine kinase-1 (ROCK1) were determined by quantitative reverse transcription–polymerase chain reaction in vessels from Ctrl, STZ, and STZ+NSC23766, 48 hours. n=3 for each group. **P*<0.05. C, Representative immunoblots (left) and densitometric analysis (right) of 4 independent experiments evaluating protein levels of ROCK-1, phospo (p)-eNOS, eNOS, p-phosphoinositide 3-kinase (PI3K), PI3K, p-protein kinase B (Akt; T473), Akt, p21 activated kinase, RhoA-GPT, RhoA, Rac1-GTP, Rac1, and β-actin in mesenteric arteries from Ctrl, STZ, and STZ+NSC23766 mice, 48 hours. n=3 for each group. **P*<0.05.

treatment with tiron alone only partly ameliorated vascular relaxation, accordingly with its scavenger effects (Figure 3E).

Given the striking reduction of ROS production and Nox activity observed after NSC23766 treatment, we aimed to determine the individual contribution of different Nox isoforms in glucose-induced ROS generation. Interestingly, the use of Nox4 inhibitor, GTK137831, significantly decreased ROS production and Nox activity in glucose-treated vessels (Figure 3C), whereas no effects were observed after treatment with Nox1 inhibitor, ML-171 (Figure 3C). We also observed that Nox4 inhibition by GTK137831 partially restored acetylcholine-induced vasorelaxation in mesenteric arteries treated with high glucose dose (Figure 3F). Interestingly, treatment of dysfunctional vessels with Nox1 inhibitor ML-171 did not improve vascular reactivity (Figure 3F). These data demonstrate a specific involvement of Nox4 isoform in glucose-induced ROS production and suggest that the observed vascular antioxidant effects of NSC23766 could be mediated, at least in part, by the inhibition of Nox4 isoform of Nox.

The beneficial effects of Rac1/ROCK1 inhibition on glucose-induced endothelial dysfunction and ROS production observed in the whole vessels were also observed in human endothelial cells (Figure S2). Collectively, these data demonstrate that ROCK1 inhibition ameliorates, in part, hyperglycemia-induced endothelial dysfunction without affecting ROS production, whereas the improvement of endothelial function observed with Rac1 inhibitor is also attributable to its inhibitor effect on Nox, primarily on Nox4 isoform, reducing ROS production.

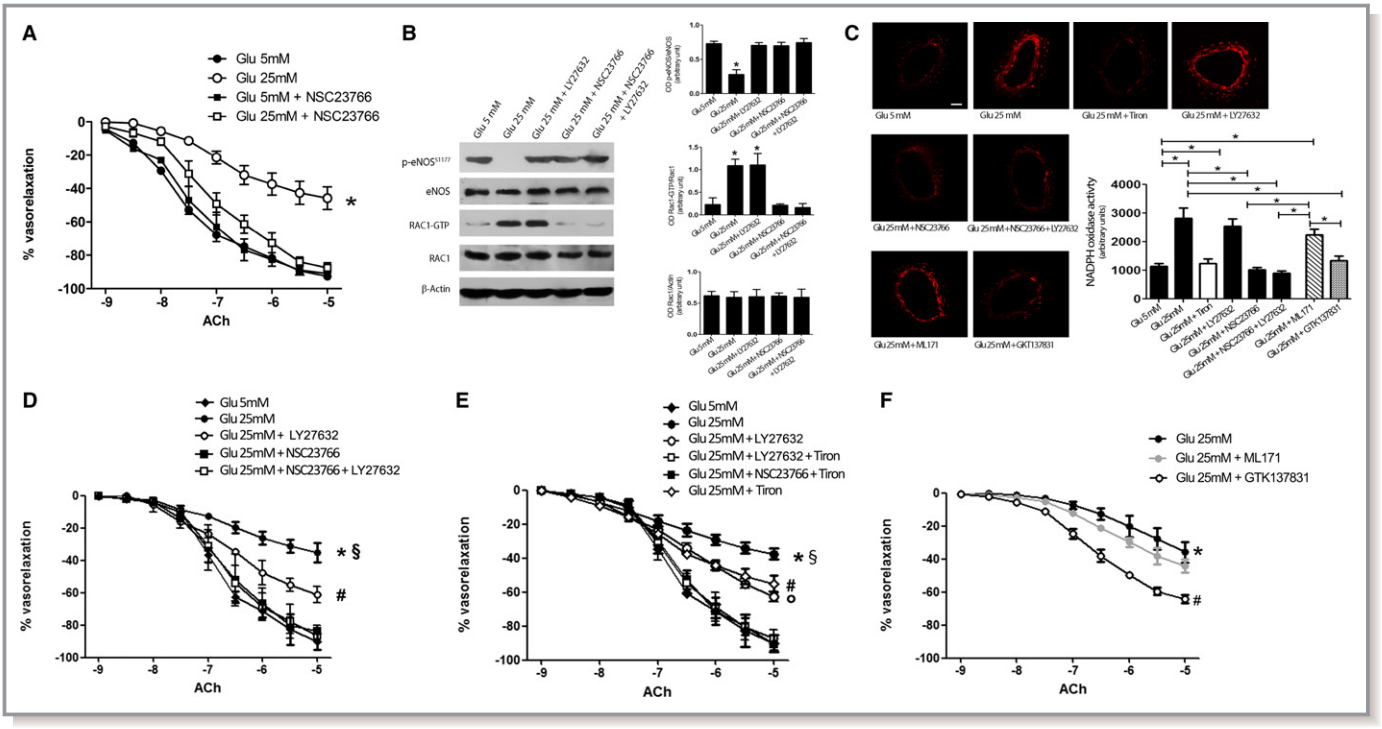

Figure 3. Interplay between RAC1 and Rho-associated coiled-coil serine/threonine kinase-1 in vessels and platelets. A, Acetylcholine (ACh) vasorelaxation in preconstricted mesenteric arteries treated with low glucose (Glu; 5 mmol/L; full circles), high glucose (Glu 25 mmol/L; empty circles), low glucose plus Rac1 inhibitor (Glu 5 mmol/L+NSC23766; full squares), and high glucose plus Rac1 inhibitor (Glu 25 mmol/L+NSC23766; empty squares). n=4 for each group. **P*<0.05 vs all. B, Representative immunoblots (left) and densitometric analysis (right) of 4 independent experiments evaluating protein levels of p-endothelial NO synthase (eNOS), eNOS, Rac1-GTP, total Rac1, and β-actin in mesenteric arteries treated with Glu 5 mmol/L, Glu 25 mmol/L, Glu 25 mmol/L plus LY27632 (Glu 25 mmol/L+LY27632), Glu 25 mmol/L+NSC23766, or Glu 25 mmol/L+NSC23766+LY27632. **P*<0.05 vs all; **P*<0.05 vs Glu 5 mmol/L, Glu 25 mmol/L+NSC23766, and Glu 25 mmol/L+NSC23766+LY27632. C, Representative images of Dihydroethidium staining to evaluate oxidative stress in mesenteric arteries treated with different stimuli/inhibitors. Columns represent the effect of NSC23766, LY27632, GKT137831, and ML-171 on nicotinamide adenine dinucleotide phosphate (NADPH)–induced lucigenin chemiluminescence in mice mesenteric arteries. Data are expressed as increase of chemiluminescence per minute in arbitrary units. n=4 for each group. **P*<0.05. D, ACh vasorelaxation in preconstricted mesenteric arteries from control treated with Glu 5 mmol/L, Glu 25 mmol/L, Glu 25 mmol/L+LY27632, Glu 25 mmol/L+NSC23766, and Glu 25 mmol/L+NSC23766+LY27632. n=4 for each group. **P*<0.05 vs Glu+LY27632; #*P*<0.05 vs Glu 5 mmol/L, Glu 25 mmol/L+NSC23766, and Glu 25 mmol/L+NSC23766+LY27632; §*P*<0.05 vs Glu 5 mmol/L, Glu 25 mmol/L+NSC23766, and Glu 25 mmol/L+NSC23766+LY27632. E, ACh vasorelaxation in preconstricted mesenteric arteries from mesenteric arteries treated with Glu 5 mmol/L, Glu 25 mmol/L, Glu 25 mmol/L+LY27632, Glu 25 mmol/L+LY27632+tiron, Glu 25 mmol/L+NSC23766+tiron, and Glu 25 mmol/L+tiron. n=3 for each group. **P*<0.05 vs Glu 25 mmol/L+LY27632; #*P*<0.05 vs Glu 5 mmol/L, Glu 25 mmol/L+LY27632+tiron, and Glu 25 mmol/L+NSC23766+tiron; §*P*<0.05 vs Glu 5 mmol/L, Glu 25 mmol/L+LY27632+tiron, and Glu 25 mmol/L+NSC23766+tiron; °*P*<0.05 vs Glu 5 mmol/L, Glu 25 mmol/L+LY27632+tiron, and Glu 25 mmol/L+NSC23766+tiron. F, ACh vasorelaxation in preconstricted mesenteric arteries from vessels treated with Glu 25 mmol/L, Glu 25 mmol/L plus ML-171 (Glu 25 mmol/L+ML-171), or Glu 25 mmol/L plus GTK137831 (Glu 25 mmol/L+GTK137831. n=5 for each group. **P*<0.05 vs Glu25 mmol/L+GTK137831; #*P*<0.05 vs Glu 25 mmol/L+ML-171.

Rac1 Inhibition Restores NO Production in High Glucose–Treated Human Platelets

Treatment of human platelets with high glucose concentration (25 mmol/L) induced a strong activation of Rac1 and a significant reduction of eNOS phosphorylation (Figure 4A). Interestingly, administration of LY27632 restored eNOS phosphorylation without affecting Rac1 activation (Figure 4A), posing Rac1 as an upstream modulator of ROCK1 signaling also in platelets. In addition, NSC23766 was also able to restore eNOS phosphorylation, in presence of high glucose, at similar levels compared with LY27632, whereas coadministration of both Rac1 and ROCK1 inhibitors did not further modify eNOS phosphorylation status (Figure 4A). These data suggested that the effect of NSC23766 on eNOS phosphorylation is mediated by ROCK1.

Reduced platelet NO production represents a crucial alteration in diabetes mellitus. As expected, we observed a dramatic impairment of NO release in the supernatants of platelets exposed to high glucose concentration compared with platelets exposed to low glucose, measured by Sievers NO analyzer (NOA280i) (Figure 4B). To confirm the specificity of

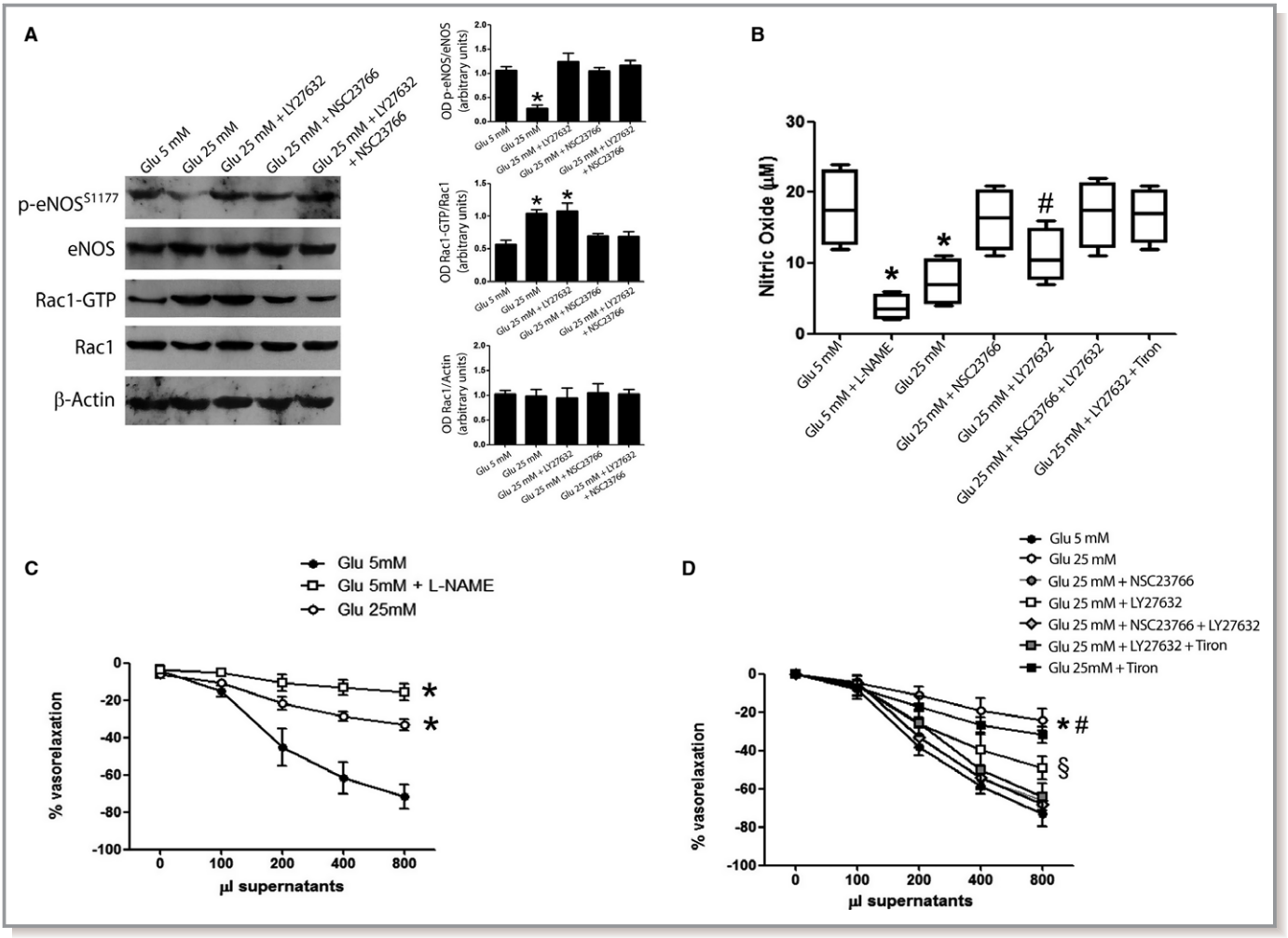

Figure 4. Rac1 inhibition restores NO release from platelets. A, Representative immunoblots (left) and densitometric analysis (right) of 4 independent experiments evaluating protein levels of p-endothelial NO synthase (eNOS), eNOS, Rac1-GTP, total Rac1, and β-actin in platelets. **P*<0.05 vs all. B, Quantitative measurement of NO levels in platelet supernatants treated with glucose 5 mmol/L (Glu 5 mmol/L), Glu 5 mmol/L+Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), glucose 25 mmol/L (Glu 25 mmol/L), Glu 25 mmol/L plus NSC23766 (Glu 25 mmol/L+NSC23766), Glu 25 mmol/L plus LY27632 (Glu 25 mmol/L+LY27632), Glu 25 mmol/L plus NSC23766 plus LY27632 (Glu 25 mmol/L+NSC23766+LY27632), and Glu 25 mmol/L plus LY27632 plus tiron (Glu 25 mmol/L+LY27632+tiron). Box plots representing the mean and the minimum and maximum values of NO amounts. n=4 for each group. **P*<0.05 vs all; $^{\#}$*P*<0.05 vs Glu 25 mmol/L+NSC23766, Glu 25 mmol/L+NSC23766+LY27632, Glu 5 mmol/L, and Glu 25 mmol/L. C, Dose-response curves of phenylephrine precontracted aorta rings to supernatants derived from human platelets treated with Glu 5 mmol/L, Glu 5 mmol/L+L-NAME, and Glu 25 mmol/L. n=4 for each group. **P*<0.05 vs Glu 5 mmol/L. D, Dose-response curves of phenylephrine precontracted aorta rings to supernatants derived from human platelets treated with Glu 5 mmol/L, Glu 25 mmol/L, Glu 25 mmol/L+NSC23766, Glu 25 mmol/L+LY27632, Glu 25 mmol/L+NSC23766+LY27632, Glu 25 mmol/L+LY27632+tiron, or Glu 25 mmol/L+tiron. n=4 for each group. **P*<0.05 vs all; §*P*<0.05 vs Glu 25 mmol/L+LY27632; §*P*<0.05 vs Glu 25 mmol/L+NSC23766; $^{\#}$*P*<0.05 vs all.

NO production, we also measured NO levels in supernatant of platelets treated with NOS inhibitor Nω-nitro-L-arginine methyl ester hydrochloride. As shown in Figure 4B, Nω-nitro-L-arginine methyl ester hydrochloride treatment completely abolished NO production in platelet supernatant. Interestingly, treatment with Rac1 inhibitor NSC23766 restored platelet NO production, whereas treatment of high glucose–stimulated platelets with ROCK1 inhibitor was able to restore only, in part, the impaired NO production (Figure 4B). Similar to what was observed in vessels, addition of tiron to high-glucose platelets treated with LY27632 further enhanced NO production to the levels observed with Rac1 inhibitor NSC23766 (Figure 4B).

Human platelet supernatant evoked a rapid dose-dependent relaxation of mouse aortic rings; this effect was NO dependent because it was abolished by eNOS inhibition with Nω-nitro-L-arginine methyl ester hydrochloride (Figure 4C). As expected, the effect on vasorelaxation induced by platelet supernatant was markedly reduced by high glucose level (25 mmol/L) compared with vessels treated with supernatant of platelets with low dose of glucose (5 mmol/L) (Figure 4C),

confirming the effects of high glucose to induce an impairment in the NO-dependent vasorelaxant mechanisms.

To evaluate the role of Rac1/ROCK1 axis in platelet-induced NO-dependent vasorelaxation, supernatants from NSC23766 or ROCK1 inhibitor LY27632-treated platelets were used on mouse aortic ring preparations. Interestingly, treatment of platelets with NSC23766 restored the ability of platelet supernatant to induce a dose-dependent relaxation of mouse aortic rings in presence of high glucose levels (Figure 4D), whereas the supernatant of LY27632-treated platelets was able to ameliorate only, in part, vasorelaxation (Figure 4D). Interestingly, the addition of tiron caused an enhancement of vasorelaxant effect observed with supernatant of LY27632-treated platelets, reaching a similar level on what was observed in presence of NSC23766 alone or NSC23766 plus LY27632 (Figure 4D). The administration of tiron alone exerted only a mild, not significant, improvement of vasorelaxation.

Taken together, these results indicated that the effect of Rac1 inhibitor on NO metabolism in presence of high glucose levels depends on the modulation of both eNOS phosphorylation and oxidative stress.

NSC23766 Reduces Platelet Aggregation Induced by High Glucose Levels

Because impairment of NO production is closely associated with an increase of platelet reactivity, we investigated the effect of Rac1 inhibitor on human platelet aggregation. As expected, platelet aggregation induced by type I collagen was enhanced after treatment with increasing concentrations of glucose (Figure 5A). Subsequently, we aimed to identify the effective concentration of NSC23766 able to modulate platelet aggregation. We performed a dose-response curve with NSC23766 in human platelets exposed to 5 and 25 mmol/L of glucose. In platelets exposed to 5 mmol/L (mimicking "normoglycemic" condition), the effective dose to reach a significant inhibition of platelet aggregation was 30 μmol/L, whereas further increase in NSC23766 dose (starting from 50 μmol/L) practically abolished any collagen-induced aggregation (Figure 5B). Interestingly, when the same dose-response curve was done exposing platelets to 25 mmol/L of glucose (mimicking "hyperglycemic" condition), an increased sensitivity to Rac1 inhibition was observed. Specifically, under this condition, the inhibitory effect of NSC23766 on platelet aggregation appeared already at the dose of 15 μmol/L, decreasing further at 30 μmol/L (Figure 5B). Similar results were obtained when platelets were stimulated with different agonists, such as arachidonic acid (0.5 mmol/L), ADP (50 mmol/L), or thrombin receptor-activating peptide (25 μmol/L) (data not shown). These results suggest that glucose per se can prime platelets, making them more susceptible to the effects of NSC23766, and that Rac1 hyperactivity played a pivotal role in the modulation of high glucose–induced platelet aggregation.

Moreover, to demonstrate that Rac1 effects on glucose-dependent platelet aggregation were not dependent by changes in platelet osmolarity, we evaluated platelet aggregation after increasing dose of osmotic-control mannitol with and without NSC23766. The administration of mannitol did not change platelet reactivity, confirming the specific role of glucose to induce platelet hyperaggregation (Figure S3). Accordingly, platelet Rac1 levels did not show any change after mannitol treatment (Figure S3).

Finally, we investigated the potential role of ROCK1 in modulation of platelet aggregation during hyperglycemic conditions. Differently from what was observed in vessels, ROCK1 inhibition by LY27632 in platelets did not affect platelet aggregation in presence of both high glucose or low glucose concentrations (data not shown), confirming the marginal role of ROCK1 in modulation of platelet aggregation, as observed before.[25]

Platelets From Diabetic Patients Show Increased Levels of Activated Rac1

To translate our results into a clinical setting, we evaluated active Rac1 levels in platelets isolated from diabetic patients and control subjects without diabetes mellitus (Table). Platelets from diabetic patients showed higher levels of activated Rac1 compared with control samples (Figure 6A and 6B).

It is well known that in diabetes mellitus, platelets are hyperreactive, with intensified adhesion, activation, and aggregation.[26] Thus, we evaluated the level of activated Rac1 in platelets from diabetic patients accordingly with their reported percentage of glycated hemoglobin. Interestingly, our pull-down assay showed a higher level of active Rac1 in platelets from patients with elevated percentage of glycated hemoglobin (10%) compared with those with lower glycated hemoglobin (7%), indicating a correlation between diabetes mellitus status and Rac1 activation (Figure 6A and 6B).

Moreover, to evaluate the effect of diabetes mellitus on platelets, we evaluated platelet aggregation in diabetic patients. As expected, under basal condition, the aggregation induced by collagen was enhanced in platelets from diabetic patients compared with control subjects (Figure 6C, left panel). Next, we evaluated the efficacy of NSC23766 to inhibit collagen-induced platelet activation in diabetic patients. Interestingly, to obtain a significant reduction in diabetic platelet aggregation, a higher dose of NSC23766 was necessary compared with control platelets (60 in comparison to 30 μmol/L already effective in platelets from control subjects) (Figure 6C, middle panel).

Although platelets exposed to increasing concentration of glucose exhibit an increased sensitivity to Rac1 inhibition

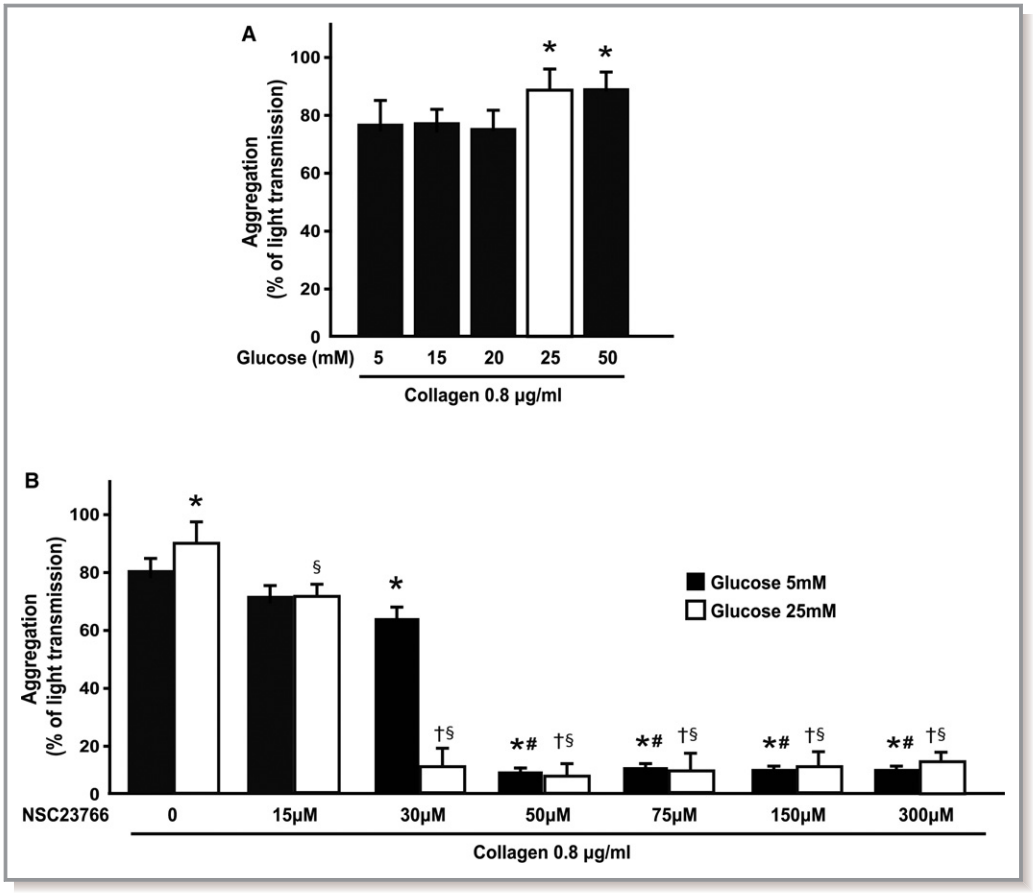

Figure 5. NSC23766 inhibits glucose-induced platelet hyperaggregation. A, Quantification of platelet aggregation presented as percentage of light transmission of platelets from control (CTRL) subjects treated with increasing concentrations of glucose (Glu). n=6 independent experiments from individual subjects. **P*<0.05 vs glucose 5 mmol/L. B, Quantification of platelet aggregation presented as percentage of light transmission of platelets from CTRL subjects treated with increasing concentrations of NSC23766 at 5 and 25 mmol/L Glu. n=4 independent experiments from individual subjects. **P*<0.05 vs Glu 5 mmol/L without NSC23766; #*P*<0.05 vs Glu 5 mmol/L with NSC23766 30 µmol/L; §*P*<0.05 vs Glu 25 mmol/L without NSC23766; †*P*<0.05 vs Glu 25 mmol/L with NSC23766 15 µmol/L and Glu 5 mmol/L with NSC23766 30 µmol/L.

(Figure 5B), higher doses of Rac1 inhibitor were necessary to reach the same levels of inhibition of aggregation in platelets isolated from diabetic patients (Figure 6C, middle panel). To rule out the potential off-target effects of NSC23766 observed when used at high concentrations,[27] we used another structurally different Rac1 inhibitor, called EHT1864. As shown in Figure S4, also with EHT1864, a higher dose of inhibitor was necessary to significantly reduce platelet aggregation in diabetic conditions compared with control subjects.

NSC23766 Exerts Additive Effect on Platelet Aggregation From Diabetic Patients Treated With Acetylsalicylic Acid

On the basis of the evidence that several diabetic patients showed a resistance to common antiplatelet drugs, such as acetylsalicylic acid (ASA), we decided to investigate the efficacy of NSC23766 treatment in isolated platelets from ASA-treated diabetic patients. In particular, we tested increasing concentrations of NSC23766 on platelets from diabetic patients treated with ASA, 100 mg/d. Interestingly, in this experimental condition, NSC23766 treatment was able to further reduce the platelet aggregation in diabetic patients already treated with ASA (Figure 6C, right panel).

Discussion

We found that pharmacological inhibition of Rac1 by NSC23766 attenuated endothelial dysfunction in experimental model of diabetes mellitus and reduced platelet hyperaggregation in diabetic patients. These novel results suggest a potential protective role of Rac1 inhibition on vascular injury and platelet hyperaggregation in diabetes mellitus.

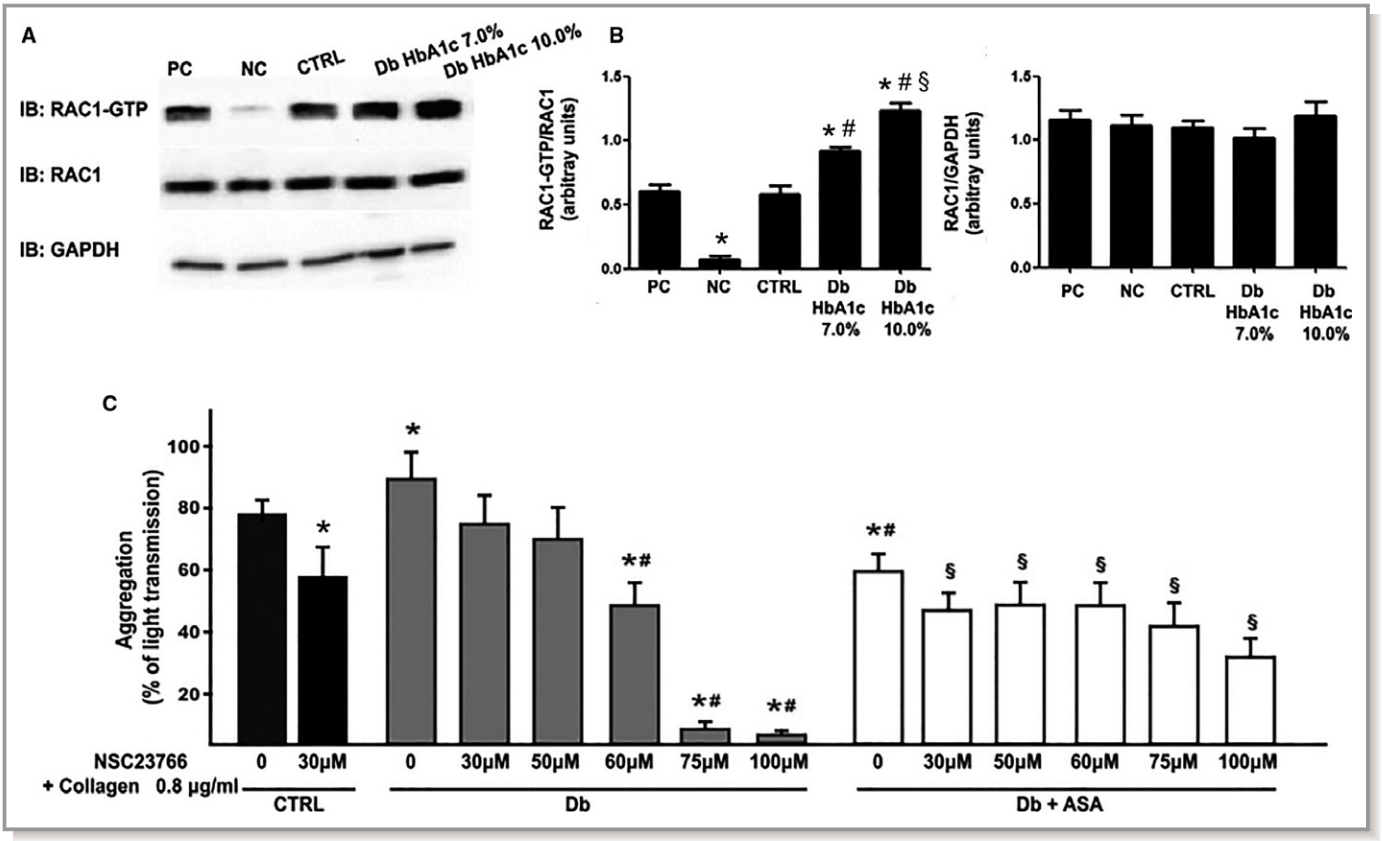

Figure 6. NSC23766 ameliorates platelet hyperaggregation in diabetes mellitus. A, Representative immunoblots of Rac1-GTP and Rac1 levels in platelets from control (CTRL) subjects or diabetic (Db) patients with different percentage of glycated hemoglobin (HbA1c). GAPDH protein levels were used for normalizing samples. B, Densitometric analysis of Rac1-GTP (left) and Rac1 (right) protein levels in platelet samples from CTRL and Db patients. n=4 independent experiments from individual subjects. **P*<0.05 vs all; #*P*<0.05 vs CTRL; §*P*<0.05 vs Db HbA1c 7.0%. C, Quantification of platelet aggregation presented as percentage of light transmission of CTRL platelets (left), Db platelets (middle), and Db platelets+acetylsalicylic acid (ASA) treated with NSC23766. n=4 independent experiments from individual subjects. **P*<0.05 vs CTRL 0 (without NSC23766); #*P*<0.05 vs Db 0 (without NSC23766); §*P*<0.05 vs CTRL, Db 0, and Db+ASA 0 (without NSC23766). IB indicates immunoblot; NC, negative control; and PC, positive control.

Rac1 is a regulatory component of Nox, which represents 1 of the major sources of ROS in the vascular wall. ROS generation is crucially involved in diabetes mellitus and diabetic complications.[28] Although many sources of ROS contribute to increased oxidative stress in diabetes mellitus (direct effect of hyperglycemia, mitochondria, and xanthine oxidase), several Nox isoforms have been found specifically upregulated in vascular wall in the presence of high glucose.[29,30] We have previously shown that Rac1 represents a crucial modulator of ROS-induced vascular dysfunction in preclinical model of diabetes mellitus.[19] Its inhibition by an adenoviral vector carrying Rac1 dominant negative mutant protects from endothelial dysfunction in experimental diabetes mellitus.[19] In the past decade, NSC23766 was identified as a small-molecule inhibitor of Rac–guanine nucleotide exchange factor–mediated activation of Rac1.[14,15] Until now, its main field of application has been cancer biology, in which Rac1 has been reported as a novel important therapeutic target in several type of malignancies.[31–35] Given the growing importance of Rac1 in the cardiovascular system, recently NSC23766 has been tested in different cardiovascular disorders.[22]

Herein, we have shown the protective effects of Rac1 inhibitor, NSC23766, on endothelial function after high glucose–induced vascular damage. Mechanistically, we demonstrated that amelioration of endothelial function via restoration of eNOS phosphorylation by Rac1 inhibition requires ROCK1 as a crucial component of Rac1 signaling in both isolated cells and vessels. Although previous studies have suggested a reduction in eNOS expression after Rac1 inhibition,[36] the modulation of Rac1 activity by NSC23766 in our study was not related to changes in its expression in both mouse vessels and human endothelial cells. Moreover, we were recently able to demonstrate that Rac1 inhibition by NSC23766 exerts a beneficial effect also in human vessels, ameliorating endothelial dysfunction.[16] These results pointed out the important role of Rac1 as a therapeutic target to improve vascular homeostasis.

Levay et al[27] have demonstrated an effect of NSC23766 as nonselective competitive antagonist of muscarinic acetylcholine receptors in neonatal rat cardiomyocytes. In our experimental model on resistance vessels, we were able to show that NSC23766 per se did not interfere with acetylcholine vasorelaxation (Figure S5). Consistently with other studies, we have demonstrated that the activation of Rac1 (Rac1-GTP) negatively modulates eNOS phosphorylation through ROCK-1 pathway.[37,38] In addition to its effect on eNOS phosphorylation, inhibition of Rac1 also blunted Nox ROS production. Hence, these data demonstrated that the amelioration of endothelial relaxation obtained by Rac1 inhibition in presence of high glucose levels depends on 2 mechanisms: the increased eNOS phosphorylation and the reduction of oxidative stress.

These data prompted us to explore the effects of Rac1 inhibitor in a mouse model of diabetes mellitus. NSC23766 treatment reduced the enhanced Rac1 activation observed in preclinical diabetes mellitus and restored acetylcholine-evoked vasorelaxation. Interestingly, amelioration of endothelial function observed after Rac1 inhibition in vessels from diabetic mice was present up to 96 hours after NSC23766 systemic administration and, accordingly, we observed a sustained inhibition of Rac1 activity in mice vessels at the same time point (Figure S6). Reduction of ROS production in diabetic vessels treated with NSC23766 can be attributed to reduction in Nox activity. Although, given the complexity of ROS production in diabetes mellitus, previously discussed, it is likely that Nox might not be the only target of Rac1 inhibition. Using pharmacological inhibition of the different Nox isoform, we were able to identify Nox4 as a critical component of NSC23766-mediated ROS suppression in vascular wall. These data will serve as a platform to pursue more in-depth mechanistic insights in Rac1/Nox4 interaction.

In addition to vascular damage, diabetes mellitus is also characterized by platelet dysfunction. Previous studies reported that high levels of glucose were able to increase platelet aggregation in vitro and were associated with ROS production.[39] Rac1 is involved in platelet actin cytoskeleton reorganization during platelet activation. A crucial feature of platelets is represented by their ability to produce NO. Our data demonstrate that high glucose levels activate Rac1 in platelets, and this effect is associated with an impaired NO release. Notably, similar to what was observed in vessels and endothelial cells, the administration of NSC23766 in platelets protects from the deleterious effect of high glucose on NO metabolism, enhancing eNOS phosphorylation, through ROCK-1 inhibition, and blunting oxidative stress. It is well known that a reduction in NO release is coupled with alteration in platelet activation. In this regard, 1 of the most striking changes that occur during platelet activation is the translocation of CD62 (P-selectin) protein to the outer platelet membrane. Therefore, the expression of CD62 on the membrane of platelets is considered to be a valuable indicator for platelet activation in different diseases, including diabetes mellitus.[40,41] To evaluate the effects of NSC23766 on platelet activation in diabetes mellitus, we performed the immunoblot analysis of CD62 expression in cytosol and membrane subcellular fraction of platelets isolated from diabetic (streptozotocin-treated) and control mice in presence and absence of NSC23766. As shown in Figure S7, membrane expression of CD62 in diabetic platelets was significantly increased. More important, in vivo administration of NSC23766 in diabetic mice abolished CD62 membrane translocation in platelets, demonstrating a significant reduction of platelet activation on Rac1 inhibition. These data suggest that the effects of NSC23766 on platelet function in diabetes mellitus are potentially beyond the expected cytoskeleton reorganization and might contribute to the beneficial effects observed in vitro and in vivo, corroborating our previous results.

Interestingly, herein we have shown that Rac1 is responsive to high glucose levels, enhancing platelet aggregation, acknowledging Rac1 as an important regulator of platelet function during hyperglycemic conditions. We demonstrated that a condition mimicking hyperglycemia in vitro increases platelet Rac1 activation. On the basis of this result, we hypothesized that increased active Rac1 platelet levels under hyperglycemic conditions could contribute to the increased platelet activity observed in diabetes mellitus. Using a specific inhibitor of Rac1 activity, NSC23766, we demonstrated that glucose-induced platelet hyperaggregation was reduced. The increased efficacy of NSC23766 to inhibit platelet aggregation in hyperglycemic state could be explained by increased Rac1 GTP levels under this condition. The effect of glucose on platelet aggregation starts to appear for concentration of glucose of 25 mmol/L and seems to be sustained even with higher concentration (50 mmol/L), suggesting the presence of a threshold for glucose-induced hyperaggregation in human platelets. Under normal glucose condition, the dose of 30 μmol/L, NSC23766 was sufficient to reduce Rac1 activity, modulating, in turn, platelet function. Although the dose used in our study was significantly lower compared with the one used elsewhere,[42] we cannot completely exclude that the potential off-target effects of NSC23766 might affect our results. Because the effect of NSC23766 on platelet aggregation, under high glucose condition, is already present for lower NSC23766 doses (15 μmol/L), this raised the question of how to separate the effect of hyperglycemia and the effect of NSC23766 on platelet aggregation. In our experiments, we chose relatively high levels of glucose (25 and 50 mmol/L) to mimic hyperglycemic conditions. Indeed, these levels of hyperglycemia are clinically relevant, because blood glucose commonly increases at ≈30 mmol/L in diabetic ketoacidosis and can reach 60 mmol/L during diabetic hyperosmolar

coma.[43] Therefore, during hyperglycemic condition, even a small increase in platelet aggregation can significantly worsen the clinical conditions. In our experiments, we recorded an increase of ≈10% in platelet aggregation on hyperglycemic stimuli using light transmission aggregometry. This increase is consistent with previous literature using the same technique,[39] although it can be smoothened by the fact that the baseline percentage of platelet aggregation in our subjects is higher compared with previous reports. We speculated that this effect can be attributable to the fact that the control population is not represented by completely "healthy" subjects because, despite being free from cardiovascular disease and diabetes mellitus, they present some cardiovascular risk factors, such as hypertension and smoking, that can contribute to the increased platelet reactivity. Collectively, data from other groups and we suggest that hyperglycemic condition produces a 10% to 20% increase in platelet aggregation after stimulation with proaggregating stimuli (ie, collagen) that can reflect, at least in part, the prothrombotic state observed in diabetic patients.

Given the known off-target effect of NSC23766,[42] it is possible to speculate that glucose can prime platelets, making them more susceptible to the effects of NSC23766. Accordingly, in our model, Rac1 GTP is specifically induced by high glucose in platelets, without osmotic effects, as demonstrated by the absence of Rac1 activation with mannitol; therefore, the mild effects of NSC23766 on platelet aggregation observed in normoglycemic state might be caused by the low levels of Rac1 GTP in platelets. Accordingly, platelets from diabetic patients showed a positive correlation between activated Rac1 and levels of glycated hemoglobin. To provide further support that Rac1 is a critical target in diabetic platelets, we used another Rac1 inhibitor, called EHT1864. Differently from NSC23766, which prevents the conversion of Rac1-GDP to Rac1-GTP by competitively blocking the binding loop of Rac1-specific guanine nucleotide exchange factors, EHT1864 is a specific allosteric inhibitor of the Rac family, resulting in dissociation of nucleotides.[44] Using 2 different Rac1 inhibitors, we further corroborate the notion that Rac1 plays a major role in platelet proaggregating status in diabetes mellitus, underlying the peculiar platelet characteristics of diabetic subjects.

It is important to underline that in condition of Rac1 hyperactivation (such as platelets from diabetic patients), a higher dose of Rac1 inhibitor was necessary to reduce platelet aggregation compared with platelets from subjects without diabetes mellitus, pointing out that diabetes mellitus affects platelet function in many ways, which are to be exclusively recapitulated by increasing the concentrations of glucose in vitro. We also found NSC23766 treatment further reduced platelet aggregation in those subjects taking ASA, identifying Rac1 inhibition as a potential future pharmacological strategy to limit platelet hyperaggregation in diabetes mellitus. Unlike diabetic patients without ASA in whom we were able to recognize a dose-response curve of Rac1 inhibitory effects on platelet aggregation, the additive effects of NSC23766 on ASA-treated diabetic platelets were not dose dependent. These effects could be attributed to the lower responsiveness of platelets already treated with ASA. In fact, inhibition of platelet aggregation by other mechanisms (such as cyclooxygenase-dependent mechanisms) could blunt the incremental inhibitory effect of Rac1 inhibition.

Study Limitations

The beneficial effects of Rac1 inhibition on hyperglycemia-induced vascular and platelet dysfunction observed ex vivo need to be confirmed in ad hoc preclinical models of thrombus formation and using tissue-specific genetic approaches to distinguish between platelet- and endothelial cell–driven effects. Nevertheless, the current findings suggest that Rac1 inhibition may be accomplished directly in vivo because of the favorable safety profile of NSC23766, as observed in our mouse model of streptozotocin-induced diabetes mellitus. Finally, the effects of LY27632 as ROCK1 inhibitor can be also attributed, in part, to the inhibition of ROCK2, although recent studies have demonstrated that ROCK1 and ROCK2 have distinct nonredundant functions and have different targets in different cell types.[45,46]

Conclusions

The results of our study address an important challenge in biological features of diabetes mellitus (namely, platelet hyperreactivity and endothelial dysfunction). We provide further evidence about the involvement of Rac1 in both vascular injury and platelet hyperaggregation induced by diabetes mellitus. Our findings could support the use of Rac1 inhibition by NSC23766 in combination with ASA for antiplatelet therapy in diabetes mellitus. Although proposing Rac1 inhibitor as an immediate therapeutic approach in diabetic patients is far beyond the scope of our work, we believe that the identification of targets/drugs able to modulate >1 function in this disease could represent the right way to move forward. In particular, the combination of beneficial effects of Rac1 inhibition on both vascular and platelet function in diabetes mellitus might represent a potential effective strategy (in the future) to increase the therapeutic compliance of these patients.

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Disclosures

None.

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Impact of chronic kidney disease on platelet aggregation in patients with acute coronary syndrome

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Aims **Chronic kidney disease (CKD) is associated with increased thrombotic events and seems to influence platelet reactivity. Conflicting results have been published on platelet response in CKD patients with stable coronary artery disease. The aim of our study was to investigate the impact of CKD on platelet aggregation in acute coronary syndrome (ACS) patients receiving dual antiplatelet therapy, included the more potent P2Y12 inhibitors.**

Methods **We enrolled 206 patients with ACS, divided in two groups, according to the presence or the absence of moderate/severe CKD. Platelet aggregation was performed with light transmission aggregometry and results are expressed as percentage of maximum platelet aggregation. High residual platelet reactivity (HRPR) was defined as maximum platelet aggregation more than 59%.**

Results **Patients with CKD [estimate glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2, $n=28$] were prevalent older, diabetic, had previous coronary revascularization. In these patients, platelet aggregation was significantly higher than in those with eGFR ≥ 60 ml/min/1.73 m^2 (ADP 10 μmol/l: 28.46 ± 26.19 vs. 16.64 ± 12.79, $P<0.001$; ADP 20 μmol/l: 30.07 ± 25.89 vs. 17.46 ± 12.82, $P<0.001$). HRPR was observed in 4.4% of patients, with higher prevalence in those with eGFR less than 60 ml/min/1.73 m^2 [21.4 vs. 1.7%, $P<0.001$, odds ratio (OR) [95% confidence interval (CI)] = 15.91 (3.71–68.17), $P<0.001$]. At multivariate analysis, after correction for baseline confounders, eGFR [adjusted OR (95% CI) = 0.95 (0.91–0.98), $P=0.007$], together with the use of clopidogrel [adjusted OR (95% CI) = 23.59 (4.01–138.82), $P<0.001$], emerged as determinants of HRPR.**

Conclusion **In patients with ACS receiving dual antiplatelet therapy, CKD is associated with an increasing ADP-induced platelet aggregation and higher prevalence of HRPR, which is mainly correlated to clopidogrel use.**

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Keywords: acute coronary syndrome, chronic kidney disease, high residual platelet reactivity, light transmission aggregometry

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Introduction

Chronic kidney disease (CKD) is a recognized predictor of adverse outcomes in patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI).[1,2] The incidence of major cardiovascular events in this population is mainly related to a higher risk of both atherothrombotic complications, including thrombotic stent occlusion and bleeding.[3–5] In patients with ACS, dual antiplatelet therapy (DAPT) has become the standard care to protect from death and ischemic events at short and long-term follow-up after PCI.[6–8] There is limited and contrasting evidence on the optimal management of DAPT in CKD patients and whether CKD should drive DAPT duration.[5,9] However, in most recent guidelines, DAPT in patients with renal dysfunction does not require any variation or dose adjustment until the late stage of CKD (stage 5), when estimate glomerular filtration rate (eGFR) is lower than 15 ml/min/1.73 m^2, and ticagrelor or prasugrel are no more recommended.[10–12] Recent studies suggest that renal failure could impact the efficacy of antiplatelet agents in CKD patients, exposing to a higher risk of adverse events.[13–15] To date, conflicting data have been reported regarding the effect of renal failure on residual platelet reactivity, and most of them are related to patients with stable coronary artery disease (CAD) receiving clopidogrel in association with aspirin.[16–19] The aim of the current study is to investigate the impact of CKD on platelet aggregation in the contemporary setting of ACS patients receiving DAPT with aspirin and a P2Y12 inhibitor,

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including the newly more potent drugs such as ticagrelor and prasugrel.

Methods

The observational study enrolled consecutive patients hospitalized from January 2015 to June 2016 at coronary care unit of University Federico II of Naples for an ACS [ST-segment elevation myocardial infarction (STEMI), Non-ST-segment elevation myocardial infarction (NSTEMI), unstable angina] and undergoing PCI. Exclusion criteria were platelet count more than 600 000/μl or less than 100 000/μl, hematocrit more than 50% or less than 25%, known blood dyscrasia or bleeding diathesis, eGFR less than 15 ml/min/1.75 m^2 or dialysis, concomitant neoplastic or immune-mediated pathologies, ongoing oral anticoagulation therapy. All patients were on DAPT with aspirin (loading dose of 150–300 mg orally or 75–150 mg intravenously, and 100 mg once a day as maintenance dose) and a P2Y12 inhibitor such as ticagrelor (180 mg loading dose, 90 mg twice daily as maintenance dose), prasugrel (60 mg loading dose, 10 mg once a day as maintenance dose) or clopidogrel (300 mg loading dose, 75 mg once a day as maintenance dose). The study was conducted according the principles of the Declaration of Helsinki. The local institutional Ethics Committee approved the study protocol and all patients gave written informed consent to participate. Baseline serum creatinine levels were assessed at the time of the PCI procedure in all patients. We retrospectively divided our population in groups, according to the eGFR, which was calculated using the Modification of Diet in renal Disease formula. CKD was defined according to the National Kidney Foundation's Classification[20] as follows: normal renal function with eGFR $\geq$ 90 ml/min/1.73 m^2, mild renal impairment with eGFR between 60 and 89 ml/min/1.73 m^2, moderate CKD with eGFR between 30 and 59 ml/min/1.73 m^2, severe CKD with eGFR less than 30 ml/min/1.73 m^2. Due to the limited number of patients with severely decreased renal function, these patients were combined with those having moderate renal failure.

Platelet aggregation was measured in all patients already on DAPT within 48 h after coronary angiography after diagnosis of ACS. Blood samples were collected immediately before the morning or evening dose of antiplatelet drug. The aggregation test time from symptom onset and from antiplatelet load was recorded. When GPIIb/IIIa inhibitors were prescribed, an interval of 18–24 h after completion of the infusion was required before platelet function testing was performed, to avoid interference with aggregation assay.[21] Light transmission aggregometry (LTA) was performed using a dual channel lumi-aggregometer (model 700; Chrono-Log, Havertown, Pennsylvania, USA), as previously described.[22,23] Platelet-poor plasma was used as reference for 100% aggregation and platelet aggregation was measured in nonadjusted platelet-rich plasma after stimulation with ADP in a final concentration of 10 and 20 μmol/l. Aggregation curves were recorded for 6 min. All measurements were obtained within 2 h of sample collection, and the results are reported as a percentage of maximum platelet aggregation (MPA). Based on the LTA findings, high residual platelet reactivity (HRPR) was defined as MPA more than 59%.[24]

Statistical analyses were performed using IBM-SPSS, version 23 (SPSS Inc, Chicago, Illinois, USA). Continuous variables were expressed as mean ± SD and compared with Student t-test. Categorical data were expressed as percentage and comparisons were made by χ^2 test. Patients were divided in two groups according to the renal function at the time of the PCI. One-way analysis of variance (ANOVA) analysis was used for comparison across groups according to the severity of CKD. Correlation between eGFR and platelet aggregation was performed using Pearson's correlation. The association between the CKD and HRPR was assessed using logistic regression analysis. Multivariate stepwise logistic regression analysis was performed to determine the independent association with HRPR. The following variable were tested on univariable analysis: age, hypertension, diabetes mellitus, dyslipidemia, current smoking, eGFR, platelet count, thrombolysis, ticagrelor, clopidogrel, prasugrel, diuretics, oral people without diabetes, insulin, betablockers, angiotensin converting enzyme inhibitors/AT1 blocker, STEMI, NSTEMI, unstable angina. Only variables with a P value less than 0.10 were then entered into the final multivariate logistic regression model (age, eGFR, platelet count, use of clopidogrel, ticagrelor or diuretics) with model selection using the backward likelihood ratio, providing odds ratio (OR) and 95% confidence intervals (CIs). Variance of inflation factor was also used to assess any multicollinearity among the variables to include into the model. Only variables with variance of inflation factor values less than 5 were included. A P value less than 0.05 was considered statistically significant.

Results

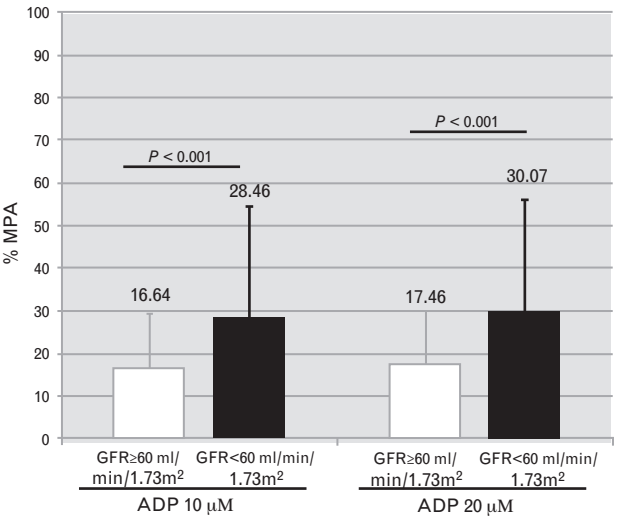
A total of 206 patients were included in the study. Baseline demographic data, clinical characteristics and laboratory data are described in Table 1. Compared with patients with normal renal function, patients with moderate-to-severe CKD (eGFR < 60 ml/min/1.73 m^2, $n = 32$) were more likely to be older ($P < 0.001$) and women ($P = 0.003$), diabetic ($P < 0.001$) and hypertensive ($P = 0.021$) but were less often smokers ($P = 0.003$). In the group of impaired renal function, a higher prevalence of previous PCI ($P = 0.042$) and coronary artery bypass surgery ($P = 0.005$) were observed, and also a more clinical instability at presentation (Killip class > 1, $P = 0.031$). These differences could be explained by the higher morbidity of CKD patients. The two groups of study

Table 1 Baseline demographic data, clinical characteristics and laboratory data according to renal function

	GFR ≥ 60 ml/min/1.73 m^2 $n = 178$	GFR < 60 ml/min/1.73 m^2 $n = 28$	*P* values
Age (year)	58.2 ± 9.6	68.4 ± 9.6	<0.001
Men	154 (87)	18 (64)	0.003
Risk factors			
Smoking	129 (73)	12 (44)	0.003
Hypertension	96 (54)	21 (78)	0.021
Dyslipidemia	76 (43)	10 (37)	0.579
Diabetes mellitus	29 (16)	16 (59)	<0.001
BMI (kg/m^2)	27.6 ± 4.2	28.3 ± 5.4	0.482
Creatinine (mg/dl)	0.86 ± 0.15	1.54 ± 0.47	<0.001
Creatinine clearance (ml/min/1.73 m^2)[a]	97.5 ± 20.9	46.4 ± 9.9	<0.001
Comorbidity			
Previous MI	25 (14)	6 (22)	0.275
Previous PCI	25 (14)	8 (30)	0.042
Previous TIA/Stroke	4 (2)	0	0.427
Previous CABG	2 (1)	3 (11)	0.002
Clinical presentation			
Killip class >1	13 (10)	6 (25)	0.031
LV EF (%)	45.4 ± 5.9	42.2 ± 9.6	0.015
RWMS	1.7 ± 0.3	1.8 ± 0.5	0.093
STEMI	117 (66)	19 (70)	0.661
NSTEMI	57 (32)	8 (30)	0.789
Unstable Angina	3 (2)	0	0.497
Cardiovascular medications			
ACEi/ARBs	104 (60)	19 (70)	0.251
Statins	172 (97)	27 (100)	0.377
Beta-blockers	145 (82)	25 (93)	0.152
Diuretics	11 (6)	5 (19)	0.028
Insulin	11 (6)	8 (31)	<0.001
Oral antidiabetics	20 (12)	8 (30)	0.011
Trombolysis	32 (18)	8 (30)	0.159
Ticagrelor	139 (78)	18 (64)	0.111
Prasugrel	30 (17)	3 (11)	0.410
Clopidogrel	9 (5)	7 (25)	<0.001
Time from symptom onset (h)	46.4 ± 15.8	48.5 ± 15.2	0.517
Time from antiplatelet load (h)	43.1 ± 14.6	44.3 ± 13.1	0.683
Periprocedural drugs			
UFH	170 (96)	27 (100)	0.293
Bivalirudin	2 (1)	0	0.579
GPIIb/IIIa inhibitors	52 (30)	11 (44)	0.145
Laboratory parameters			
Hemoglobin (g/dl)	13.9 ± 1.4	12.5 ± 1.9	<0.001
Hematocrit (%)	41.9 ± 4.5	37.8 ± 6.1	<0.001
Platelet count (×10^3/ml)	226.9 ± 64.1	261.8 ± 100.3	0.018

Values are *n* (%) or mean ± SD. ACEi, angiotensin converting enzyme inhibitors; ARB, AT1 blocker; CABG, coronary artery bypass graft; GFR, glomerular filtration rate; LV EF, left ventricle ejection fraction; MI, myocardial infarction; NSTEMI, non-ST-segment elevation myocardial infarction; PCI, percutaneous coronary intervention; RWMS, regional wall motion score; STEMI, ST-segment elevation myocardial infarction; TIA, transient ischemic attack; UFH, unfractionated heparin. [a]Estimated creatinine clearance was calculated according to MDRD formula.

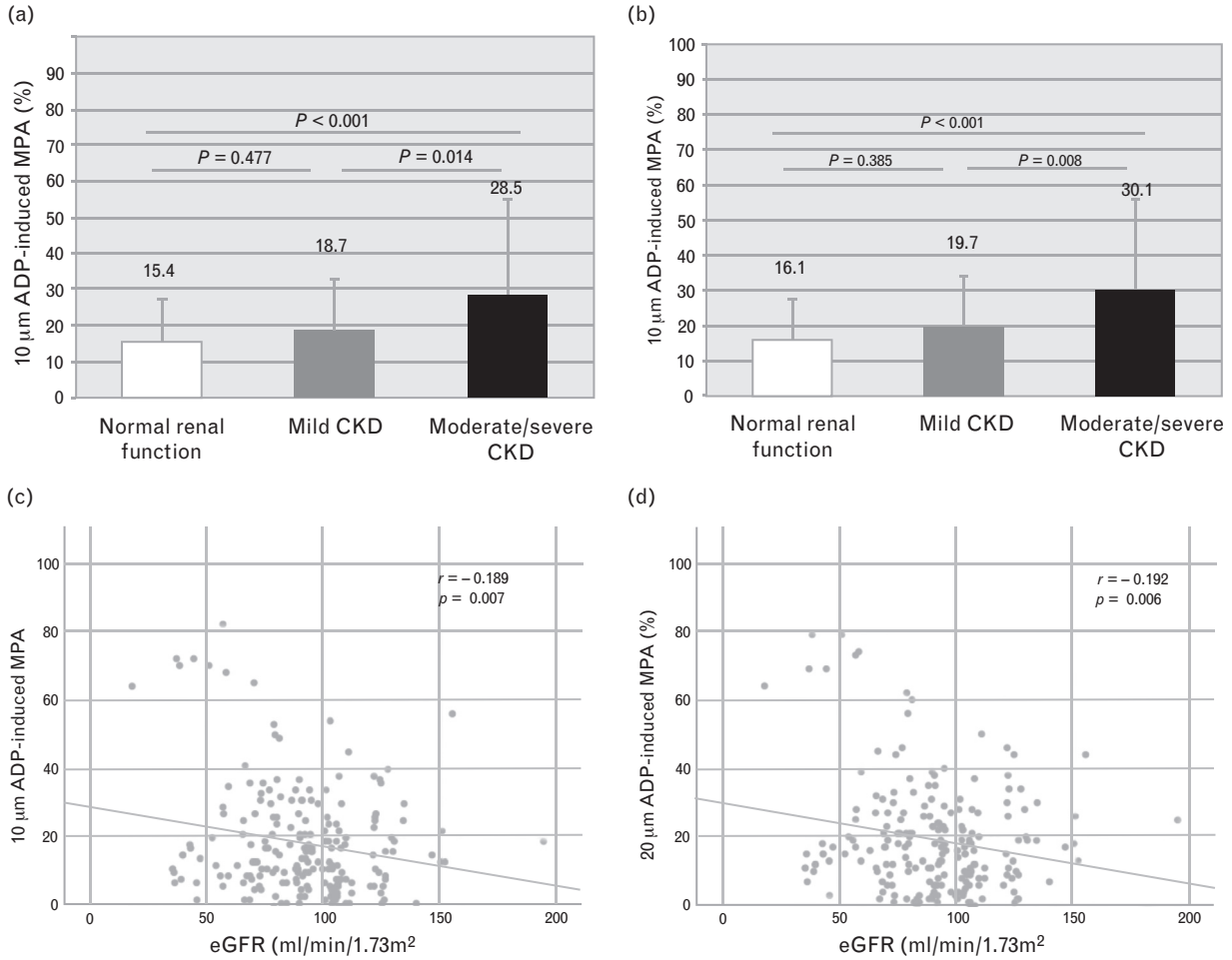
Fig. 1



Percentage of maximum platelet aggregation after stimulation with ADP 10 μmol/l and ADP 20 μmol/l according to renal function.

did not differ in term of cardiovascular medication, except for the use of insulin ($P < 0.001$), oral antidiabetics ($P = 0.011$), diuretics ($P = 0.028$) and clopidogrel ($P = 0.002$). Moreover, patients with CKD had lower haemoglobin, haematocrit ($P < 0.001$) and higher platelet count ($P = 0.018$) then those with preserved renal function. Fig. 1 shows the mean value and SD of MPA induced by ADP in the two groups. Interestingly, platelet aggregation was significantly higher in patients with eGFR less than 60 ml/min/1.73 m^2 than in those with eGFR ≥ 60 ml/min/1.73 m^2 (ADP 10 μmol/l: 28.46 ± 26.19 vs. 16.64 ± 12.79, $P < 0.001$; ADP 20 μmol/l: 30.07 ± 25.89 vs. 17.46 ± 12.82, $P < 0.001$) (Fig. 1). Indeed, the ANOVA, performed to evaluate the magnitude of platelet reactivity among the three stages of renal function, demonstrated significantly lower ADP-induced platelet aggregation in patients with normal renal function and mild CKD compared with those with moderate/severe CKD (Fig. 2a and b). Conversely, no significant differences in platelet aggregation were seen between patients with normal and mild CKD (Fig. 2a and b), despite a trend towards an increasing percentage of MPA can be observed with advancing stages of renal failure (Fig. 2c and d). As confirmation, at linear regression analysis a weak but significant correlation was observed between eGFR and ADP-induced platelet reactivity, at both ADP dosage (ADP 10 μmol/l: $r = -0.189$, $P = 0.007$; ADP 20 μmol/l: $r = -0.192$, $P = 0.006$) (Fig. 2 c and d) HRPR was observed in nine patients (4.4%) with higher prevalence in those with CKD less than 60 ml/min/1.73 m^2 [21.4 vs. 1.7%, $P < 0.001$, OR (95% CI) = 15.91 (3.71–68.17), $P < 0.001$] (Fig. 3a and b) At multivariate analysis, after correction for baseline confounders, eGFR together with the use of clopidogrel emerged as strongest determinants of HRPR [eGFR: adjusted OR (95% CI) = 0.95 (0.91–0.98), $P = 0.007$; clopidogrel: adjusted OR (95% CI) = 23.59 (4.01–138.82), $P < 0.001$] (Table 2). When patients on DAPT with clopidogrel were excluded from the analysis and only patients treated with new P2Y12 inhibitors (ticagrelor and prasugrel) were considered, no significant differences in terms of MPA were seen between the groups of moderate/severe CKD and normal renal function (ADP 10 μmol/l: 18.86 ± 19.40 vs. 15.42 ± 11.20%, $P = 0.230$; ADP 20 μmol/l: 19.81 ± 17.17 vs. 16.52 ± 11.74%, $P = 0.254$).

Fig. 2



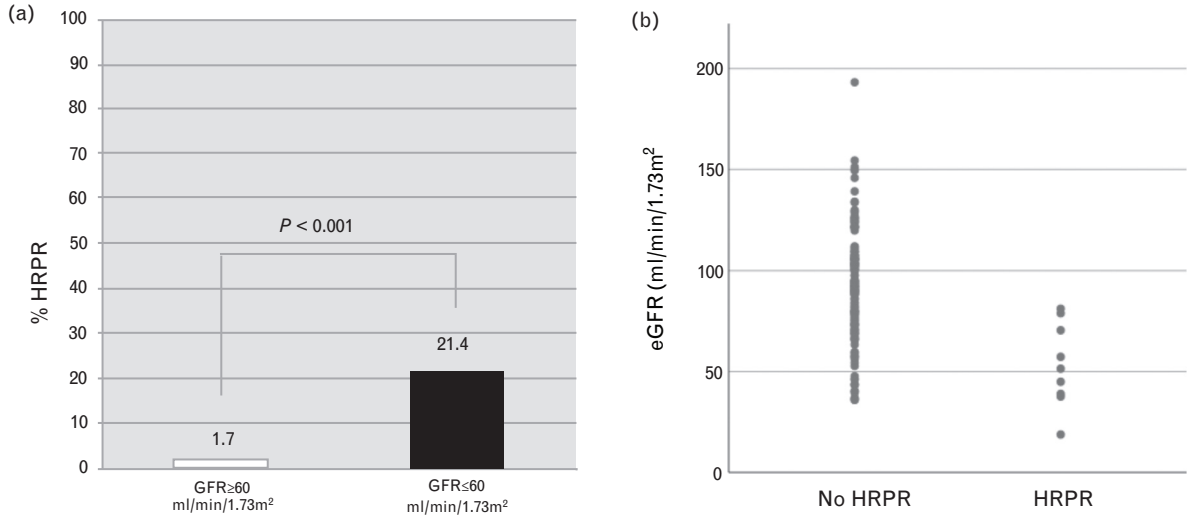
Bar graph showing the percentage of maximum platelet aggregation induced by ADP 10 μmol/l (a) and ADP 20 μmol/l (b) in patients with estimate glomerular filtration rate at least 90 ml/min/1.73 m^2 (normal), 60–89 ml/min/1.73 m^2 (mild chronic kidney disease), and less than 60 ml/min/1.73 m^2 (moderate/severe chronic kidney disease). Correlation between estimate glomerular filtration rate and platelet aggregation induced by ADP 10 μmol/l (c) and ADP 20 μmol/l (d).

Discussion

The current study demonstrates that in patients with ACS treated with DAPT, the presence of moderate to severe CKD is associated with higher degrees of residual platelet reactivity compared with patients with normal or mildly reduced renal function. After adjustment for baseline confounders, eGFR remains inversely associated with HRPR, together with the use of clopidogrel. When only patients in therapy with the new P2Y12 inhibitors are considered in the analysis, no significant increasing of platelet aggregation is detected in CKD group, indicating that the use of clopidogrel mainly drives the HRPR observed in patients with impaired kidney function.

Chronic kidney disease has demonstrated to be an independent predictor of myocardial infarction (MI), stroke and all-cause mortality.[25–28] In patients with ACS, in particular, chronic renal failure have shown at 1-year follow-up to increase occurrence of ischemic events more than major bleeding complications, especially when severe and when is concomitant with anemia.[29] The elevated cardiovascular morbidity and mortality related to CKD is partly explained with a more aggressive atherosclerotic disease and a greater risk of thrombotic events, compared with general population. In addition, percutaneous treatment of coronary disease in stable and acute patients with moderate or severe renal disease is associated with an increased in-hospital and long-term mortality.[30–32] This underscores the need for effective antiplatelet therapy in this setting of patients. Some studies have suggested that an impaired renal function might affect platelet function, reducing the efficacy of antiplatelet agents and contributing with this mechanism to the higher thrombotic risk that affect CKD patients. Angiolillo *et al.*[16] demonstrated in diabetic patients with CAD a reduced clopidogrel-induced antiplatelet effect

Fig. 3



Bar graph showing the prevalence of high residual platelet reactivity at ADP-induced platelet aggregation according to renal function (a). Scatter plot showing correlation between estimate glomerular filtration rate and high residual platelet reactivity (b).

and a greater prevalence of HRPR. This evidence was further confirmed by Breet *et al.*[17], that described a higher magnitude of ADP-induced platelet reactivity in patients with moderately/severely decreased eGFR with stable CAD undergoing coronary intervention. In their study, they failed to demonstrate an association between the presence of HRPR and clinical outcomes at 1-year follow-up between patients with and without CKD, suggesting that multiple comorbidities might contribute to higher mortality and composite endpoint (death, MI, stent thrombosis and stroke) observed in CKD cohort of patients.[17] Similarly, Zhu *et al.*[33] applied modified thromboelastrography to evaluate HRPR in 6745 patients with different CKD stages, receiving DAPT with aspirin and clopidogrel after PCI. Though HRPR for ADP was correlated with the decline of eGFR, no significant difference was observed for major adverse cardiovascular and cerebrovascular events between patients with and without HRPR.[33] Conversely, an association with HRPR and short-term adverse clinical events in patients with renal failure undergoing PCI was showed by Mangiacapra *et al.*[19], who found both high and low platelet reactivity as strongest predictors of both ischemic and bleedings events. In support of this evidence, a recent meta-analysis showed in CKD patients treated with clopidogrel higher prevalence of HRPR, associated with increased hazard of ischemic events and worse outcome compared with patients with normal platelet reactivity.[34]

Most of the study available explored the effect of renal failure on platelet function in patients with stable CAD undergoing PCI, usually after loading dose of clopidogrel. To date, little is known about the impact of CKD on platelet reactivity in the early phases of an ACS, specially when the new potent P2Y12 inhibitors (i.e. prasugrel and ticagrelor) are used. A recent retrospective study on patients treated with clopidogrel and ticagrelor showed the absence of any relationship between chronic renal failure and HRPR.[35] However, the analysis included either patient with ACS or undergoing elective PCI, and platelet aggregation assay was performed with Multiplate (Roche AG, Grenzach-Wyhlen, Germany), a whole blood test, at 30–90 days from the coronary intervention. Thus, this population cannot be considered

Table 2 Univariate and multivariate analysis of the parameters independently associated with high residual platelet reactivity

Parameter	Univariate OR (95% CI)	*P* value	Multivariate OR (95% CI)	*P* value
Age	1.23 (1.10–1.37)	<0.001	–	–
eGFR	0.94 (0.91–0.97)	<0.001	0.95 (0.91–0.98)	0.007
Platelet count	1.01 (1.00–1.02)	0.064	–	–
Ticagrelor	0.08 (0.02–0.38)	0.002	–	–
Clopidogrel	35.4 (7.70–162.72)	<0.001	23.59 (4.01–138.82)	<0.001
Diuretics	16.92 (1.55–30.90)	0.011	–	–

CI, confidence interval; eGFR, estimate glomerular filtration rate; OR, odds ratio.

representative of the acute phase, when it's commonly known that platelets are more reactive and a poor antiplatelet drug responsiveness and HRPR could have a greater influence on clinical outcome, owing to interplay between activated platelets and ruptured plaque or the stent strut. Conversely, in stable cardiovascular patients, considering the lower endothelial thrombogenicity and lower platelet activation status, the detection of poor platelet response to antiplatelet drug may be less critical.[36]

In the current study, the analysis of platelet aggregation was performed with LTA within 48 h of diagnosis of ACS and confirmed that, also in the acute setting, there is a strong association between renal failure and platelet hyperactivity. Moreover, patients included in the study were treated with all P2Y12 inhibitors currently available, included the newest and most powerful prasugrel and ticagrelor, that have become, according to the recent guidelines, of first choice in this setting of patients.[8,10–11] In our population, clopidogrel emerged as a strong determinant of HRPR, together with renal function. Also this data appears to be in line with the previous studies, that reported in CKD patients an higher on-clopidogrel platelet reactivity.[16,17,34] Conversely, when patients treated with clopidogrel were excluded from the analysis, platelet aggregation didn't different significantly between patients with normal and impaired renal function, indicating that, probably, the poorer response to antiplatelet agents described in CKD patients was mainly attributable to clopidogrel, rather than to the newer P2Y12 inhibitors. Indeed, recent evidences demonstrated in CKD patients treated with ticagrelor or prasugrel lower platelet reactivity levels compared with clopidogrel.[37] High levels of circulating procoagulant factors, upregulation of the P2Y12 signalling pathway, increased thrombin generation, abnormalities of nitric oxide synthesis, together with a poor bioavailability of clopidogrel's active metabolite due to a reduced activity of the hepatic cytochrome P450 system, have been proposed to explain platelet hyperreactivity and HRPR among patients with renal dysfunction.[38] In the current study, at univariate analysis, patients with CKD treated with ticagrelor seemed to have lower rates of HRPR. This result could partly explain the greater reduction in total adverse ischemic events and mortality demonstrated in a subanalysis of PLATelet inhibition and patient Outcomes trial on a subgroup of patients with ACS affected by CKD ($eGFR < 60\,ml/min/1.73\,m^2$) in therapy with ticagrelor compared with clopidogrel.[39] Accordingly, these finding could support the use of ticagrelor over clopidogrel in the setting of patients with impaired renal function undergoing PCI for an acute ischemic event.

Our study showed the lack of significant difference in platelet response between patients with normal and those with mild CKD, despite a trend towards and increasing platelet reactivity was seen, while a significant difference was detected between the groups of mild and moderate/severe CKD. Probably this result can be referred to the small cohort of patients of each group, or rather can suggest the presence of a threshold of kidney function, below which an impairment of platelet function occurs. This observation, actually, is in agreement with previous clinical studies, that demonstrated worse outcomes in patient with more advanced stages of CKD.[40,41]

A limitation of our study is that the small number of patients with severe renal failure ($eGFR < 30\,ml/min/1.73\,m^2$) and the exclusion of patients with eGFR less than $15\,ml/min/1.73\,m^2$ or on haemodialysis do not allow to extrapolate the results of the current study to patients with severe and very severe CKD. Moreover, we didn't investigate the effect of contrast administration during angiography on kidney function or potential impact on platelet aggregation. The prevalent use of clopidogrel in the group of patients with CKD represent a limitation that might explain the higher incidence of HRPR in that study group. Furthermore, the higher profile risk observed in the CKD patients might have contributed to the increased platelet aggregation observed. The wide SD in MPA values and the large CIs in the regression analysis might limit the clinical applicability of the study results and the clinical impact of clopidogrel-induced HRPR in patients with renal dysfunction. Finally, we didn't report data regarding clinical follow-up, thus we could not investigate whether HRPR reported in CKD patients could affect long-term outcome.

Conclusion

In patients with ACS receiving DAPT, chronic renal failure is associated with an increasing ADP-induced platelet aggregation, which is most detected at higher stages of renal dysfunction, and with a higher prevalence of residual platelet reactivity, demonstrating a poorer responsiveness to antiplatelet drugs, in particular to clopidogrel. This finding might have important clinical implications given the higher prothrombotic tendency and incidence rate of adverse cardiovascular events in CKD patients, and could guide the choice of the best antiplatelet therapy in this setting of patients.

Acknowledgements

Conflicts of interest

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Effects of Carvedilol Versus Metoprolol on Platelet Aggregation in Patients With Acute Coronary Syndrome: The PLATE-BLOCK Study

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Platelet aggregation plays a pivotal role in acute coronary syndrome (ACS). In this setting, β-blockers (BBs) are used to counteract the effects of catecholamines on heart. Circulating catecholamines can also potentiate platelet reactivity, mainly through α_2- and β_2-adrenoceptors on human platelets' surface, thus BB may affect platelet aggregation; however, the effects of different BBs on platelet aggregation in contemporary-treated patients with ACS have been poorly investigated. One hundred patients with ACS on dual antiplatelet therapy with aspirin and ticagrelor were randomized to receive treatment with carvedilol, a nonselective BB (n = 50), or metoprolol, a selective β_1-blocker (n = 50), at maximum tolerated dose. Light transmission aggregometry was performed at randomization (T0) and at 30-day follow-up (T30), and the results were expressed as a percentage of maximum platelet aggregation (MPA). The primary end point was epinephrine-induced MPA at 30 days. Patients were predominantly men (80%), and mean age was 57.3 ± 9.7 years. The 2 randomized groups were well balanced for baseline characteristics. At T0, mean MPA was similar between the groups (18.96 ± 9.05 vs 18.32 ± 9.21 with 10 μM epinephrine, 14.42 ± 9.43 vs 15.98 ± 10.08 with 20 μM adenosine diphophate (ADP), and 13.26 ± 9.83 vs 14.30 ± 9.40 with 10 μM ADP for carvedilol and metoprolol, respectively, all p = NS). At 30 days, platelet aggregation induced by epinephrine was significantly lower in the carvedilol group than in the metoprolol group (23.52 ± 10.25 vs 28.72 ± 14.37, p = 0.04), with a trend toward the lower values of ADP-induced MPA (20 μM ADP 19.42 ± 13.84 vs 24.16 ± 13.62, p = 0.09; 10 μM ADP 19.12 ± 12.40 vs 22.57 ± 13.59, p = 0.19). In conclusion, carvedilol, a nonselective BB, reduces residual platelet reactivity in patients with ACS compared with the selective BB, metoprolol. **(Am J Cardiol 2018;122:6–11)**

Platelet aggregation plays a pivotal role in the pathogenesis of ischemic events during and after acute coronary syndrome (ACS).[1–3] Myocardial ischemia is associated with a high activity of the sympathetic nervous system, which is reflected by increased plasma levels of epinephrine and norepinephrine. In patients with ACS, β-blocker (BB) drugs are important to counteract the effects of catecholamines on heart, and many compounds are available (some with selective β_1-adrenoceptor blockade and some with nonselective α- and β-inhibition properties[4]), but there is no molecule recommended over the other.[5–7] Besides their effects on heart, circulating catecholamines have also demonstrated to affect platelet reactivity in different manners, such as potentiating the proaggregant effect of other substances, influencing the response to antiplatelet agents, directly interacting with platelets' surface adrenergic receptors (α_{2A} subtype is the most abundant, but β_2 type is also present).[8–12] Additionally, nonselective BB seem also able to decrease plasma catecholamine levels more than selective ones,[13] and their lipophilicity can increase the ability to indirectly affect platelet aggregation by a chemical interaction with platelet's cell membrane.[14] A recent meta-analysis suggested that nonselective lipophilic BB reduced platelet aggregation more effectively than selective nonlipophilic BB, but included studies were well outdated (mainly conducted in 1970s to 1980s), and none of them included patients with ACS treated with contemporary dual antiplatelet therapy (DAPT).[8] The aim of our study was to compare the effects of the nonselective BB, carvedilol, with the selective metoprolol on platelet aggregation induced by epinephrine and adenosine diphosphate (ADP) in the contemporary setting of patients with ACS receiving DAPT with aspirin and ticagrelor.

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Methods

The PLATE-BLOCK study is an investigator-initiated, single-center, open-label, prospective randomized trial. Consecutive patients hospitalized for an ACS (ST-segment elevation myocardial infarction [STEMI], non–ST-segment elevation myocardial infarction [NSTEMI], unstable angina) presented at the coronary care unit of University Federico II of Naples, undergoing acid acetylsalicylic and ticagrelor treatment and percutaneous coronary intervention, were screened. Exclusion criteria were as follows: age <18 years; contraindication to BB therapy; ongoing prasugrel, ticlopidine, or clopidogrel therapy; creatinine clearance <30 ml/min; moderate to severe anemia (hemoglobin <10 mg/dl); platelet count >600,000/mm^3 or <130,000/mm^3; hematocrit >50% or <25%; known blood dyscrasia or bleeding diathesis; concomitant neoplastic or immune-mediated pathologies; ongoing oral anticoagulation therapy. Patients were treated with aspirin (loading dose of 150 to 300 mg orally or 75 to 150 mg intravenously, and 100 mg once a day as maintenance dose) and ticagrelor (180 mg loading dose, 90 mg twice daily as maintenance dose). All patients eligible for enrollment who accepted to participate provided written informed consent and were randomly assigned to carvedilol or metoprolol treatment at maximum tolerated dosage. Randomization occurred using concealed table that was previously generated using Research Randomizer (https://www.randomizer.org). The study complied with the Declaration of Helsinki. The local institutional ethics committee approved the study protocol, and all patients gave written informed consent to participate. The trial protocol is registered within ClinicalTrials.gov, number NCT02809820.

Clinical events occurring within 30 days were recorded. Any death, unless an unequivocal noncardiovascular cause could be established, was defined as death from cardiovascular causes. Myocardial infarction was defined in accordance with the third universal definition proposed in 2012.[15] Stent thrombosis was defined according to the Academic Research Consortium criteria.[16] Stroke was defined as focal loss of neurologic function caused by an ischemic or hemorrhagic event, with residual symptoms lasting at least 24 hours or leading to death. Bleeding was defined according to Thrombolysis In Myocardial Infarction (TIMI) and Bleeding Academic Research Consortium criteria.[17]

Samples for platelet function testing were taken at baseline (within 48 hours after coronary angiography after diagnosis of ACS) and at 30 days. The measurements were performed in the morning just before the administration of the morning ticagrelor dose. When GPIIb/IIIa inhibitors were prescribed, an interval of 18 to 24 hours after completion of the infusion was required before platelet function testing was performed, to avoid interference with aggregation assay. Platelet aggregation was measured by light transmission aggregometry (LTA) using a dual channel lumi-aggregometer (model 700; Chrono-Log, Havertown, Pennsylvania). Platelet-rich plasma was prepared from blood that was drawn from the antecubital vein by venipuncture into 3.8% trisodium citrate (w/v) Vacutainer blood collection tubes. Platelet-rich plasma was obtained by centrifugation of blood at 180 *g* at 25°C for 10 minutes. Platelet-poor plasma was obtained by centrifugation of the rest of the blood at 1,500 *g* at 25°C for 10 minutes.[3] Platelet aggregation was monitored at 37°C with constant stirring (1,200 rpm) and measured as the increase in light transmission for 6 minutes, with the addition of epinephrine (10 μM) and ADP (10 and 20 μM) as a proaggregatory stimulus. All measurements were obtained within 2 hours of sample collection, and the results are reported as percentage of maximum platelet aggregation (MPA).

The primary end point of the study was to evaluate the effects of metoprolol versus carvedilol after 30 days of treatment on platelet aggregation induced by 10 μM epinephrine, in patients with ACS on DAPT. Epinephrine test was elected to be the primary end point given the anticipated effects of BB on reducing catecholamine-induced platelet aggregation. Secondary end points were the evaluation of ADP-induced platelet aggregation (10 and 20 μM) and adverse clinical events, including ischemic and bleeding complications at 30 days.

The primary hypothesis of the study is that the % MPA with epinephrine at 30 days will be reduced in the carvedilol group compared with the metoprolol group. Based on previous studies, standard deviation (SD) of MPA is quite variable, and probably different timing, different drugs, and different methodologies contribute to this.[18,19] Assuming an SD of at least 8% and that an absolute 5% would be a clinically relevant difference in the % MPA induced by epinephrine, a sample size of at least 100 subjects (50 for each group) would detect a true difference between groups with statistical power ≥80% at an alpha significance level of 0.05. The planned sample size was then increased up to 120 to allow occurrence of new contraindications or adverse events or incomplete aggregometry data. Variables were expressed as absolute numbers and percentage or mean ± SD. Comparisons were made by chi-square test or Student *t* test, as appropriate. Statistical analyses were performed using IBM-SPSS, version 23 (SPSS Inc, Chicago, Illinois).

Results

Between June 2016 and December 2016, 204 patients with ACS (STEMI, NSTEMI, and unstable angina) were assessed for eligibility. Of these, 84 did not meet the study entry criteria or refused to consent, whereas 120 provided their written informed consent to participate in the study; of these, 111 were randomized (metoprolol, n = 55; carvedilol, n = 56), representing the enrolled population. A total of 100 patients (metoprolol, n = 50; carvedilol, n = 50) were the primary population and finally analyzed (Figure 1). Baseline clinical characteristics are shown in Table 1. The mean age was 57.3 ± 9.7, and the majority of patients were males and smokers, 18% had diabetes, and 16% had history of myocardial infarction. At clinical presentation, 62% had STEMI, with an average left ventricle ejection fraction at transthoracic echocardiogram of about 45% and a relatively stable hemodynamic profile. The 2 randomized groups were homogenous in terms of cardiovascular risk factors, routine laboratory variables, or medications (Table 1). Angiographic and procedural characteristics of the 2 study groups are shown in Table 1. The culprit lesion was the left anterior descending artery in half of patients, and 1/3 of patients had multivessel disease. The majority of patients received percutaneous coronary intervention with stent implantation and had a final TIMI 3 flow.

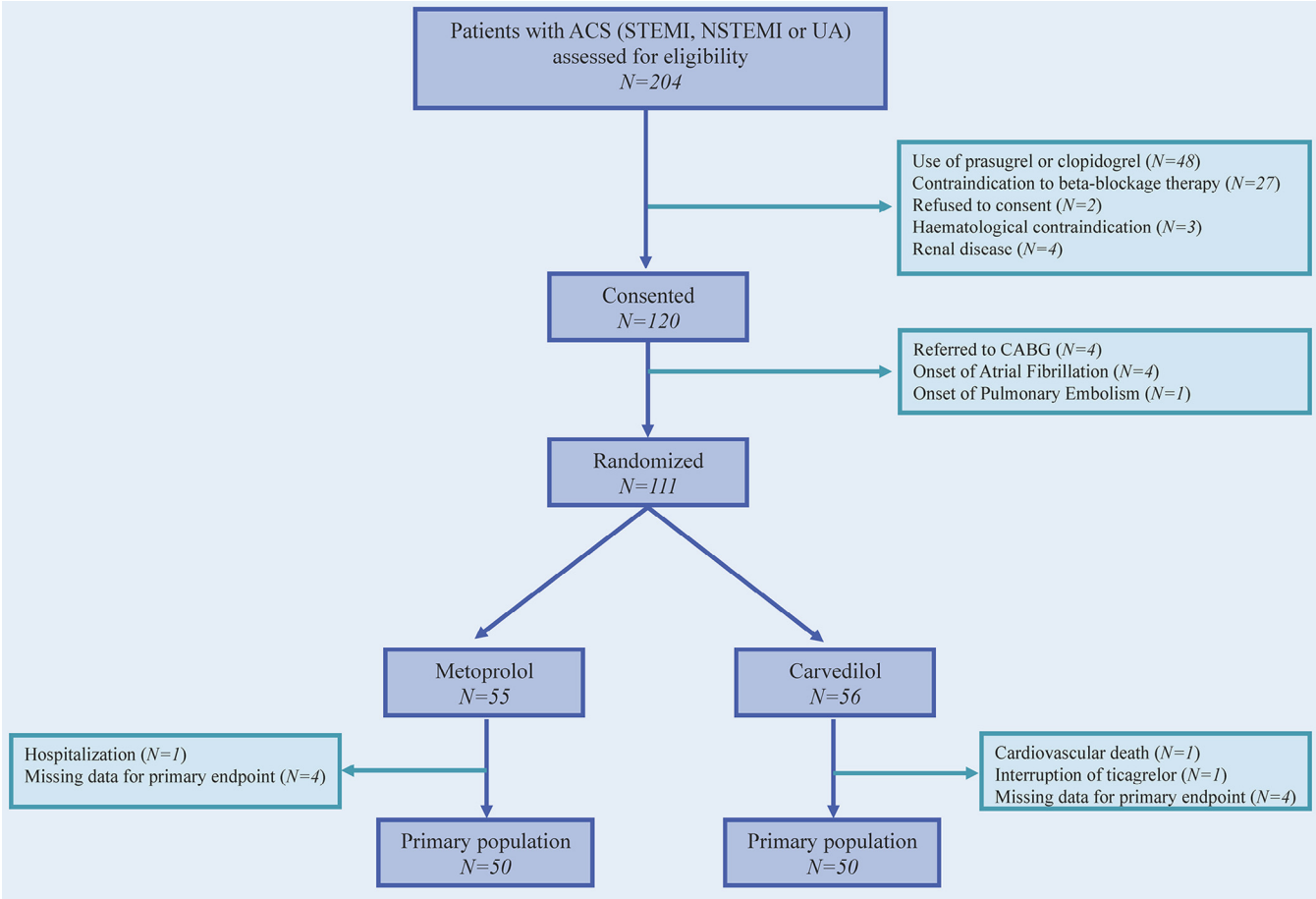

Figure 1. Flowchart of the study. ACS, acute coronary syndrome; STEMI = ST-segment elevation myocardial infarction; NSTEMI = non–ST-segment elevation myocardial infarction; UA = unstable angina.

Table 2 lists the mean value and SD of MPA induced by epinephrine and ADP in the 2 groups, at baseline (T0) and at 30 days (T30). At baseline, results of the platelet assessment were comparable between the 2 groups, with the expected platelet inhibition as a result of the DAPT. At T30, the results of MPA were significantly higher than baseline in the 2 groups for all proaggregative stimuli. Interestingly, patients in the carvedilol group showed an epinephrine-induced MPA at T30 significantly lower than that observed in the metoprolol population (23.5 ± 10.2 vs 28.7 ± 14.3, p = 0.040) (Figure 2). In secondary analyses, when ADP was used as proaggregative stimulus, a trend toward reduction in the carvedilol group compared with metoprolol group was observed and was more pronounced with higher ADP concentration (10 μM ADP 19.12 ± 12.40 vs 22.57 ± 13.59, p = 0.19; 20 μM ADP 19.42 ± 13.84 vs 24.16 ± 13.62, p = 0.088). Overall, no patient, but one in the metoprolol group, showed a high on-treatment residual platelet reactivity (MPA >59% with ADP stimulation).

Clinical outcomes at 30 days, in terms of ischemic and bleeding end points, are shown in Table 2. No death, myocardial infarction, or urgent revascularization occurred. Two patients in the carvedilol group underwent a new hospitalization within 30-day follow-up, both of them for heart failure. Notably, 1 minor bleeding was observed in the metoprolol group (blood loss from pre-existing hemorrhoids) without need for modification of DAPT and no major bleeding events were reported.

Discussion

There is evidence that BB can inhibit the catecholamine-induced platelet aggregability, but there is limited evidence regarding the role of specific BB agents to affect platelet activity and there are no data on ACS patients treated with contemporary DAPT including aspirin and a new more potent P2Y12 inhibitor. To our knowledge, this is the first randomized, open-label study on patients with ACS receiving aspirin and ticagrelor to compare carvedilol, a nonselective BB, with metoprolol, a selective β_1-blocker. We found that carvedilol significantly reduced residual platelet aggregation 30 days after the index event compared with metoprolol. Notably, this benefit was additional to DAPT and was observed despite the therapy with a new potent P2Y12 inhibitor (i.e., ticagrelor). This finding might have important clinical implications in the daily practice when choosing the type of BB agent to be used in this setting of patients.

BBs competitively inhibit the myocardial effects of circulating catecholamines and reduce myocardial oxygen consumption by lowering heart rate, blood pressure, and myocardial contractility. They are recommended for secondary

Table 1
Baseline, angiographic, and procedural characteristics according to beta-blockage therapy

Variable	Carvedilol (n = 50)	Metoprolol (n = 50)	P value
Age (years)	57.8 ± 9.5	56.8 ± 9.8	0.600
Men	41 (82%)	39 (78%)	0.617
Smoker	36 (72%)	36 (72%)	1.000
Hypertension	29 (58%)	25 (50%)	0.422
Hypercholesterolemia*	23 (46%)	17 (34%)	0.221
Diabetes Mellitus	10 (20%)	8 (16%)	0.603
BMI (kg/m^2)	27.9 ± 4.9	28.5 ± 4.5	0.596
Previous MI	10 (20%)	6 (12%)	0.275
Previous PCI	9 (18%)	6 (12%)	0.401
Previous TIA/Stroke	1 (2%)	1 (2%)	0.977
Previous coronary bypass	2 (4%)	1 (2%)	0.558
Creatinine (mg/dl)	0.92 ± 0.23	0.87 ± 0.16	0.199
Creatinine clearance (ml/min)†	100.8 ± 32.4	109.8 ± 31.7	0.165
COPD	8 (16%)	7 (14%)	0.779
Clinical presentation			
Killip class >1	7 (14%)	7 (14%)	1.000
LVEF (%)	45.0 ± 6.3	44.9 ± 5.7	0.921
RWMS	1.6 ± 0.3	1.7 ± 0.3	0.559
Heart rate (bpm)	71.8 ± 9.1	74.4 ± 10.6	0.196
Systolic BP (mm Hg)	122.6 ± 17.2	123.0 ± 17.4	0.921
Diastolic BP (mm Hg)	79.1 ± 9.3	76.7 ± 11.3	0.256
STEMI	31 (62%)	31 (62%)	1.000
NSTEMI	18 (36%)	19 (38%)	0.836
Unstable angina pectoris	1 (2%)	0 (0%)	0.315
Laboratory parameters			
Hemoglobin (g/dl)	13.8 ± 1.5	14.0 ± 1.5	0.520
Hematocrit (%)	41.7 ± 4.6	41.8 ± 4.1	0.984
Platelet count (×10^3/ml)	210.2 ± 56.1	230.2 ± 62.9	0.097
Cardiovascular medications			
Proton Pump Inhibitors	49 (98%)	50 (100%)	0.315
ACEi/ARBs	25 (50%)	32 (64%)	0.157
Statins	49 (98%)	48 (96%)	0.558
Diuretics	3 (6%)	2 (4%)	0.630
Insulin	4 (8%)	2 (4%)	0.371
Oral antidiabetics	8 (16%)	6 (12%)	0.509
Thrombolysis	5 (10%)	2 (4%)	0.240
Randomized beta-blocker dosage			
Mean (standard deviation)	13.5 ± 6.5	98.0 ± 26.6	-
Median (range)	12.5 (6.25–25)	100 (50–200)	-
Periprocedural antithrombotics			
UFH	49 (98%)	47 (94%)	0.307
Bivalirudin	0 (0%)	1 (2%)	0.315
Glycoprotein IIb/IIIa inhibitors	15 (30%)	20 (40%)	0.366
Culprit coronary artery			
Left main	0 (0%)	1 (2%)	0.315
Left anterior descending artery	23 (46%)	29 (58%)	0.230
Left circumflex	12 (24%)	7 (14%)	0.202
Right coronary artery	13 (26%)	13 (26%)	>0.999
Multi-vessel coronary disease	13 (26%)	17 (34%)	0.383
TIMI flow pre-PCI			
0	20 (40%)	16 (32%)	0.405
1	5 (10%)	7 (14%)	0.538
2	12 (24%)	9 (18%)	0.461
3	13 (26%)	18 (36%)	0.280
TIMI flow post-PCI			
0	0 (0%)	0 (0%)	-
1	0 (0%)	0 (0%)	-
2	1 (2%)	0 (0%)	0.315
3	49 (98%)	50 (100%)	0.315
Procedural details			
Stent	47 (94%)	49 (98%)	0.307
Diameter of stent (mm)	3.11 ± 0.47	3.12 ± 0.35	0.828
Length of stent (mm)	21.93 ± 7.37	20.31 ± 7.01	0.270

Values are n (%) or mean ± SD.

ACEi = angiotensin-converting enzyme inhibitors; ARB = angiotensin receptor blocker; BMI = body mass index; BP = blood pressure; COPD = chronic obstructive pulmonary disease; HR = heart rate; LVEF = left ventricle ejection fraction; MI = myocardial infarction; NSTEMI = non–ST-segment elevation myocardial infarction; PCI = percutaneous coronary intervention; RWMS = regional wall motion score; STEMI = ST-segment elevation myocardial infarction; TIA = transient ischemic attack; TIMI = Thrombolysis In Myocardial Infarction; UFH = unfractionated heparin.

* Hypercholesterolemia refers to patients receiving lipid-lowering therapy at baseline (before index event).

† Estimated creatinine clearance was calculated according to Cockcroft-Gault formula.

Table 2
Results of platelet aggregation induced by epinephrine and ADP with LTA and 30-day clinical outcomes

	Carvedilol (n = 50)	Metoprolol (n = 50)	p
Epinephrine 10 µmol/L			
T0	18.96 ± 9.05	18.32 ± 9.21	0.727
T30	23.52 ± 10.25*	28.72 ± 14.37*	0.040
ADP 10 µmol/L			
T0	13.26 ± 9.83	14.30 ± 9.40	0.590
T30	19.12 ± 12.40*	22.57 ± 13.59*	0.190
ADP 20 µmol/L			
T0	14.42 ± 9.43	15.98 ± 10.08	0.426
T30	19.42 ± 13.84*	24.16 ± 13.62*	0.088
Clinical outcomes			
Death	0	0	-
Myocardial Infarction	0	0	-
Stent Thrombosis	0	0	-
Stroke	0	0	-
Urgent TVR	0	0	-
Intracranial bleeding	0	0	-
TIMI major bleed	0	0	-
TIMI minor bleed	0	1 (2%)	0.315
BARC type 3 or 5	0	0	-
BARC type 2	0	1 (2%)	0.315
Hospitalization	2 (4%)	0	0.153

Values are expressed as % of maximum platelet aggregation (MPA) ± SD.

* p <0.05 versus T0.

BARC = Bleeding Academic Research Consortium; TIMI = Thrombolysis In Myocardial Infarction; TVR = target vessel revascularization.

prevention in patients with ACS, regardless of reperfusion therapy, given their beneficial effects on prognosis.[5–7] Nowadays, many BB compounds are available, with different pharmacologic profiles. Generally, BBs without intrinsic sympathomimetic activity are suggested, especially β-1 blockers such as sustained release metoprolol succinate, bisoprolol, or the β-1 and α-1 blocker carvedilol, which are also the ones demonstrating mortality benefits in patients with heart failure and systolic dysfunction.[5] Among them, often β-1 blockers

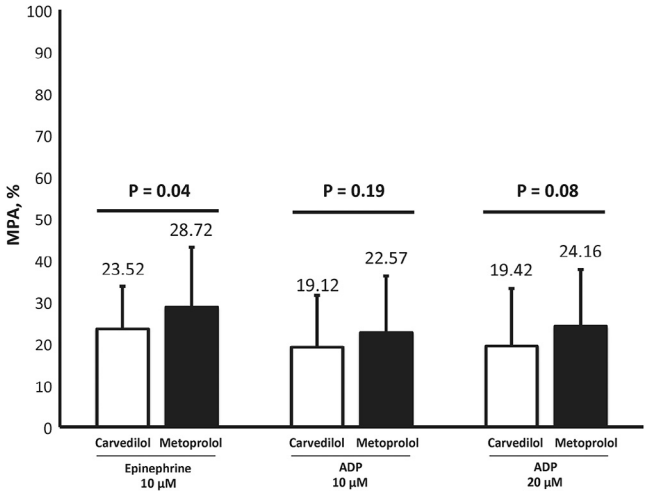

Figure 2. Maximum platelet aggregation 30 days after acute coronary syndrome according to the randomized treatment with carvedilol or metoprolol. MPA = maximum platelet aggregation.

are chosen to reduce pulmonary complications related to bronchospasm; however, there is no preferential guideline recommendation of 1 molecule over the other;[5–7] thus, our study could be relevant to provide useful insights on this topic and to help guide the selection of the optimal BB type. The early administration of BB agents in myocardial infarction is supported by several studies that demonstrated favorable effect in reducing blood pressure, heart rate, arrhythmias, and improving left ventricle systolic function. Different studies and a recent meta-analysis have suggested that, besides these well-known beneficial clinical effects, BBs could exert their protective action by also inhibiting platelet aggregation.[8–10]

The effects of circulating catecholamines on platelet reactivity have been extensively investigated, reporting an increased ADP- and collagen-induced platelet aggregation in conditions of elevated adrenergic system activity, such as myocardial infarction and angina. Catecholamines are supposed to exhibit their proaggregating effects, interacting mainly with α_{2A} adrenoceptors, whose stimulation determines the inhibition of adenylate cyclase through G_i protein.[11,20] Platelet surface also exhibits a small amount of β_2-receptor, whose activation results in AMP cyclic (cAMP) formation, which is known to inhibit platelet aggregation through mechanisms involving Ca^{++}.[12] β_2-receptor could be targeted by nonselective BB, with a consequent inhibition of platelets cAMP formation, decrease of calcium availability, and in turn platelet activation. Conversely, the use of selective β_1-blockers would protect from this mechanism, as suggested by Winther et al[21] that showed higher levels of plasma and platelet cAMP and lower platelet aggregation in patients treated with metoprolol compared with propranolol. However, compared with placebo, metoprolol did not show any reduction of ADP-induced platelet aggregation in patients with myocardial infarction.[22] Therefore, the antiplatelet effect of BB is only partially explained by the direct interaction with the platelet adrenoceptors, but there are also other indirect mechanisms. Indeed, it is known that BBs are able to decrease plasma levels of catecholamines, with a more pronounced effect of nonselective compounds.[23,24] In particular, in patients with heart failure, the long-term therapy with carvedilol but not metoprolol reduced coronary sinus norepinephrine levels.[13]

In addition, some nonselective BBs, such as carvedilol and propranolol, have shown a membrane-stabilizing effect that affects Ca^{++} availability in the platelets and inhibits platelet aggregation.[14,25] Petrikova et al[26,27] investigated the antiplatelet activity of carvedilol and showed that, besides the antagonistic effects on α-adrenoceptors, carvedilol, thanks to its lipophilicity, inhibits platelet aggregation and thromboxane B2 formation through the interaction with membrane macromolecules, such as phospholipids, ion channels, enzymes, and other molecules.

In accordance with the available data, our study demonstrates that patients receiving carvedilol showed lower epinephrine-induced platelet aggregation than those treated with metoprolol after ACS. Based on the results of LTA, carvedilol was not able to significantly reduce ADP-induced platelet aggregation. Also, this finding is in accordance with previous studies[27,28] that demonstrated an in vitro dose-dependent reduction of aggregation when epinephrine was used as stimulus, whereas carvedilol was least effective in platelets stimulated with ADP even at high concentrations.

Notably, the baseline level of platelet aggregation is lower than T30 values. This finding seems to be consistent with pharmacodynamic studies of patients treated with ticagrelor, which showed that, after 6 weeks of treatment, the inhibitor effect on platelet aggregation measured with LTA was slightly lower than 24 hours after the loading dose.[19] In our study, baseline aggregation was performed within 24 to 48 hours since the coronary angiography, so platelet aggregation could be still influenced by different kinds of medications.

This study explored platelet aggregation and was not powered to assess clinical events. However, the 2 BBs investigated are routinely used in the daily practice and none of them is expected to significantly impact on ischemic and bleeding events compared with the other. Additionally, it is well known that DAPT with more potent P2Y12 inhibitors is the standard of care after ACS and percutaneous coronary intervention,[29,30] and platelet reactivity is associated with thrombotic events; thus, its reduction could be beneficial in reducing thrombotic complications.[2] Our data were focused on ticagrelor-based DAPT and cannot be extended to patients receiving different DAPT regimens (i.e., prasugrel or clopidogrel) or single antiplatelet therapy associated with anticoagulation therapy (i.e., patients with atrial fibrillation or mechanic valves).

In conclusion, our study showed that in patients with ACS receiving contemporary DAPT with aspirin and ticagrelor, the use of carvedilol, a nonselective BB, is associated with a reduced residual platelet aggregation compared with metoprolol, a selective β_1-blocker. This finding might have important clinical implications, given the enhanced adrenergic signaling in the setting of ACS and its known association with platelet reactivity, thrombotic events, and long-term outcomes. However, further studies are needed to evaluate the translational effect of this benefit on clinical outcomes.

Disclosures

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Part V

Discussion and conclusions

Discussion

Part I. Feasibility and reference ranges for longitudinal strain and myocardial work indices in EACVI NORRE study population.

An optimal application of clinical echocardiography implies an accurate definition of 'normality', on the basis of which abnormalities can be detected (51). Currently, available echocardiographic 'reference values' that define 'normality' are mostly based on cross-sectional observations and refer to earlier studies with wide variability of sample sizes, selection criteria, definition of 'healthy individuals', performance and/or reading approaches, or statistical analyses, and often obtained using old technologies (52-54). The need to develop echocardiographic reference limits has become even more evident with the advent and introduction in routine clinical practice of new methodologies such as 2D and 3D STE and MW. Based on this perspective, the NORRE (Normal Reference Ranges for Echocardiography) Study, a large prospective multicentre study performed in 22 laboratories accredited by the European Association of Cardiovascular Imaging (EACVI) and in one American laboratory, have enrolled 734 healthy subject over a wide range of ages (25–75 years old) with the aim to provide a set of 'normal values' using both conventional and advanced echocardiographic techniques (55). In our study (chapter 2), we provided normal reference limits for 2D and 3D measurement of LA function, using vendor-independent software, and examined the influence of age, gender, and vendor on the reference ranges. The use of a vendor-independent software allowed to obtain homogeneous measurements irrespective of the echocardiographic equipment used to acquire data. We found that LA reservoir and conduit function decreased with age while pump function increased. This finding may be explained by age-related changes in LV diastolic performance from normal to diastolic dysfunction grade 1. In fact, we also demonstrated an increase in LA stiffness, an index that has been reported as a sensitive marker of diastolic dysfunction. Moreover, all indices of reservoir function and LA strains had no gender and vendor differences. The comparable values of

LA strain independently of the machine used to acquire LA images reported in our study would support a more extensive use of LA strain and other indices of LA function as an early and sensitive marker of diastolic dysfunction.

Actually, STE software can assess layer-specific strain, thus allowing the measurement of epicardial, mid-myocardial, and endocardial longitudinal strain. The absence of differences between vendors for layer-specific strain values makes this technique a useful tool for feasibility, accuracy, and reproducibility.(56) Given the promising application of this new STE method, in our study (chapter 3) we provided normal references values for both genders in a wide range of ages. We found higher values of all layer-specific strains in women than in men, without age dependency. Moreover, an increasing gradient of layer-specific strain values from epicardial towards endocardial layer was shown. The mechanism underlying these findings remains unclear, but we suggested to be secondary to the ability of the endocardial fibres to stretch more potently compared to the epicardial fibres during end-diastole (57), resulting in higher LS values in endocardial layer.

In the same population of healthy adults subjects, we tried to establish normal reference limit for MW indices (chapter 4) and to examine the influence of age and gender on normal reference ranges. We showed that global work index (GWI) and global constructive work (GCW) changed with age, but only in women, while no differences were found in men. Conversely, no differences were found according to age in global wasted work (GWW) and global work efficiency, but lower and higher values, respectively in women than in men. These results seemed to be strictly correlated to differences in systolic and diastolic blood pressure, even if still in the normal range, between women and men. The high impact of blood pressure on MW indices have already been observed in previous studies in hypertensive patients, that showed a significant increased on GWI when compared to controls, despite a normal GLS. (26) This result would suggest that GWI increase is the expression of the higher energy level at which the LV works to compensate the increased afterload, and that GLS in not able to detect. Thus in clinical practice, MW could play a promising role in the serial assessment

of patients with or at risk of developing CV disease as in pathological conditions characterized by increased afterload.

Due to growing interest in MW, its correlations with LV dimensions, standard and advanced 2D parameters of LV systolic function, and indices of diastolic function have also been explored (chapter 5). In the large population of healthy adults subject enrolled in the NORRE study we did not find a strong correlation between MW indices and LV size, whilst significant correlation was demonstrated between both circumferential and radial strain with GCW. These findings, thus, highlight as likely all the components of myocardial deformation contribute to generate MW, so it, and in particular GCW, could be supposed to globally reflect LV mechanics and performance. In our analysis, GWI and GCW were also significantly correlated with parameters that traditionally reflect LV systolic performance (EF, stroke volume, cardiac output etc). Conversely, correlation of MW with parameters of diastolic function was really poor. Our data, hence, support the role of MW as a reliable parameter of myocardial systolic performance, in addition to traditional and strain ones. To date, its application has been tested in patients candidate to cardiac resynchronization therapy (22), or suffered from CAD (23-24), hypertensive and dilated cardiomyopathy (26). Based on these results, MW appears to be a promising tool in several other pathological conditions.

Part II. Multiparametric approach in the diagnosis, prognosis and treatment of aortic stenosis

The management of patients with asymptomatic AS has continued to challenge clinicians. While evidence supports the intervention in those with LVEF <50%, a conservative approach is indicated for asymptomatic patients with severe AS and preserved systolic function (28,58). In our project of thesis, we observed how, in absence of symptoms, other echocardiographic parameters should be considered in the risk assessment of severe AS patients. Data on a large registry of 1375 patients with moderate to severe AS followed in 10 heart valve clinics in Europe, Canada, and the United States

(chapter 6), showed that the mean 4-year overall survival rates under medical management was 86%, with a crude rate of sudden death very low (0.65%). Within this population, however, those patients with severe AS at entry, age, peak aortic jet velocity of 5 m/s or greater, and LVEF less than 60% were independently associated with CV death. Interestingly, the negative effect of peak aortic jet velocity remained significantly associated with CV death also after AVR. Moreover, in patients with moderate AS at entry who progressed to severe AS and were referred for AVR, the baseline variables predicting worse outcomes were directionally similar (peak aortic jet velocity of 3.0m/s or greater and LVEF less than 60%). These data make us reflect on the current indication to AVR and suggest that adjusting the cutoff for LVEF (less than 60% instead of less than 50%) would be reasonable, as well as consider a peak aortic jet velocity of 5 m/s or greater as an indication to replacement regardless of symptoms, due to its strong correlation with worse prognosis.

The clinically silent phase of severe AS is associated with a relatively low risk of sudden death, ranging from 0.25% to 1.7% per year. (59-60) However, it's known that, for patients with severely stenotic aortic valve, once symptoms develop, annual mortality rate raises up to 30%, which makes the early recognition of symptoms and timely referral to intervention really critical. In this context, exercise echocardiography has proven to be an effective support in the risk stratification and decision making of patients with aortic valve disease (chapter 7). Indeed, almost one-third of patients with severe AS exhibit exercise-limiting symptoms; these patients have worse outcomes. Beside its important role in unmasking symptoms, incremental prognostic value has been also attributed to some exercise Doppler echocardiographic findings: an increase in mean transaortic gradient by >20 mmHg, systolic blood pressure drop, inadequate increase in LVEF or GLS reduction during exercise, and exercise-induced pulmonary hypertension.

Many efforts have been spent to find markers or early sign of myocardial disfunction able to ascertain the symptomatic status and outcome of patients with AS. Firstly, we focused on the contribution of epicardial adipose tissue (EAT) and late gadolinium-enhancement fibrosis, quantified

by CMR in a subset of 118 patients with moderate to severe AS (chapter 8). We found that the degree of myocardial remodeling, represented by LV fibrosis and BNP release, was significantly associated with symptom onset, but did not predict outcome. Conversely, EAT volume was significant associated with the occurrence of events. Thus, these two CMR parameters showed distinct, but complementary diagnostic significance.

Myocardial fibrosis is associated with impairment of GLS, which, in turn, has demonstrated to predict prognosis. Given that longitudinal function is largely governed by the subendocardial myocardial fibres, that are affected first by the increased wall stress associated with AS, we hypothesized that endocardial LS would be a more sensible marker of myocardial disfunction in severe AS patients (chapter 9). Yet, we explored differences in multilayer LS according to symptomatic status, demonstrating for the first time that endocardial LS is more affected than epicardial LS in patients with AS, and even more in the advanced phases of the disease, when symptoms occur. In addition, endocardial LS, but not epicardial LS, was independently associated with CV outcomes.

If on one hand LS has demonstrated to be an appropriate marker of early, subclinical LV dysfunction, on the other hand its load dependency can affect its diagnostic accuracy, too (61-62). An increase in afterload, in fact, may lead to misinterpretation of the true contractile function due to a strain reduction. MW has been recently proposed as a new approach to explore myocardial performance balanced by afterload, through an estimation of non-invasive LV pressure during a cardiac cycle. Since with this method LV pressure is estimated from systolic blood pressure (SBP) measured with a cuff manometer, it has not been validated in pathologic condition such as AS, in which LV peak systolic pressure is higher than SBP. In our study, we proposed to estimate peak systolic LV pressure as the sum of SBP and mean transaortic pressure gradient. Hence, we retrospectively analysed 283 patients with AS and preserved LVEF and evaluated the correlation of MW indices with AS severity parameters and LV hypertrophy (chapter 10). Compared to an age- and

sex-matched control group, AS patients showed lower values of GLS, but associated with a significant increase of GWI, GCW and GWW, without affecting the efficiency. These modifications seemed to be correlated to the severity of AS, low-flow state and increased global LV afterload, but not on the grade of LV hypertrophy. We supposed that the increase of GWI and GCW observed in AS reflected the higher energy level required by the LV pump work against increased arterial afterload. A similar finding was observed by Chan et al in a subgroup of patients with advanced grades of hypertension. (26). Comparable with AS patients, in those with uncontrolled hypertension LV remodeling is accompanied with higher LV end-systolic stiffness, that allows to enhance myocardial contractility, reflecting the ability of the LV to pump against a given pressure with higher level. Thus, GWI increase represents and index of the enhanced myocardial contractility in a remodeled LV, characterized, in the initial phases of the disease, by preserved EF and eventually decreased GLS. At a later stage, we narrowed the analysis on 170 patients with asymptomatic moderate to severe AS (chapter 11), which were stratified according a staging cardiac scheme, that takes into account myocardial structural changes, hemodynamics parameters and indices of myocardial dysfunction (63). Interestingly, we observed a significant reduction of GWI in the advanced stages of the disease (Stage 3-4), expression of an impaired contractile performance of the cardiomyocytes, with a trend of reduction also of GCW. At the same time though, a value of GWI lower than 1866 mmHg% resulted predictive of CV mortality. Hence we postulated that the evaluation of MW indices may allow a better phenotyping of asymptomatic patients at higher risk of developing cardiovascular events during follow-up.

At this point, we extended our research on the modification and prognostic role of deformation parameters in AS candidates to TAVI. A retrospective analysis on 62 patients with severe AS and preserved LVEF undergoing TAVI (chapter 12) revealed that immediately post-TAVI, LV reverse remodeling and functional reserve recruitment was detected by deformation parameters, but strain improvement was not uniform: early significant recovery of longitudinal strain was found in basal lateral and anteroseptal segments, while regional radial strain at the level of papillary muscle.

Interestingly, only segments that improved most significantly in longitudinal and radial directions early after intervention showed correlations with prognosis. So, given the evidence that not only global but also indices of segmental LV function before and early after TAVI may affect patient prognosis, regular assessment of regional radial and longitudinal strain might be used as a signal to select patients for earlier intervention. Instead, a different LV reverse remodeling was observed in patients with radiation-associated AS (chapter 13). A comparison between 33 AS patients with prior mediastinal radiotherapy (XRT) and 136 without history of cancer, undergoing TAVI, revealed that since before intervention, patients with prior XRT have more marked impairment of LV systolic function, detected by a decrease in layer-specific LV strain. After TAVR, recovery of heart function was better in patients with lone AS, in whom a significant increase of global, endocardial and epicardial LS was demonstrated. Conversely, in the presence of radiation cardiomyopathy, myocardial recovery was significantly impaired, with no post-procedural improvement in LS. Further studies, with a larger cohort of patients and longer follow-up are needed to better evaluate clinical implication of these findings.

Based on emerging evidences, a multiparametric approach, that integrates the assessment of structural cardiac abnormalities by multi imaging and biomarker profiles, is advisable for a best formulation of a follow-up and management plan of each patient with aortic stenosis.

Part III. Ischemia-driven coronary revascularization: how stress echocardiography can make difference.

Based on the latest evidence in the setting of acute (ACS) and chronic coronary syndrome (CCS) (40), a new concept of CAD as a dynamic process, progressive and serious even in clinically silent periods have emerged. In this scenario, a successful myocardial revascularization, aimed at minimizing residual ischemia, relieving symptoms and reducing the risk of future CV events, plays a crucial role. In presence of multivessel coronary artery disease, the question of whether, when and how patients should undergo complete revascularization remains still debated. Given the relevance

of the topic, in chapter 14 we provided an overview of recent evidence and current indication to perform a complete revascularization in patients with ACS or CCS and multivessel disease. As evidenced in the current guidelines, an ischaemia-driven revascularization is the most encouraged approach. In this context, non-invasive functional imaging for myocardial ischaemia has a key role in diagnosis of CAD in patients with high clinical likelihood. Among non-invasive functional tests, DSE is considerable a reliable tool, associated with high accuracy for the detection of flow-limiting coronary stenosis (41-42). Nevertheless, we decided to test whether the application of STE during peak phase of DSE could increase accuracy in the diagnosis of myocardial ischemia, in order to overcome the limit of the test (operator- and image quality- dependence) (chapter 15). This multicenter, prospective study demonstrated that STE is feasible even at the highest heart rate reached during peak stress. Furthermore, the analysis of regional LS of the myocardial segments perfused by the three major coronary arteries revealed that the addition of strain to visual assessment of wall motion abnormalities is able to improve accuracy in prediction of significant stenosis of left anterior descending coronary artery. This results was even more impressive in those patients with CCS and wall motion abnormalities at rest. Conversely, in presence of left circumflex or right coronary arteries significant stenosis, LS was less accurate than visual wall motion assessment at peak stress. Based on these results, STE could be considered an additional tool available to the clinician useful in the discrimination of inducible ischemia in a specific setting of patients.

Part IV. Platelet and microvascular function

Endothelial dysfunction and abnormal platelet function represent the main determinants of the vascular accidents in patients affected by diabetes mellitus, hypercholesterolemia and hypertension (64). The use of drugs able to inhibit platelet activation, ROS production and increase NO release in endothelium is pivotal in limiting vascular damage and the incidence of thrombotic events. In this

regard, aiming to investigate if nutraceutical (NUT) compounds have positive effect on endothelial and platelet function, we conducted an interventional, single center, randomized, single-blind study in 28 consecutive patients with cardiovascular risk factors comparing two NUT combination (chapter 16). We therefore demonstrated that LopiGLIK compound, which is a combination of berberine, red yeast rice powder and extract of *Morus alb*a, significantly improves endothelium-mediated CFR after 30 days, an effect that we showed to be independent of LDL-cholesterol changes and other covariates including age, and is not mediated by significant changes in platelet aggregation. The beneficial effect of LopiGLIK on the endothelium of coronary arteries seemed to be mainly due to *Morus alba*, a component able to act on endothelial nitric oxide synthase (eNOS) signaling, enhancing NO bioavalability also in a population with a variable amount of CV risk factors and a wide age range (33–78 years). Despite *Morus alba* has demonstrated to significantly inhibit arterial thrombosis in vivo due to antiplatelet activity tested in experiments on rats (65-66), this action has not been confirmed in our study population. Conversely, in diabetes mellitus patients we identified a new molecular target able to limit platelet aggregation (chapter 17). Rac1 is a protein involved in ROS production, responsible of an increased oxidative stress and endothelium dysfunction of diabetic patients (67). In addition to its role in ROS generation, Rac1 is involved in platelet actin cytoskeleton reorganization, modulating, in turn, platelet aggregation (68). In our study, we demonstrated that a pharmacological inhibitor of Rac1, named NSC23766, attenuated endothelial dysfunction in experimental model of diabetes mellitus and reduced platelet hyperaggregation in diabetic patients. More interestingly, additional role of Rac1 inhibition in reducing platelet aggregation was observed in diabetic patients treated with acetyl salicylic acid (ASA), overcoming the ASA resistance often observed in this setting of patients. These novel results suggest a potential protective role of Rac1 inhibition on vascular injury and platelet hyperaggregation in diabetes mellitus.

The identification of the determinants of residual platelet reactivity in patients treated with antiplatelet therapy is of pivotal importance in the prevention of thrombotic CV and cerebrovascular

events. This aspect becomes even more prominent after an ACS treated with percutaneous coronary intervention, when the interplay between activated platelets and ruptured plaque or the stent strut may enhance the risk of stent thrombosis or a new ischemic event. In this scenario, we investigated if chronic kidney disease (CKD) could contribute to platelet reactivity. in ACS patients early after percutaneous revascularization (chapter 18). In a cohort of 206 ACS patients treated with dual antiplatelet therapy (DAPT), the presence of moderate to severe CKD was associated with higher degrees of residual platelet reactivity compared with patients with normal or mildly reduced renal function. More precisely, we found that platelet hyperreactivity observed in patients with CKD was mainly driven by the use of clopidogrel: when the analysis was restricted to patients in therapy with the new P2Y12 inhibitors, no significant difference in platelet aggregation was detected between patients with normal and impaired renal function. Thus, according the results of our study, the use of the novel, more potent P2Y12 inhibitors should be advocated in presence of real dysfunction to counteract the higher prothrombotic tendency and incidence rate of adverse CV events typical of this setting of patients.

Although platelet inhibition obtained with the use of ticagrelor and prasugrel in ACS patients is efficacy in reducing thrombotic risk, other compounds used in the treatment of coronary syndromes could interfere, enhancing or limiting the antiplatelet effect.

We conducted a randomized, open label study in ACS patients receiving aspirin and ticagrelor (chapter 19), to compare carvedilol, a nonselective beta-blocker drug, with metoprolol, a selective β_1-blocker. We found that carvedilol significantly reduced residual platelet aggregation 30 days after the index event compared with metoprolol, providing an additional benefit to the dual antiplatelet therapy, observed despite the use of a new potent P2Y12 inhibitor (i.e. ticagrelor). This finding might have important clinical implications in the daily practice when choosing the type of beta-blocker agent to be used in this setting of patients.

Conclusions

In this long lasting research journey we a) provided normal references values for LA strain, LV multilayer strain and MW indices from the large healthy population of NORRE study; b) demonstrated that in asymptomatic AS followed in heart valve center the risk of sudden death is low, but the rate of all-cause and CV mortality increase in those with severe stenosis at baseline, peak aortic jet velocity of 5.0 m/s or greater or LVEF less than 60%; c) evaluated the additional prognostic role of advanced technique (EAT volume, layer specific strain, radial strain and GWI) in patients with aortic stenosis, from asymptomatic status to TAVI intervention; d) demonstrated the accuracy of global and regional LS performed during dobutamine stress echocardiography in predicting significant stenosis of left descending coronary artery; e) investigated how hyperglycemia, hypercholesterolemia and uremia affect endothelial and platelet function, detecting new compounds able to counteract their pathological effects.

All these studies, albeit ranging in a very broad field of research, share the use of innovative and advanced tool (strain parameters, MW indices, value of platelet aggregation) that have demonstrated a promising role in the diagnosis of physiopathological mechanism underlying valvular and ischemic heart disease and in the prognostic stratification. Further and larger studies are needed to definitely attest their adding value, in order to include their use in the routine clinical practice.

List of abbreviations

ACS = acute coronary syndrome

AS = aortic stenosis

CAD = coronary artery disease

CCS = chronic coronary syndrome

CMR = cardiac magnetic resonance

CKD = chronic kidney disease

CV = cardiovascular

DAPT = dual antiplatelet therapy

EAT = epicardial adipose tissue

GCW = global constructive work

GLS = global longitudinal strain

GWI = global work index

GWW = global wasted work

PACS = peak atrial contraction strain

PALS = peak atrial longitudinal strain

PSL = pressure-strain loops

ROS = reduce reactive oxygen species

RV = right ventricle

STE = speckle tracking echocardiography

TAVI = transcatheter aortic valve implantation

LA = left atrium

LV = left ventricular

MW = myocardial work

MVD = microvascular disease

XRT = mediastinal radiotherapy

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Echocardiography and the European Association of Cardiovascular Imaging. Eur Heart J Cardiovasc Imaging. 2015 Mar;16(3):233-70.

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55. Lancellotti P, Badano LP, Lang RM, et al. Normal Reference Ranges for Echocardiography: rationale, study design, and methodology (NORRE Study). Eur Heart J Cardiovasc Imaging. 2013 Apr;14(4):303-8.
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66. Kim DS, Ji HD, Rhee MH, Sung YY, Yang WK, Kim SH, et al. Antiplatelet activity of Morus alba leaves extract, Mediated via Inhibiting granule secretion and Blocking the Phosphorylation of Extracellular-Signal-Regulated Kinase and Akt. Evid Based Complement Alternat Med. 2014:639548.

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Curriculum vitae

PERSONAL INFORMATION

FEDERICA ILARDI

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fedeilardi@gmail.com

Sex Female | Date of birth 24/11/1986 | Nationality Italian

Cardiologist

JOB POSITION

International PhD Programme in Cardiovascular Pathophysiology and Therapeutics

AOU Federico II di Napoli, Italy - CHU Sart Tilman, Liège, Belgium

WORK EXPERIENCE

From 2013 to 2016

First operator in Vascular Pathology Laboratory

Department of Cardiology, Cardiac Surgery and Cardiovascular Emergency (Prof. G. Esposito)
Federico II University, Naples, Italy;

From 2014 to 2016

First operator in Echocardiography Laboratory

Interdepartimental Laboratory of Cardiac Imaging (Prof. M. Galderisi)
Federico II University, Naples, Italy;

From 2016 to 2018

First operator in Stress Echocardiography Laboratory

Cardiology Department and Heart Valve Clinic (Prof. P. Lancellotti)
CHU Sart Tilman, Liège, Belgium

From 1st November 2017 to now

Clinical and Translational Research - PhD Programme in Cardiovascular Pathophysiology and Therapeutics

AOU Federico II di Napoli, Italy - CHU Sart Tilman, Liège, Belgium

From 1st April 2020 to now

Scientific Collaboration for Clinical and Translational Cardiovascular Research

Mediterranea Cardiocentro - Napoli

EDUCATION AND TRAINING

2017 - 2018

Certification in Adult Transthoracic Echocardiography

European Association of Cardiovascular Imaging (EACVI)

From 2016 to 2017

Fellowship in Echocardiography
Cardiology Department and Heart Valve Clinic
CHU Sart Tilman, Liège, Belgium

From 2012 to 2017

Resident in Cardiovascular Disease
Federico II University, Naples, Italy;

2015

Certification in Vascular Ultrasound
Italian Society of Vascular Diagnostics, SIDV-GIUV

From 2008 to 2012

Internship in Laboratory of Cardiology and Molecular Biology
Department of Advanced Biomedical Science
Federico II University, Naples, Italy;

From 2005 to 2011

Graduation in Medicine and Surgery
Federico II University, Naples, Italy;

PERSONAL SKILLS

Mother tongue(s): Italian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C1	C1	C1	C1
French	B2	B2	B2	B2	B1

Levels: A1/2: Basic user - B1/2: Independent user - C1/2 Proficient user
Common European Framework of Reference for Languages

Communication skills

Good communication skills gained through experience as Speaker in National and International meetings.

Organisational / managerial skills

- Sub-investigator in the following clinical studies: The Evaluation of Bococizumab (PF-04950615;RN316) in Reducing the Occurrence of Major Cardiovascular Events in High Risk Subjects (**SPIRE-1**); The Evaluation of Bococizumab (PF-04950615; RN316) in Reducing the Occurrence of Major Cardiovascular Events in High Risk Subjects (**SPIRE-2**); Outcomes Study to Assess STatin Residual Risk Reduction With EpaNova in HiGh CV Risk PatienTs With Hypertriglyceridemia (**STRENGTH**).
- Leadership and good team-leading skills gained as President of the Rotaract Club Napoli Sud-Ovest (2011-12).
- Good organisational skills gained as Secretary for 3 years of the Rotaract Club Napoli Sud-Ovest.

Job-related skills

- Recognized advanced statistical and epidemiological competence.
- Recognized advanced Molecular Biology and Translational Medicine.

Computer skills

- Good command of Microsoft Office™ tools; Apple applications;
- Basic expertise in SPSS statistical package.

HONOURS AND AWARDS

- 2015 Winner of Moderated Poster Presentation ESC Congress.
- 2020 Winner Best Scientific Publication Under 40, SIECVIrtual-Congresso delle Regioni.

COMMISSIONS OF TRUST

- 2017 - Reviewer, Plos One
- 2020 – Reviewer, Cardiovascular Ultrasound
- 2020 – Reviewer, Scientific Reports
- 2020 – Reviewer, The International Journal of Cardiovascular Imaging
- 2020 – Reviewer, Clinical Therapeutics
- 2020 – Reviewer, Diagnostics (Basel)

MEMBERSHIPS

- Form 2012 Member, Italian Society of Cardiology, Working group on "Echocardiography"
- From 2012 Member, European Society of Cardiology, Working group on "Cellular biology of the heart"
- From 2014 Member, European Association of Cardiovascular Imaging (EACVI)
- From 2014 Member, European Association of Percutaneous Coronary Intervention (EAPCI)
- From 2018 Member, ESC Council on Hypertension
- From 2018. Board member, Italian Cardiologist of Tomorrow (ICOT)
- From 2019. Member, ESC Council on Valvular Heart Disease
- From 2020. Member, Italian Society of Cardiovascular Imaging (SIECVI)

TRAINING COURSES

- 2017 - EACVI webinar on Stress echo in non ischemic disease, EACVI-ESC
- 2017 - Adult Basic Life Support – Defibrillation (BLS-D) training course
- 2015 - Teaching course "Transesophageal Echocardiography/3D", Italian Society of Echocardiography (SIEC)
- 2014 - Cardiopulmonary Resuscitation and Automated External Defibrillator (CPR&AED) Refresher Seminar – European Resuscitation Council

List of Publications

1. Perrino C, Scudiero L, Petretta MP, Schiattarella GG, De Laurentiis M, **Ilardi F**, Magliulo F, Carotenuto G, Esposito G. "*Total occlusion of the abdominal aorta in a patient with renal failure and refractory hypertension: a case report*" Monaldi Arch Chest Dis. 2011 Mar;76(1):43-6.
2. Perrino C, Gargiulo G, Pironti G, Franzone A, Scudiero L, De Laurentis M, Magliulo F, **Ilardi F**, Carotenuto G, Schiattarella GG, Esposito G. *Cardiovascular effects of treadmill exercise in physiological and pathological preclinical settings*. Am J Physiol Heart Circ Physiol. 2011. Jun;300(6):H1983-9. Epub 2011 Apr 13. Review
3. Di Serafino L, Scudiero L, De Laurentis M, **Ilardi F**, Magliulo F, Carotenuto G, Perrino C, Esposito G "*Drug-Eluting Stent for the Treatment of Early Fistula Failure*" International Journal of Clinical Medicine, volume 2 - numero 3 – Luglio 2011.
4. Franzone A, De Laurentis M, Ferrone M, Scudiero L, Carotenuto C, Magliulo F, **Ilardi F**, Serino F, Carbone A, Visco E, Esposito G "*Nuove strategie per la sostituzione percutanea della valvola aortica*" Giornale italiano di Cardiologia pratica, Anno 10 - Numero 3 - Luglio-Dicembre 2011
5. **Ilardi F**, Magliulo F, Gargiulo G, Schiattarella GG, Carotenuto G, Serino F, Ferrone M, Visco E, Scudiero F, Carbone A, Perrino C, Trimarco B, Esposito G. *Endovascular treatment of carotid artery stenosis: evidences from randomized controlled trials and actual indications*. Monaldi Arch Chest Dis. 2011 Dec;76(4):183-91. Review.
6. Perrino C, Schiattarella GG, Magliulo F, **Ilardi F**, Carotenuto G, Gargiulo G, Serino F, Ferrone M, Scudiero F, Carbone A, Trimarco B, Esposito G. *Cardiac Side Effects of Chemotherapy: State of Art and Strategies for a Correct Management.* Curr Vasc Pharmacol. 2012 May 8.
7. Perrino C, Schiattarella GG, Sannino A, Pironti G, Petretta MP, Cannavo A, Gargiulo G, **Ilardi F**, Magliulo F, Franzone A, Carotenuto G, Serino F, Altobelli GG, Cimini V, Cuocolo A, Lombardi A, Goglia F, Indolfi C, Trimarco B, Esposito G. *Genetic deletion of uncoupling protein 3 exaggerates apoptotic cell death in the ischemic heart leading to heart failure*. J Am Heart Assoc. 2013 May

20;2(3):e000086.

8. Schiattarella GG, Perrino C, Magliulo F, **Ilardi F**, Serino F, Trimarco V, Izzo R, Amato B, Terranova C, Cardin F, Militello C, Leosco D, Trimarco B, Esposito G. *Statins and the elderly: recent evidence and current indications*. Aging Clin Exp Res. 2012 Jun;24(3 Suppl):47-55. Review
9. Schiattarella GG, Magliulo F, D'Alise G, Mannacio V, **Ilardi F**, Trimarco B, Esposito G, Cirillo P. *The pitfalls of managing thrombosis of an Absorb™-treated bifurcation*. Int J Cardiol. 2014 Jul 1;174(3):e93-5.
10. Esposito G, Schiattarella GG, Perrino C, Cattaneo F, Pironti G, Franzone A, Gargiulo G, Magliulo F, Serino F, Carotenuto G, Sannino A, **Ilardi F**, Scudiero F, Brevetti L, Oliveti M, Giugliano G, Del Giudice C, Ciccarelli M, Renzone G, Scaloni A, Zambrano N, Trimarco B. *Dermcidin: a skeletal muscle myokine modulating cardiomyocyte survival and infarct size after coronary artery ligation.* Cardiovasc Res. 2015 Sep 1;107(4):431-41.
11. **Ilardi F**, Santoro C, Cirillo P, Esposito G, Trimarco B, Galderisi M. *Quantitative detection of inducible ischemia during dobutamine stress by speckle tracking echocardiography: A dream comes true.* Int J Cardiol. 2016 Oct 1;220:357-9.
12. Esposito R, **Ilardi F**, Schiano Lomoriello V, Sorrentino R, Sellitto V, Giugliano G, Esposito G, Trimarco B, Galderisi M. *Identification of the main determinants of abdominal aorta size: a screening by Pocket Size Imaging Device*. Cardiovasc Ultrasound. 2017 Jan 13;15(1):2.
13. Bernard A, Addetia K, Dulgheru R, Caballero L, Sugimoto T, Akhaladze N, Athanassopoulos GD, Barone D, Baroni M, Cardim N, Hagendorff A, Hristova K, **Ilardi F**, Lopez T, de la Morena G, Popescu BA, Penicka M, Ozyigit T, David Rodrigo Carbonero J, van de Veire N, Stephan Von Bardeleben R, Vinereanu D, Luis Zamorano J, Martinez C, Magne J, Cosyns B, Donal E, Habib G, Badano LP, Lang RM, Lancellotti P. *3D echocardiographic reference ranges for normal left ventricular volumes and strain: results from the EACVI NORRE study*. Eur Heart J Cardiovasc Imaging. 2017 Apr 1;18(4):475-483.
14. Sugimoto T, Dulgheru R, Bernard A, **Ilardi F**, Contu L, Addetia K, Caballero L, Akhaladze N,

Athanassopoulos GD, Barone D, Baroni M, Cardim N, Hagendorff A, Hristova K, Lopez T, de la Morena G, Popescu BA, Moonen M, Penicka M, Ozyigit T, Rodrigo Carbonero JD, van de Veire N, von Bardeleben RS, Vinereanu D, Zamorano JL, Go YY, Rosca M, Calin A, Magne J, Cosyns B, Marchetta S, Donal E, Habib G, Galderisi M, Badano LP, Lang RM, Lancellotti P. *Echocardiographic reference ranges for normal left ventricular 2D strain: results from the EACVI NORRE study*. Eur Heart J Cardiovasc Imaging. Eur Heart J Cardiovasc Imaging. 2017 May 1;18(8):833-840.

15. Lancellotti P, Galderisi M, Edvardsen T, Donal E, Goliasch G, Cardim N, Magne J, Laginha Sugimoto T, Dulgheru R, Marchetta S, **Ilardi F**, Contu L, Go YY, Lancellotti P. *What Does 3D Echocardiography Add to 2D Echocardiography in the Assessment of Mitral Regurgitation?* Curr Cardiol Rep. 2017 Aug 24;19(10):90. Review.
16. S, Hagendorff A, Haland TF, Aaberge L, Martinez C, Rapacciuolo A, Santoro C, **Ilardi F**, Postolache A, Dulgheru R, Mateescu AD, Beladan CC, Deleanu D, Marchetta S, Auffret V, Schwammenthal E, Habib G, Popescu BA. *Echo-Doppler estimation of left ventricular filling pressure: results of the multicentre EACVI Euro-Filling study*. Eur Heart J Cardiovasc Imaging. 2017 Sep 1;18(9):961-968.
17. **Ilardi F**, Sugimoto T, Dulgheru R, Marchetta S, Contu L. *Exercise echocardiography in valve disease,* Continuing Cardiology Education, 2018. https://doi.org/10.1002/cce2.64, Review.
18. Schiattarella GG, Carrizzo A **Ilardi F**, Damato A, Ambrosio MT, Madonna M, Trimarco V, Marino M, De Angelis E, Settembrini S, Perrino C, Trimarco B, Esposito G, Vecchione C. *Rac1 Modulates Endothelial Function and Platelet Aggregation in Diabetes Mellitus.* J Am Heart Assoc. 2018 Apr 6;7(8).
19. **Ilardi F**, Gargiulo G, Schiattarella GG, Giugliano G, Paolillo R, Menafra G,De Angelis E, Scudiero L, Franzone A, Stabile E, Perrino C, Cirillo P, Morisco C, Izzo R, Trimarco V, Esposito G. *Effects Of Carvedilol Versus Metoprolol On Platelet Aggregation in Patients With Acute Coronary Syndrome: The PLATE-BLOCK Study*. Am J Cardiol. 2018 Jul 1;122(1):6-11

20. Sugimoto T, Robinet S, Dulgheru R, Bernard A, **Ilardi F**, Contu L, Addetia K, Caballero L, Kacharava G, Athanassopoulos GD, Barone D, Baroni M, Cardim N, Hagendorff A, Hristova K, Lopez T, de la Morena G, Popescu BA, Penicka M, Ozyigit T, Rodrigo Carbonero JD, van de Veire N, Von Bardeleben RS, Vinereanu D, Zamorano JL, Go YY, Marchetta S, Nchimi A, Rosca M, Calin A, Moonen M, Cimino S, Magne J, Cosyns B, Galli E, Donal E, Habib G, Esposito R, Galderisi M, Badano LP, Lang RM, Lancellotti P. *Echocardiographic reference ranges for normal left atrial function parameters: results from the EACVI NORRE study*. Eur Heart J Cardiovasc Imaging. 2018 Jun 1;19(6):630-638
21. Lancellotti P, Magne J, Dulgheru R, Clavel MA, Donal E, Vannan MA, Chambers J, Rosenhek R, Habib G, Lloyd G, Nistri S, Garbi M, Marchetta S, Fattouch K, Coisne A, Montaigne D, Modine T, Davin L, Gach O, Radermecker M, Liu S, Gillam L, Rossi A, Galli E, **Ilardi F**, Tastet L, Capoulade R, Zilberszac R, Vollema EM, Delgado V, Cosyns B, Lafitte S, Bernard A, Pierard LA, Bax JJ, Pibarot P, Oury C. *Outcomes of Patients With Asymptomatic Aortic Stenosis Followed Up in Heart Valve Clinics.* JAMA Cardiol. 2018 Nov 1;3(11):1060-1068.
22. Davin L, Nchimi A, **Ilardi F**, Dulgheru R, Marchetta S, Gach O, Marechal P, Cimino S, Bruyère PJ, Georgiopoulos A, Dibato JE, d'Amico G, Galderisi M, Parisi V, Oury C, Lancellotti P. *Epicardial Adipose Tissue and Myocardial Fibrosis in Aortic Stenosis Relationship With Symptoms and Outcomes: A Study Using Cardiac Magnetic Resonance Imaging.* JACC Cardiovasc Imaging. 2019 Jan;12(1):213-214.
23. Esposito R, Sorrentino R, Giugliano G, Avvedimento M, Paolillo R, Santoro C, Scalamogna M, Esposito M, **Ilardi F**, Rozza F, Esposito G, Galderisi M, Trimarco V. *Different age-independent effects of nutraceutical combinations on endothelium-mediated coronary flow reserve*. Immun Ageing. 2018 Nov 22;15:30.
24. Manganaro R, Marchetta S, Dulgheru R, **Ilardi F**, Sugimoto T, Robinet S, Cimino S, Go YY, Bernard A, Kacharava G, Athanassopoulos GD, Barone D, Baroni M, Cardim N, Hagendorff

A, Hristova K, López-Fernández T, de la Morena G, Popescu BA, Penicka M, Ozyigit T, Rodrigo Carbonero JD, van de Veire N, Von Bardeleben RS, Vinereanu D, Zamorano JL, Rosca M, Calin A, Moonen M, Magne J, Cosyns B, Galli E, Donal E, Carerj S, Zito C, Santoro C, Galderisi M, Badano LP, Lang RM, Oury C, Lancellotti P. *Echocardiographic reference ranges for normal non-invasive myocardial work indices: results from the EACVI NORRE study*. Eur Heart J Cardiovasc Imaging. 2019 May 1;20(5):582-590.

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