

ORIGINAL INVESTIGATIONS

Lipid Accumulation in Hearts Transplanted From Nondiabetic Donors to Diabetic Recipients



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ABSTRACT

BACKGROUND Early pathogenesis of diabetic cardiomyopathy (DMCM) may involve lipotoxicity of cardiomyocytes in the context of hyperglycemia. There are many preclinical studies of DMCM pathogenesis, but the human evidence is still poorly understood.

OBJECTIVES By using a nondiabetic mellitus (non-DM) heart transplanted (HTX) in diabetes mellitus (DM) recipients, this study conducted a serial study of human heart transplant recipients evaluating cardiac effects of diabetic milieu (hyperglycemia and insulin resistance) on lipotoxic-mediated injury. We evaluated cardiomyocyte morpho-pathology by serial biopsies of healthy implanted hearts in DM recipients during 12-month follow-up from HTX. Because metformin reduces ectopic lipid accumulation, we evaluated the effects of the drug in a nonrandomized subgroup.

METHODS The DMCM-AHEAD (Diabetes and Lipid Accumulation and Heart Transplant) prospective ongoing study (NCT03546062) evaluated 158 first HTX recipients (82 non-DM, 76 DM of whom 35 [46%] were receiving metformin). HTX recipients were undergoing clinical standard evaluation (metabolic status, echocardiography, coronary computed tomography angiography, and endomyocardial biopsies). Biopsies evaluated immune response, Oil Red-O staining, ceramide, and triacylglycerol levels. Lipotoxic factors and insulin resistance were evaluated by reverse transcriptase-polymerase chain reaction.

RESULTS There was a significant early and progressive cardiomyocyte lipid accumulation in DM but not in non-DM recipients ($p = 0.019$). In the subgroup receiving metformin, independently from immunosuppressive therapy that was similar among groups, lipid accumulation was reduced in comparison with DM recipients not receiving the drug (hazard ratio: 6.597; 95% confidence interval: 2.516 to 17.296; $p < 0.001$). Accordingly, lipotoxic factors were increased in DM versus non-DM recipients, and, relevantly, metformin use was associated with fewer lipotoxic factors.

CONCLUSIONS Early pathogenesis of human DMCM started with cardiomyocyte lipid accumulation following HTX in DM recipients. Metformin use was associated with reduced lipid accumulation independently of immunosuppressive therapy. This may constitute a novel target for therapy of DMCM. (J Am Coll Cardiol 2020;75:1249–62)

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ABBREVIATIONS AND ACRONYMS

BMI	= body mass index
CHD	= coronary heart disease
CT	= computed tomography
DM	= diabetes mellitus
DMCM	= diabetic mellitus cardiomyopathy
EMB	= endomyocardial biopsy
HF	= heart failure
HOMA-IR	= Homeostatic Model Assessment for Insulin Resistance
HR	= hazard ratio
HTX	= heart transplantation
IRS	= insulin receptor substrate
ISHLT	= International Society for Heart Lung Transplantation
PPAR	= peroxisome proliferator-activated receptor
SREBP-1c	= sterol regulatory element-binding transcription factor 1c
TAPSE	= tricuspid annular plane systolic excursion

Patients with type 2 diabetes mellitus (DM) have an increased risk of myocardial dysfunction leading to heart failure (HF) (1). Despite optimal medical therapy, many patients with DM proceed to end-stage HF requiring heart transplantation (HTX) (2).

Pathogenesis of human DM cardiomyopathy (DMCM) is still under active investigation (3). The early cardiac damage of DMCM can occur in the absence of coronary heart disease (CHD), valvular diseases, as well as hypertension and/or dyslipidemias (4). However, most patients with DM have hypertension and dyslipidemias when they present HF in the absence of CHD. Both insulin resistance and hyperglycemia are each independent risk factors for the development of DMCM (5-7) in which lipotoxic injury within cardiomyocytes has been implicated (8,9).

Major pathogenic hypotheses are derived from translational models (3,10), with human evidence being far less developed. To gain relevant insights in the early pathogenesis of

human DMCM, we developed a serial study of human heart transplant recipients. We evaluated the effects of diabetes on early metabolic myocardial mechanisms involved in the initiation and progression of DMCM. Then, we studied modifications of healthy transplanted hearts in DM recipients for 12-month follow-up according to International Society for Heart Lung Transplantation (ISHLT) guidelines (11) that include endomyocardial biopsies (EMBs). Because metformin reduces ectopic fat accumulation in patients with DM both in monotherapy (12) and in combination with other antidiabetic drugs (13,14), we investigated lipid accumulation following HTX in a nonrandomized subgroup of DM recipients receiving metformin.

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METHODS

PATIENTS. From January 2010, we conducted a prospective ongoing study (DMCM-AHEAD study, NCT03546062) under ALCOA (Attributable, Legible,

Contemporaneous, Original and Accurate) integrity protocols on patients who underwent first orthotopic HTX in the referring center of Region Campania at Monaldi Hospital with a follow-up of 12 months (15,16). The study was approved by the Ethical Committee (protocol 438) and patients gave written informed consent. The surgical technique used was previously described in detail (15,16).

The study population was divided into 2 main groups based on pre-transplantation DM diagnosis according to American Diabetes Association guidelines (17). Patients with DM for at least 6 months before HTX, with optimal glycemic control (HbA1c ≤ 7.0), body mass index (BMI) < 30 kg/m², without severe secondary end-organ disease (retinopathy, neuropathy, or nephropathy) were included. These selection criteria are based on strictly regulated guidelines for registration on the waitlist for HTX, according to ISHLT guidelines (11). Within the DM group, patients who never used metformin were classified as “no-metformin patients with DM” whereas patients with DM who already used metformin for at least 6 months before HTX and continued throughout the follow-up were classified as “metformin patients with DM.” Patients with EMB specimens consistent with ISHLT Grade 2R considered positive for rejection, positive donor-specific antibodies, increased T4/T8 ratio, positive immunoglobulin (Ig)M and/or IgG cytomegalovirus antibodies, and with post-HTX diabetes were excluded from the study. Patients underwent immunosuppression therapy (11,18) by induction with polyclonal antilymphocyte antibodies and maintenance therapy mainly based on cyclosporine or tacrolimus, in association with mycophenolate mofetil, everolimus, and prednisone.

CLINICAL, ECHOCARDIOGRAPHIC, STRESS TEST, AND COMPUTED TOMOGRAPHY EVALUATIONS. The evaluations were recorded after HTX at week 1, week 12, week 24, and week 48 (clinical and instrumental evaluation and glycemic control, i.e., fasting glycemia and HbA1c) (19,20). Systemic insulin resistance, as Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), was evaluated before HTX (before starting immunosuppressive therapy) and at week 48 after HTX, during immunosuppressive therapy. Coronary computed tomography (CT) angiography and myocardial scintigraphy evaluation test were

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recorded at week 48. The following outcomes were recorded: mortality (cerebrovascular, cardiovascular, infections, rejection, post-operative hemorrhage, and other), renal dysfunction (creatinine >2 mg/dl), and acute heart rejection.

HEART BIOPSIES. EMBs were obtained either as routine surveillance protocol or as a diagnostic tool for cases with allograft dysfunction and for clinically suspected rejection. The biopsy schedule was performed weekly for the first month, every 2 weeks for the next month, once for the next 4 weeks, once for the next 6 weeks, then every 3 months for the next 2 years, and afterward every 6 months for the next years.

ENDOMYOCARDIAL BIOPSIES PROCEDURES, COMPLICATIONS, AND REJECTION EVALUATION. Tissue analysis. Lipid accumulation in cardiomyocytes. EMBs procedures for tissue analysis. EMBs were embedded in optimum cutting temperature Tissue-Tek O.C.T. (Sakura Europa) in aluminum molds, frozen quickly in liquid nitrogen, and stored at -80°C . Frozen sections with 10- μm thickness were obtained in a cryostat, dried at room temperature for 60 min, fixed in 10% formaldehyde for 10 min, and then frozen and again dehydrated for 60 min.

Oil Red-O evaluation. Sections were placed in 100% propylene glycol for 3 min, stained with a solution of Oil Red-O preheated for 8 min at 60°C , differentiated in 85% propylene glycol for 3 min, washed in tap water for another 3 min, and mounted with glycerin (21). Positive control was performed from fatty adipose tissue of the breast as described in the literature (Supplemental Figure 1). Digital pictures were morphometrically evaluated by computer-assisted image analysis (ZEN 2.5 pro software, ZEISS, Zaventem, Belgium) (Supplemental Figure 2).

Triacylglycerol and ceramide measurements in heart tissues. A solution (500 μl) of 2 mM NaCl/20 mM EDTA/50 mM sodium phosphate buffer, pH 7.4, was added to myectomy samples removed from the left ventricular septum. Then, 10 μl of homogenate was mixed with 10 μl of tert-butyl alcohol and 5 μl of Triton X-100/methyl alcohol mixture (1:1 v/v) for the extraction of lipids. Triacylglycerol was measured with Triglyceride Assay Kit (Quantification ab65336; Abcam, Cambridge, Massachusetts). Ceramide was measured with Human Ceramide ELISA Kit (MBS7254089, Biosource, BlueBell, Pennsylvania).

Molecular analysis. Insulin receptor substrate (IRS)-1, IRS-2, sterol regulatory element-binding transcription factor (SREBP)-1c, peroxisome proliferator-activated receptor (PPAR)- γ , and PPAR- α were analyzed by reverse transcriptase-polymerase

chain reaction (because the material of biopsies was not sufficient to evaluate protein levels by Western Blot or mass spectrometry) as well as double staining immunofluorescence (Supplemental Appendix).

STATISTICAL ANALYSIS. The biopsies were handled and read blinded to diabetes status. Data were expressed as mean \pm SD for continuous variables, and as percentages for categorical variables. To evaluate differences between baseline and follow-up clinical characteristics, paired Student's *t*-tests were used for continuous variables. Fisher exact test was used to evaluate differences for categorical variables. Multiple regression analyses were used to examine the influence of HOMA-IR, hypertension, and dyslipidemia on triacylglycerol and ceramide myocardial levels as well as to identify the association of triacylglycerol and ceramide myocardial levels with left ventricular ejection fraction, E/e' and tricuspid annular plane systolic excursion (TAPSE) (20). Cox regression was used to estimate adjusted hazard ratios (HRs) and define lower and upper confidence limits for associations of diabetes and metformin treatment with myocyte lipid accumulation. Data were analyzed with SPSS software version 23 (IBM Corp., Chicago, Illinois). A *p* value <0.05 was considered statistically significant.

RESULTS

BASELINE CHARACTERISTICS AND OUTCOMES AT 1-YEAR FOLLOW-UP. Characteristics of patients and study population selection. Characteristics of HTX recipients and donors are in Table 1. A total of 189 recipients, eligible for the study, were divided into 2 groups: those without ($n = 103$, 54%) and those with ($n = 86$, 45%) DM. No significant difference was found in 1-year mortality between groups (8 patients without DM, 7.8%; 5 patients with DM, 5.9%). No significant difference was seen at 1-year rejection (5 patients without DM, 4.8%; 3 patients with DM, 3.5%) and infection (3 patients without DM, 2.9%; 2 patients with DM, 2.3%). Finally, 5 healthy recipients developed new-onset diabetes (we will evaluate this issue in another dedicated study). Therefore, the final study population that completed the study and follow-up included 158 recipients (82 non-DM and 76 DM) (Figure 1). Taking into account their immunosuppressive treatment, 35 DM recipients (46%) receiving metformin and 41 (54%) recipients did not receive the drug. Compared with patients without pre-transplant DM, patients who had pre-transplant diabetes spent significantly more time in the hospital during the first 1 year after HTX. The mean time hospitalized was 22 days (median, 16 days; maximum,

TABLE 1 Baseline and Follow-Up Clinical Characteristics of the Study Population

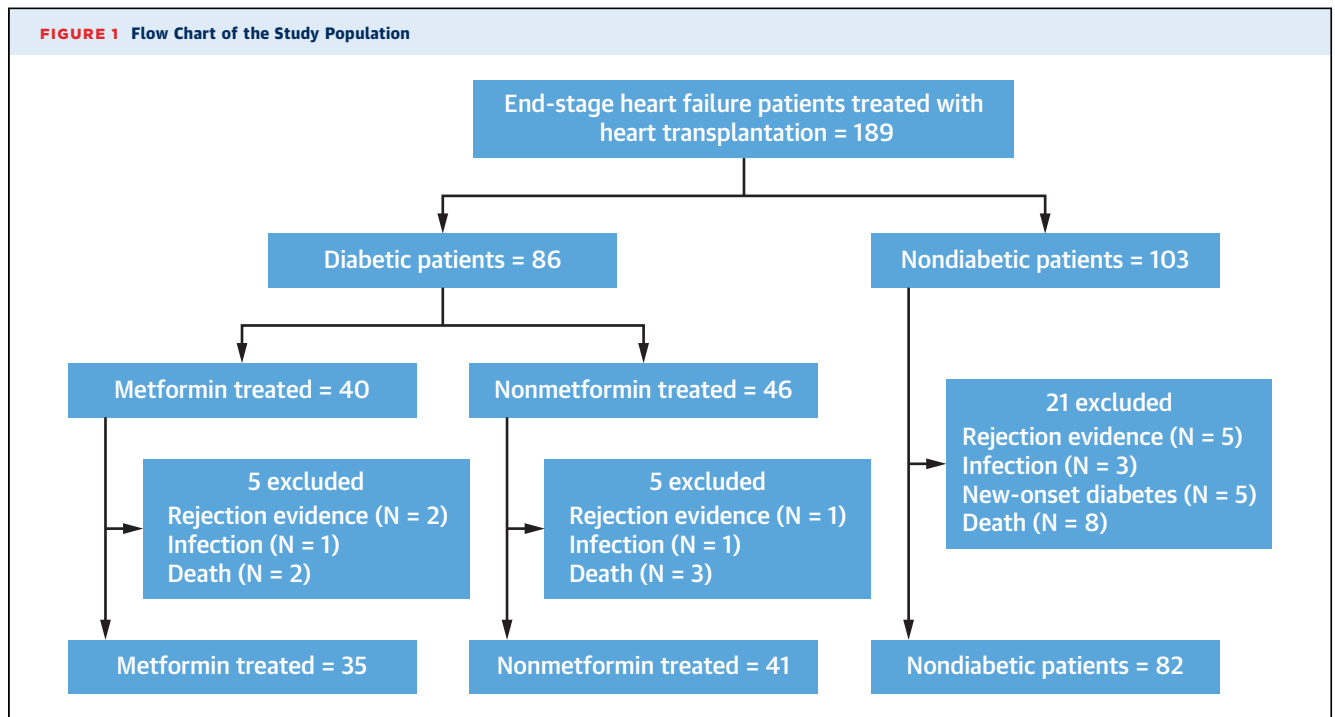
	Nondiabetic		Patients			No Metformin		Diabetic Patients		
	Basal (n = 82)	Follow-Up (n = 82)	p Value	Basal (n = 41)	Follow-Up (n = 41)	p Value	Basal (n = 35)	Follow-Up (n = 35)	p Value	
Mean age, yrs	50.9 ± 5.8	/	/	51.6 ± 5.7	/	/	51.8 ± 6.6	/	/	
Male, %	63 (76.8)	/	/	33 (80.5)	/	/	27 (77.1)	/	/	
BMI, kg/m ²	25.1 ± 1.4	24.6 ± 1.2	0.019	27.2 ± 1.5*	25.9 ± 1.4*	0.001	27.3 ± 1.8*	26.2 ± 1.8*	0.016	
Etiology of heart failure										
Ischemic cardiomyopathy	45 (54.9)	/	/	22 (53.7)	/	/	19 (54.3)	/	/	
Dilated cardiomyopathy	34 (41.5)	/	/	17 (41.5)	/	/	14 (40.0)	/	/	
Other	3 (3.6)	/	/	2 (4.8)	/	/	2 (5.7)	/	/	
Risk factors										
Hypertension	25 (30.5)	/	/	14 (29.3)	/	/	10 (28.6)	/	/	
Dyslipidemia	29 (35.4)	/	/	12 (34.1)	/	/	12 (34.3)	/	/	
Family history of CAD	28 (34.1)	/	/	17 (41.5)*	/	/	16 (45.7)*	/	/	
Smoking history	12 (14.6)	/	/	6 (14.9)	/	/	5 (14.3)	/	/	
Laboratory analyses										
Plasma glucose, mg/dl	90.7 ± 5.4	93.5 ± 8.2	0.010	139.4 ± 26.4*	118.7 ± 13.1*	0.001	141.2 ± 18.8*	117.6 ± 17.4*	0.001	
HbA1c, %	4.7 ± 0.5	5.0 ± 0.5	0.010	6.5 ± 1.1*	6.0 ± 0.7*	0.038	6.6 ± 0.6*	6.2 ± 1.2*	0.035	
Cholesterol, mg/dl	153.5 (139.75-163.5)	149 (142-165)	0.263	177 (155-195)*	162 (153.6-178)*	0.018	173 (162-182)*	168.2 (149.6-180)*	0.046	
LDL-cholesterol, mg/dl	93 (83.9-98)	86 (79.75-100)	0.112	95 (83-114.8)	89.2 (81.2-100.4)	0.970	92 (80-112.4)	88 (85-95)	0.857	
HDL-cholesterol, mg/dl	41 (40-42)	41 (38-42)	0.065	40 (39-41)	41 (39.5-42)	0.783	41 (36-43)	41 (38-43)	0.509	
Triglycerides, mg/dl	112.5 (107.5-119)	110 (100-120)	0.007	179 (143.5-200)*	169 (148.6-178)*	0.043	183 (174-198)*	165 (155-186)	0.005	
Creatinine, mg/dl	1.0 ± 0.21	1.0 ± 0.31	0.550	1.1 ± 0.31	1.1 ± 0.41	0.744	1.1 ± 0.54	1.1 ± 0.88	0.712	
Antidiabetic therapy										
Insulin	/	/	/	13 (31.7)	/	/	8 (22.9)	/	/	
DPP-IV inhibitor	/	/	/	13 (31.7)	/	/	6 (17.1)	/	/	
GLP-1 agonist	/	/	/	2 (4.9)	/	/	2 (5.7)	/	/	
Sulfonylureas	/	/	/	7 (17.1)	/	/	3 (8.6)	/	/	
Glinides	/	/	/	12 (29.3)	/	/	4 (11.4)	/	/	
Acarbose	/	/	/	5 (12.2)	/	/	3 (8.6)	/	/	
Donor data										
Mean age, yrs	30.7 ± 11.1	/	/	30.1 ± 13.7	/	/	30.9 ± 16.9	/	/	
Male, %	51 (62.9)	/	/	25 (61.1)	/	/	21 (60.0)	/	/	
BMI, kg/m ²	26.4 ± 1.2	/	/	26.1 ± 1.3	/	/	26.3 ± 1.1	/	/	
Donor ischemic time, min	101.4 ± 37.2	/	/	102.9 ± 30.5	/	/	103.1 ± 33.8	/	/	

Values are mean ± SD, n (%), or median (25th to 75th percentile). *p < 0.01 versus nondiabetic patients.
/ = not available; BMI = body mass index; CAD = coronary artery disease; DPP-IV = Dipeptidyl peptidase IV; GLP = glucagon-like peptide; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

73 days) for recipients who had pre-transplant DM and 17 days for recipients who did not (median, 13 days; maximum, 68 days). Patients with DM were more likely to have CHD as reason for HTX (Table 1). None of the other baseline characteristics differed significantly between groups, most notably the pre-operative levels of creatinine and cholesterol. Antidiabetic therapy of patients with pre-transplant DM are reported in Table 1. Diabetes duration was 14.8 ± 2.8 years in the nonmetformin group and 15.0 ± 2.1 years in the metformin group (p = 0.802). None of the DM HTX had diabetic complications. Before HTX and during the follow-up, patients with DM evidenced an optimal glucose and lipid control (Table 1). As expected, plasma glucose levels and systemic insulin

resistance (HOMA-IR) were higher in the patients with DM compared with patients without DM both at baseline and follow-up (Figure 2). In patients with DM, plasma glucose levels and systemic insulin resistance (HOMA-IR) were higher in the DM compared with diabetic patients receiving metformin both at baseline and follow-up (Figure 2). Interestingly, at follow-up, systemic insulin resistance increased in all patients, probably because of immunosuppressive therapy.

Clinical outcomes at 1-year follow-up. After HTX, echocardiographic in-hospital evaluation showed a normal ejection fraction, slight alterations in the diastolic phase and in right ventricular function throughout the studied population without

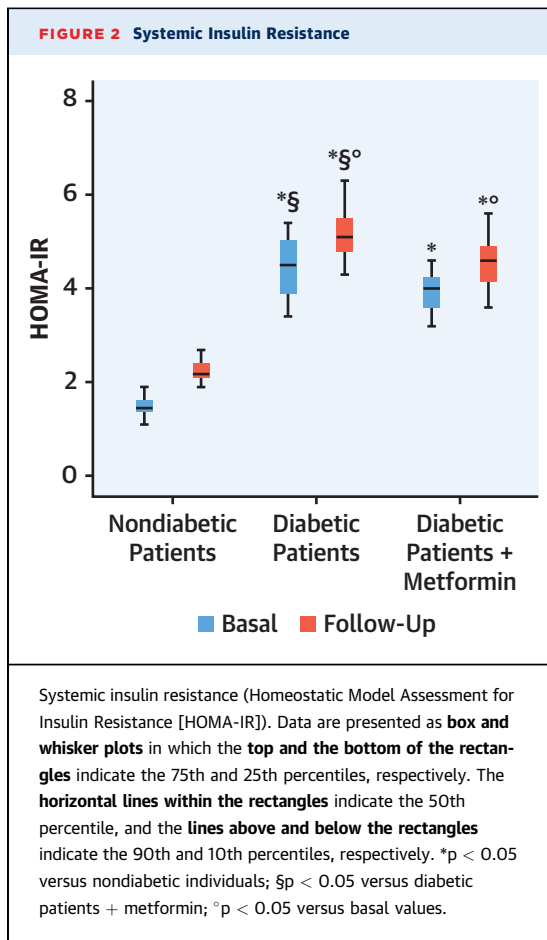


significant differences between DM and non-DM (Figure 3). After 12 months of follow-up, there was an impairment of both left and right ventricular function with a significant reduction of ejection fraction and TAPSE in DM versus non-DM recipients ($p < 0.05$) (Figure 3). E/e' ratio showed a lower reduction in DM recipients (Figure 3). Relevantly, the worsening of cardiac function was less evident in the DM subgroup receiving metformin. At 1-year follow-up, coronary CT angiography evaluations evidenced absence of coronary lesions in transplanted heart both in DM and non-DM recipients (Supplemental Figure 3). Finally, myocardial perfusion by single-photon emission CT showed absence of inducible ischemia in all patients (not shown).

LIPID ACCUMULATION IN CARDIOMYOCYTES.

Although ectopic fat accumulations are well known in pericardium, liver, and muscle tissues of patients with DM, data describing the progression of intra-myocyte lipid accumulation in human diabetic hearts are lacking. Therefore, we compared myocardium of healthy transplanted hearts in recipients with and without DM by analyzing 474 EMBs for histological and molecular analyses until the 48th week after HTX. EMBs were divided into the following categories: basal (1 to 4 weeks), intermediate (5 to 12 weeks), and final (13 to 48 weeks). As reported in Figure 4, none of the basal EMBs analyzed in both

groups of recipients contained myocardial lipid accumulation. This was confirmed both by the triacylglycerol and ceramide measurements in heart tissues (at cardiomyocyte level), which showed the similar content of lipid in DM and non-DM recipients. Interestingly, during the intermediate follow-up period, the EMBs of 11 DM recipients (28.6%) showed cardiomyocyte lipid accumulation, whereas none of the EMBs from non-DM and only 2 (5.7%) of DM recipients treated with metformin had fat accumulation in the heart tissue. Interestingly, both triacylglycerol and ceramide contents were higher in specimens from DM compared with non-DM recipients or those receiving metformin ($p < 0.01$) (Figure 5). Remarkably, EMBs of 36 DM recipients (87.8%) showed myocyte lipid accumulation, whereas none of the EMBs from non-DM recipients had fat accumulation, and a residual number of 20% receiving metformin showed lipid accumulation at final follow-up (13 to 48 weeks). In DM recipients receiving metformin, lipid accumulation was both delayed and reduced in comparison with DM recipients not receiving the drug (HR: 6.597; confidence interval: 2.516 to 17.296; $p < 0.001$) (Figure 4). This phenomenon stressed the concept that the subgroup of DM recipients who used metformin was similar to that of non-DM group. Likewise, triacylglycerol and ceramide contents was significantly higher in specimens from DM than in specimens of recipients



without DM and/or DM receiving metformin (Figure 5). Regression analysis showed a relationship between myocyte ceramide contents and HOMA-IR in the non-DM and in the 2 subgroups of DM recipients (Figure 6). Myocyte ceramide contents (dependent variable) changed when HOMA-IR levels (independent variables) were varied, whereas the other independent variables were held fixed. Regression analysis showed a relationship between heart triacylglycerol and ceramide contents and ejection fraction and TAPSE values at 1-year follow-up in the non-DM and in the 2 subgroups of DM recipients (Supplemental Figure 4). Next, regression analysis showed that the values of ejection fraction, E/e' , and TAPSE (dependent variables) changed when both triacylglycerol and ceramide heart contents (independent variables) were varied while the other independent variables were held fixed. Indeed, regression analysis showed that both systolic and diastolic heart functions (dependent variables) were reduced when myocardial lipid content (independent variables) increased (Supplemental Figure 4).

MOLECULAR ANALYSIS OF VENTRICULAR SPECIMENS.

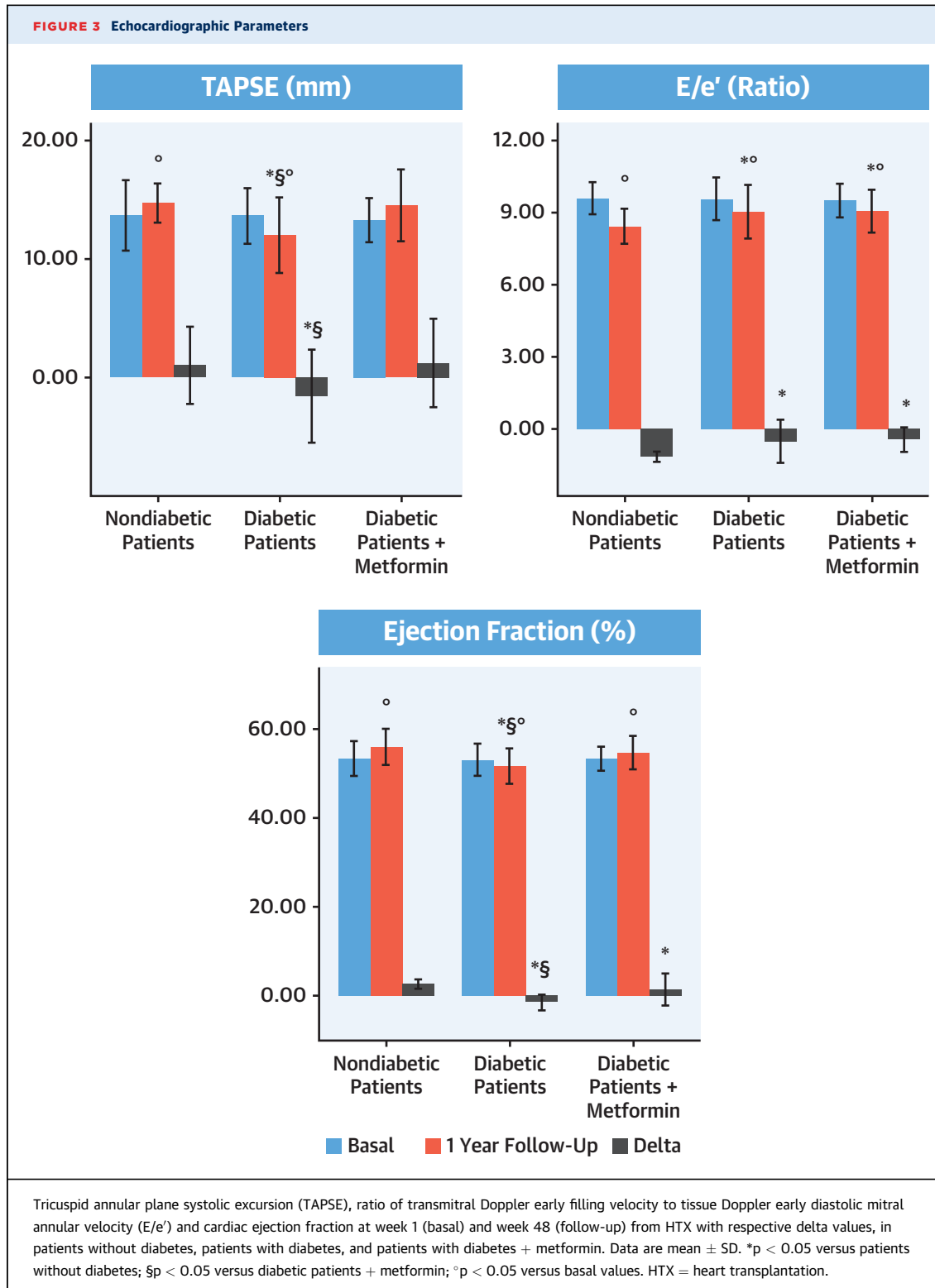
Immunofluorescence analysis evidenced that IRS-1, IRS-2, SREBP-1c, PPAR- α , and PPAR- γ were expressed in the cardiomyocytes (data not shown). SREBP-1c, PPAR- α , and PPAR- γ expression and insulin resistance markers in heart EMBs of the basal period showed similar levels in both subgroups of DM and non-DM recipients (Supplemental Figures 5 to 8). Remarkably, during the intermediate period, these lipogenic factors increased in specimens from DM compared with non-DM recipients and/or those receiving metformin (Supplemental Figures 5 to 8). These differences persisted until 12 months (Supplemental Figures 5 to 8), indicating progression of lipid accumulation together with lipogenic factors in DM recipients. Both myocardial insulin resistance markers (IRS-1 to -2) and lipogenic factors (SREBP-1c and PPAR- γ) were related to systemic insulin resistance (HOMA-IR) (Supplemental Figures 5 to 8).

FINDINGS IN RESIDENT EXPLANTED HEARTS WITH END-STAGE HF.

Although the analysis of explanted resident hearts provided no new information on the progression of DMCM, to gain insights into pathophysiology of end-stage HF during diabetes, lipid accumulation was observed in explanted hearts from patients with DM regardless of the cause of HF, whereas the same myocardial alterations were detected in only 3 biopsies from patients without DM (Figure 7). When lipogenic factors were analyzed, IRS-1 and IRS-2 levels were reduced in cardiomyocytes of explanted hearts of patients with DM compared with patients without DM ($p < 0.05$), indicating a greater insulin resistance in diabetic end-stage HF (Supplemental Figure 9). Lipogenic factors (SREBP-1c, PPAR- α , and PPAR- γ) were higher in cardiomyocytes of explanted hearts of DM compared with non-DM recipients ($p < 0.01$) (Supplemental Figure 9).

DISCUSSION

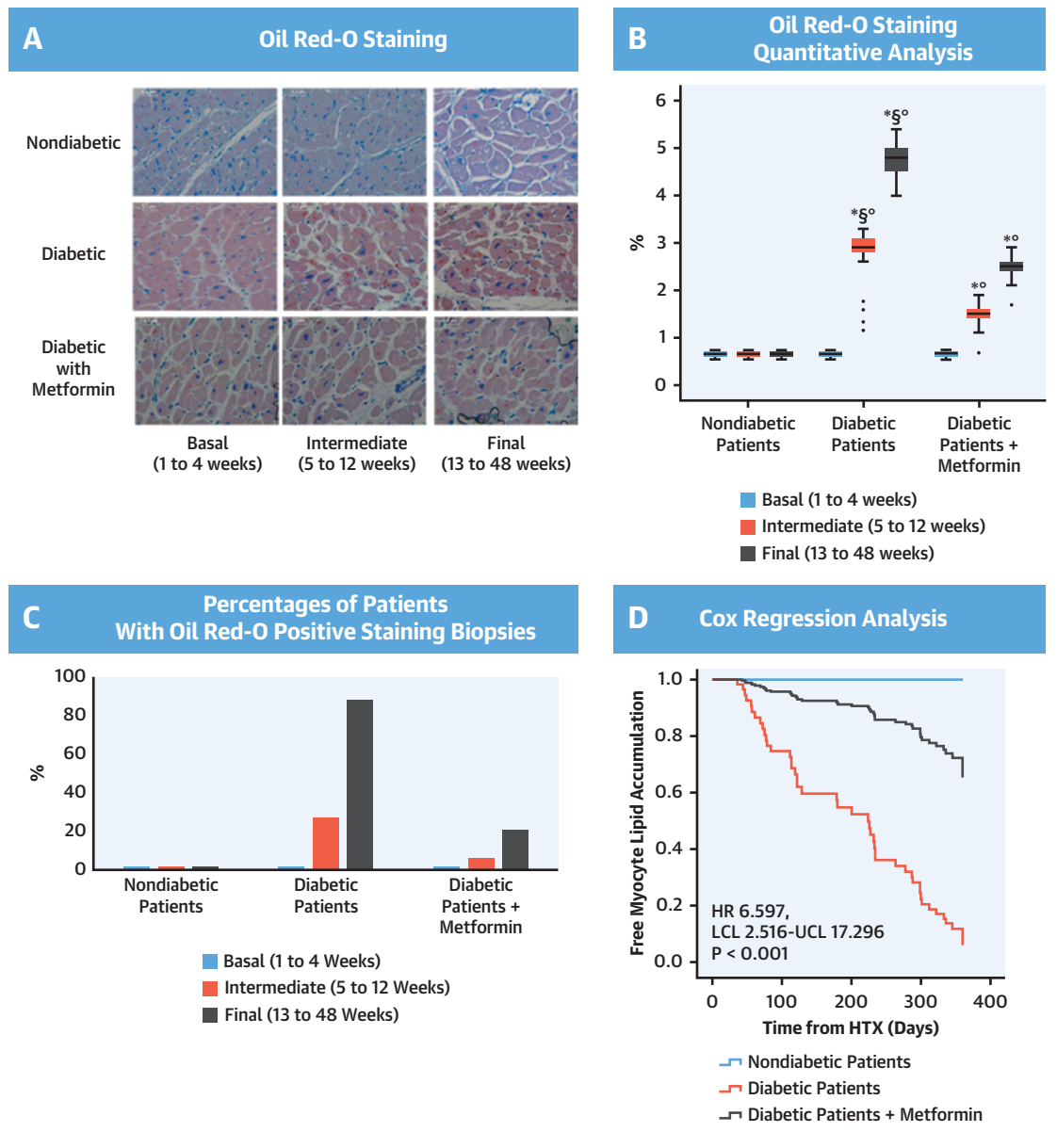
In our experimental clinical conditions, the major findings of the present report are as follows: 1) the development of a serial study of human heart transplant recipients to evaluate DMCM; 2) the evidence of early involvement of lipid cardiomyocyte accumulation in DMCM; 3) the decreased lipid cardiomyocyte accumulation associated with concomitant use of metformin; and 4) HOMA-IR values correlated with the myocyte lipid accumulation (triacylglycerol and ceramide), independently from immunosuppression (Central Illustration). Moreover, although there is some evidence of intramyocardial lipid accumulation in the experimental and human



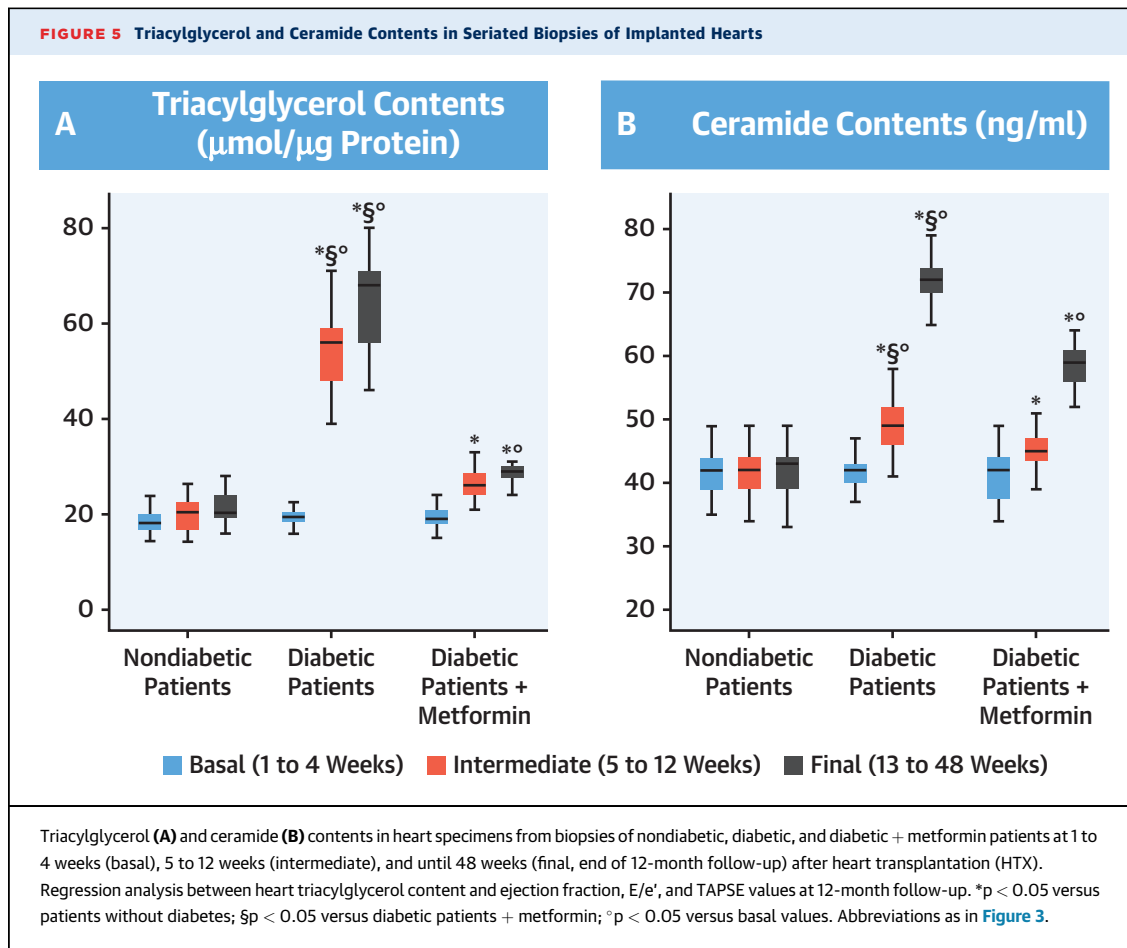
diabetic heart (3,22), our data show for the first time, that regardless of the pathogenic causes of HF, explanted hearts from patients with DM had intra-myocyte lipid accumulation.

Progression of cardiomyocyte lipid accumulation in human DMCM. Although DMCM was first reported 50 years ago (23), to date, our knowledge of the human pathogenic mechanisms inducing the onset and

FIGURE 4 Myocardial Lipid Infiltration in Seriated Biopsies of Implanted Hearts



(A) Oil Red-O staining (magnification $\times 40$) in heart specimens from biopsies of patients without diabetes, patients with diabetes, and patients with diabetes + metformin recipients at 1 to 4 weeks (basal), 5 to 12 weeks (intermediate), and until 48 weeks (final, end of 12-month follow-up) after HTX. **(B)** Percentage values of lipid accumulation positive cells from biopsies of patients without diabetes, patients with diabetes and patients with diabetes + metformin recipients at 1 to 4 weeks (basal), 5 to 12 weeks (intermediate), and until 48 weeks (final) after HTX (ZEN 2.5 pro software). **(C)** Percentages of patients with Oil Red-O positive staining biopsies from patients without diabetes, patients with diabetes, and patients with diabetes + metformin recipients at 1 to 4 weeks (basal), 5 to 12 weeks (intermediate) and until 48 weeks (final, end of 12-month follow-up) after HTX. **(D)** Cox regression analysis to estimate adjusted hazard ratio (HR) and define lower and upper confidence limits (95% LCL and 95% UCL) for associations of diabetes and metformin treatment with myocyte lipid accumulation, including the following candidate covariables: age, sex, body mass index, dyslipidemia, hypertension, smoking habits, glucose, HbA1c, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, polyclonal antilymphocyte antibodies, cyclosporine, tacrolimus, mycophenolate mofetil, everolimus, and prednisone mean dosage for each patient during follow-up. Data are mean \pm SD. * $p < 0.05$ versus patients without diabetes; § $p < 0.05$ versus diabetic patients + metformin; $^{\ddagger}p < 0.05$ versus basal values. HTX = heart transplantation.

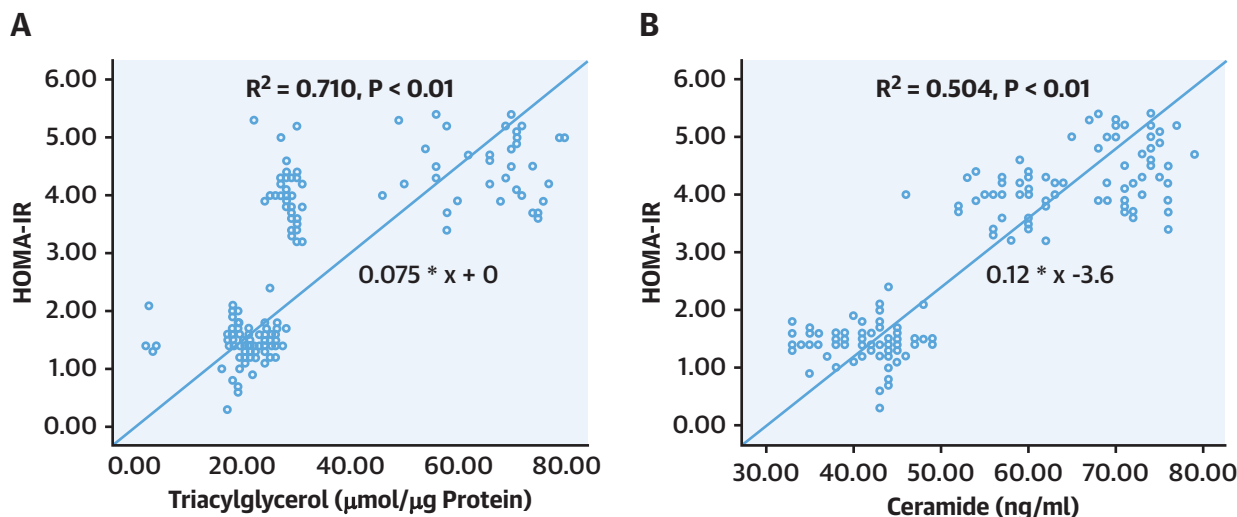


progression of cardiac dysfunction come from translational models (3,10,24-27). Our serial study of human heart transplant recipients would be useful to fill this gap. In fact, we investigated the early detrimental effects of diabetes on myocardial pathophysiological mechanisms involved in the progression of human DMCM. Our data in resident hearts were consistent with studies using Oil Red-O staining of explanted hearts at the time of HTX, which demonstrated cardiac steatosis in diabetic patients with severe HF (28,29). Moreover, our study established that cardiac lipid accumulation is a pathogenic event unrelated to the other pathogenic events of end-stage HF (ischemic, idiopathic, infective, and rheumatic). This finding supports further investigations into the effects of diabetic milieu on pathogenetic mechanisms of the main causes of HF. However, further studies on a larger population will be needed to support these observations.

Cardiomyocyte lipid accumulation and clinical outcomes. We established that metabolic derangement begins early in transplanted healthy hearts of patients with DM but not in non-DM recipients, as

proved by cardiomyocyte lipid accumulation already after 3 months from HTX but not during the first EMBs after HTX. Therefore, the diabetic milieu is able to promptly alter the cardiomyocyte lipid metabolism. Moreover, we showed that accumulation of myocardial lipid triglycerides in patients with DM was associated with cardiac dysfunction, independently of BMI, heart rate, and blood pressure. Consistently, triacylglycerol and ceramide contents were related both with early diastolic and systolic dysfunction observed in DM recipients after 12 months from HTX. Furthermore, these alterations were independent from CHD (as showed by negative coronary CT angiography and electrocardiogram stress test). Myocardial insulin receptor levels (IRS-1 and IRS-2) were lower in healthy transplanted hearts in DM recipients compared with non-DM recipients. Moreover, molecular analysis of SREBP1c and PPAR systems showed higher expression in cardiomyocytes of DM recipients. Because HOMA-IR was correlated both with lipogenic factors (SREBP-1c and PPAR-γ) and cardiomyocyte lipid accumulation, we suggest that the metabolic milieu of DM

FIGURE 6 Regression Analysis Between HOMA-IR and Triacylglycerol and Ceramide Myocardial Contents



Regression analysis evidences a relationship between myocyte triacylglycerol (A) and ceramide (B) contents and HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) in the nondiabetic and in the 2 subgroups of diabetic recipients. **Blue circles** represent patients based on triacylglycerol or ceramide and HOMA-IR levels. The **blue line** represents the linear regression line.

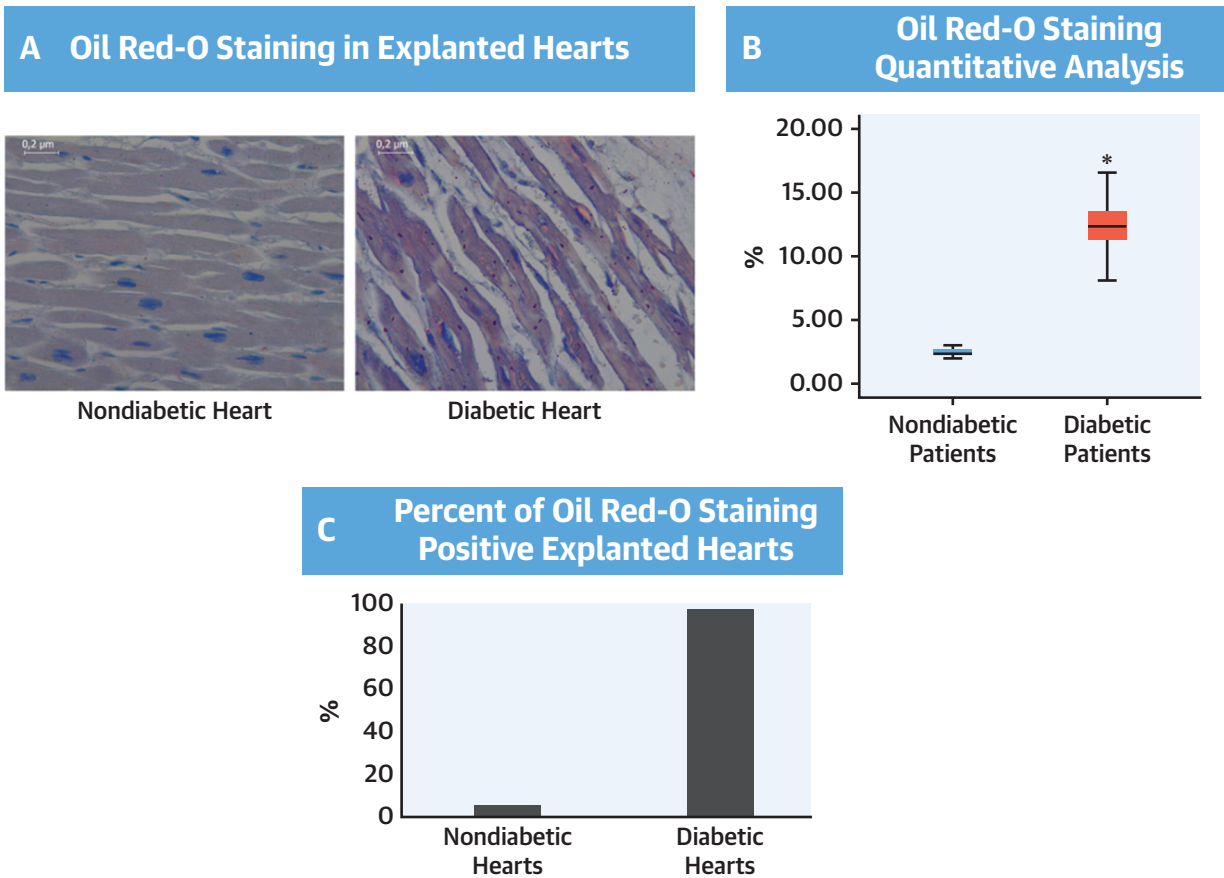
recipients had a detrimental role in the early progression of DMCM. Accordingly, the differences among lipogenic factors are in line with experimental data on DMCM (3,6,7). Nevertheless, the resistant tissues that are subject to hyperinsulinemia/insulin resistance could contribute to the inflammatory milieu (30). On the other hand, hyperinsulinemia/insulin resistance could lead to fibrosis often deleterious for muscle and other tissue. Furthermore, evidence supports, also, the importance of glucotoxicity in these molecular mechanisms, because there is such a tight correlation between defective insulin secretion and rising glucose levels (31,32). Thus, DMCM may be one of the consequences of a toxic environment. Furthermore, the main candidates for DMCM are hyperglycemia, per se, and the toxic milieu associated with the metabolic syndrome (33). Thus, hypertension, dyslipidemia, and the other nonglycemic components of the metabolic syndrome probably contribute to the pathogenesis of DMCM. Because of the nature of our study, it may not be possible to ultimately separate hyperglycemia from the other metabolic syndrome components. Although we observed a significant relationship between HOMA-IR and myocardial lipid accumulation (triacylglycerol and ceramide levels) by a multiple regression analysis including as independent variables as well as hypertension and dyslipidemia, we cannot exclude that the components of the metabolic syndrome contribute to the pathogenesis of DMCM. Metabolic syndrome and

DMCM are complex diseases that adversely influence cardiac function, sharing many pathophysiological mechanisms. Multiple mechanisms are involved in both conditions and multiple potential therapeutic targets might be found in the future.

Cardiomyocyte lipid accumulation and metformin.

We show that reduction of myocardial lipid accumulation in DM recipients was associated with metformin therapy. Thus, patients with concomitant use of metformin show a blunted effect of diabetic milieu on healthy hearts transplanted in the DM recipient. Human data suggest that metformin reduces both insulin resistance and ectopic lipid accumulation (12-14,34,35). Moreover, there is accumulating data suggesting that metformin affects lipid metabolism, and PPAR and SREBP signaling in skeletal muscle, subcutaneous adipose tissues, and hepatocytes (36,37). We observed a blunted reduction of myocardial insulin receptors and PPAR and SREBP signaling in healthy hearts transplanted in DM recipients receiving metformin. The decreased myocardial lipid accumulation may be related to metformin use and significant reduction in systemic insulin resistance, as indicated by reduced HOMA-IR levels. However, the possible benefits of metformin might also be mediated by less insulin resistance or other mechanism(s) induced by metformin. In fact, benefits of metformin include improvement in pathophysiological components of the metabolic syndrome (sub-clinical inflammation, endothelial dysfunction and

FIGURE 7 Lipid Infiltration in Explanted Hearts



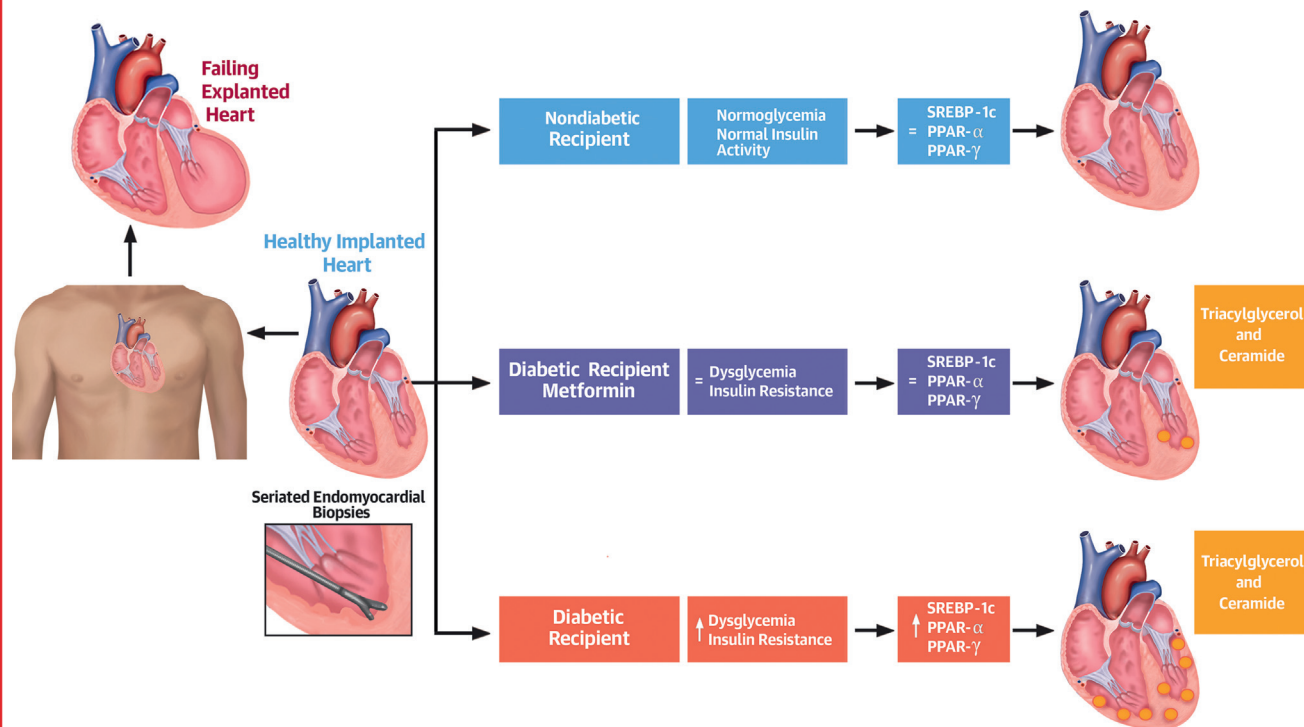
(A) Oil Red-O staining in heart specimens from explanted hearts of diabetic and nondiabetic patients. **(B)** Percent values of lipid accumulation positive cells from nondiabetic and diabetic explanted hearts (ZEN 2.5 pro software). * $p < 0.05$ versus patients without diabetes. **(C)** Percentages of explanted hearts with Oil Red-O positive staining.

nonalcoholic fatty liver disease), lipid-lowering properties, antihypertensive properties, and anti-neoplastic potential (38).

STUDY LIMITATIONS. First, our real-life study is not a multicenter study and thus we need to extend our observations to a larger cohort of randomized patients. Second, immunosuppressive therapy per se could affect molecular mechanisms of DMCM. However, we observed that progressive accumulation of myocardial lipid triglycerides in DM recipients was independent of immunosuppressive therapy by Cox regression analysis, also including covariables such as polyclonal antilymphocyte antibodies, cyclosporine, tacrolimus, mycophenolate mofetil, everolimus, and prednisone (mean dosage). Third, although de novo diabetic status, related to immunosuppressive drugs (39-41), shares many clinical pathogenic events with

DM (i.e., insulin resistance and decompensated insulin release, hypertriglyceridemia, obesity, hypertension and low-grade inflammation), the underlying pathogenic mechanisms might be different. Therefore, potentially confounding data from patients with de novo diabetes were excluded from the study. Yet, HOMA-IR as index of systemic insulin resistance could have some limitations (42,43). In fact, HOMA-IR does seem to estimate hepatic insulin resistance much better than peripheral insulin resistance. Moreover, the limitation of the HOMA-IR should be considered in individuals with very low BMI, high fasting glucose levels, and mixed-race populations. In this regard, our study population, due to the strict selection criteria for HTX (11,15,16,44), had similar BMI, fasting glucose levels, and patients were Caucasian. Finally, because there was no randomization of metformin treatment, comparison of the

CENTRAL ILLUSTRATION Lipid Accumulation in Hearts Transplanted from Nondiabetic Donors to Diabetic Recipients: Study Protocol



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Unique real-life study showing the role of myocardial lipid accumulation in early events of human diabetic cardiomyopathy. Diabetic-related insulin resistance, peroxisome proliferator-activated receptor (PPAR) system leads to increased myocardial triglyceride accumulation with subsequent early impairment of cardiac function. Metformin blunts lipid accumulation and PPAR involvement in diabetic recipients.

patients receiving and not receiving metformin cannot be assumed to be definitive, although highly suggestive. In the small numbers available, lack of significance between the differences does not mean lack of significant differences.

CONCLUSIONS

Our study evaluates early stage of human DMCM. The opportunity to study serial cardiac biopsies demonstrated, in our experimental conditions, the relative rapid progression of early pathogenic events of DMCM linked to cardiac lipid accumulation. The use of imaging software may improve objective evaluation of lipid accumulation in diabetic recipients (45). Relevantly, metformin use was associated with slower progression of such detrimental effects. This is particularly relevant because of the actual trend for the wide use of metformin also in pre-diabetic status (46).

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Endomyocardial biopsies from diabetic recipients of hearts from nondiabetic donors exhibit early lipid accumulation and lipotoxic factor expression that may contribute to the early pathogenesis of diabetic cardiomyopathy. Treatment with metformin therapy reduces lipid accumulation.

TRANSLATIONAL OUTLOOK: Future studies should target lipid accumulation in an effort to prevent the early development of diabetic cardiomyopathy and heart failure after cardiac transplantation in patients with diabetes mellitus.

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KEY WORDS CVD, diabetes, diabetic cardiomyopathy, heart transplantation

APPENDIX For supplemental figures, please see the online version of this paper.