Impairment of the Akt pathway in transplanted Type 1 diabetic hearts is associated with post-transplant graft injury[†]

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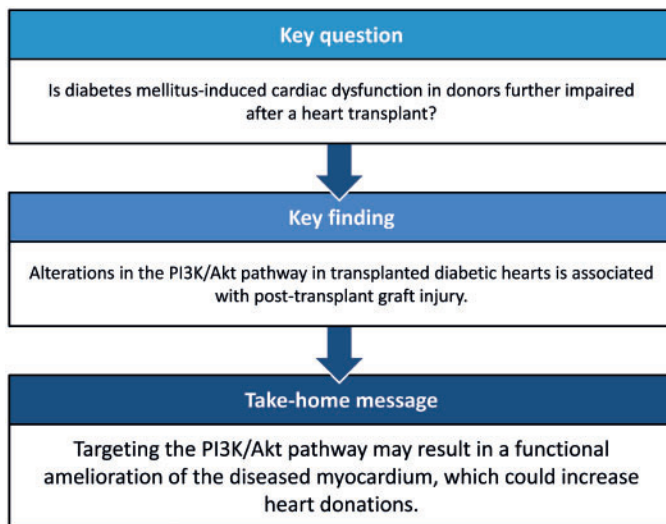[a] Department of Cardiac Surgery, Heidelberg University Hospital, Heidelberg, Germany
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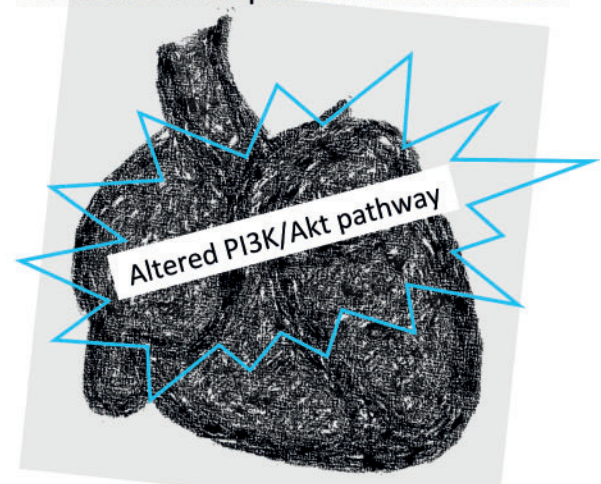[d] Department of Internal Medicine III, Molecular and Translational Cardiology, Heidelberg University Hospital, Heidelberg, Germany

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Key question

Is diabetes mellitus-induced cardiac dysfunction in donors further impaired after a heart transplant?

Key finding

Alterations in the PI3K/Akt pathway in transplanted diabetic hearts is associated with post-transplant graft injury.

Take-home message

Targeting the PI3K/Akt pathway may result in a functional amelioration of the diseased myocardium, which could increase heart donations.

Untreated transplanted diabetic heart

Altered PI3K/Akt pathway

Abstract

OBJECTIVES: The use of 'marginal' hearts, such as from donors with diabetes mellitus (DM), could offer an opportunity to expand the donor pool in cardiac transplantation. Previous studies have shown that the phosphatidylinositol-3-kinase (PI3K)/Akt pathway is altered after ischaemia/reperfusion injury in the diabetic myocardium. We hypothesized that DM-induced cardiac dysfunction in donors is further impaired after heart transplantation and that PI3K/Akt-pathway alterations may be one of the underlying pathomechanisms.

METHODS: In the donor rats, DM was induced with a single dose of streptozotocin. Non-diabetic rats only received citrate buffer. After 8 weeks, the donor left ventricular (LV) cardiac function was measured. Then, the hearts were heterotopically transplanted into non-diabetic recipients. We evaluated LV graft function 1.5 h after transplantation via a Millar catheter system at different LV volumes. Histological analyses were performed, and the expression of 84 genes involved in PI3K/Akt signalling was profiled.

RESULTS: DM was associated with significantly decreased LV contractility and impaired relaxation. After transplantation, in the DM group, the grafts' systolic function (LV systolic pressure 112 ± 31 vs 155 ± 60 mmHg; dP/dt_{max} 2676 ± 896 vs 3584 ± 1779 mmHg/s, $P < 0.05$) and diastolic function (dP/dt_{min} 924 ± 205 vs 1748 ± 512 mmHg/s, $P < 0.05$) were significantly reduced at an intraventricular volume of 170 μl.

The expression of 10 genes involved in PI3K/Akt signalling, as well as the phosphorylated Akt/total Akt protein expression ratio, were significantly down-regulated in the diabetic heart after transplantation.

CONCLUSIONS: DM-induced cardiac dysfunction is further impaired after transplantation. Targeting the PI3K/Akt pathway may result in a functional amelioration of the *a priori*-diseased myocardia, which could increase the number of potential cardiac donors.

Keywords: Diabetes mellitus · Ischaemia/Reperfusion · Heart transplantation · Phosphatidylinositol-3-kinase/Akt pathway · Gene profiling

INTRODUCTION

Heart transplantation is a well-recognized treatment option for end-stage heart failure. The discrepancy between waiting lists and performed operations has increased dramatically. Therefore, an expansion of the donor pool is essential, and efforts have been made to make use of so-called 'appropriate marginal donors'. Marginal donors include elderly donors, donors with hepatitis C virus positivity, an ejection fraction less than 45%, a donor:recipient weight ratio <0.7 [1], a donor history of alcoholism or donor with diabetes mellitus (DM). In the USA, 20 348 primary adult heart transplants were performed between January 2000 and December 2010, as reported in the United Network for Organ Sharing (UNOS) [2]. Among these, 492 (2.4%) patients received a donor heart from a diabetic donor. DM, a chronic, progressive metabolic disorder caused by impaired insulin production or insulin resistance, leads to hyperglycaemia. It is well known that Type 1 diabetes, due to the autoimmune-mediated destruction of the β-cells of the islets of Langerhans in the pancreas, is characterized by insulinopenia, hyperglycaemia and an often unaffected lipid profile. DM can affect cardiac structure and function in the absence of changes in blood pressure and coronary artery disease, a condition called diabetic cardiomyopathy. There is evidence of changes in the extracellular matrix with increased cardiac fibrosis [3], excessive generation of reactive oxygen species [4], as well as cardiac inflammation [5], characterized by increased levels of proinflammatory cytokines, which may play a role in the manifestation of diabetic cardiomyopathy. Additionally, during heart transplantation, myocardial ischaemia/reperfusion injury (IRI) is another key factor. Thus, any pre-existent myocardial damage due to DM may be aggravated by hypothermic preservation/warm reperfusion during heart transplantation. During reperfusion, the ischaemic myocardium is acutely subjected to several changes, including intracellular calcium overload, energy depletion, acidosis, neutrophils and the generation of reactive oxygen species [6]. Clinical studies have shown that carefully selected hearts from donors with diabetes can be used for transplantation, showing comparable outcomes to recipients who have received hearts from non-diabetic donors [2]. Others suggest that the diabetic heart is more sensitive to ischaemic injury than the non-diabetic heart [7].

A growing body of evidence indicates that the phosphatidylinositol-3-kinase (PI3K)/Akt-signal transduction pathway is generally considered to be beneficial for heart function [8]. Briefly, the binding of insulin to its receptor sequentially activates insulin receptor substrate, PI3K and its downstream target Akt (Supplementary Material, Fig. S1). Activated Akt translocates the glucose transporter (GLUT)-4 and increases glucose uptake. Insulin through PI3K activation also induces the translocation of the fatty acid transporter FAT/CD36 to enhance fatty acid uptake. Additionally, insulin binding to its receptor promotes an increase in calcium (Ca^{2+}) influx and the release of Ca^{2+} from sarcoplasmatic reticulum. Increased levels of lipid, glucose and Ca^{2+} generate adenosine triphosphate, which maintain contractile function. Activation of PI3K/Akt-dependent signalling has been shown to prevent cardiac myocyte apoptosis and to protect the myocardium from regional IRI in non-diabetic animals [9]. Additionally, individuals diagnosed with diabetes demonstrate reduced PI3K activity. Recently, it has been shown that the PI3K/Akt pathway is involved in myocardial IRI in diabetic rats [10]. However, the involvement of this pathway in potential diabetic heart donors and its implications in cardiac transplantation have not been documented.

We hypothesized that untreated DM-induced cardiac dysfunction in donors is further impaired after heart transplantation and that PI3K/Akt-pathway alterations may be one of the underlying mechanisms. We expect that the present work can expand on previous studies hoping to discover targets that can be triggered by drug therapy, with the goal of decreasing graft dysfunction after transplantation and increasing the number of available heart donors.

MATERIALS AND METHODS

See Supplementary Material for further details.

Animals

Male Lewis rats (250–350 g; Charles River, Sulzfeld, Germany) received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research, and with the 'Guide for the Care and Use of Laboratory Animals', prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). This investigation was approved by the appropriate institutional review committees (G19/14).

Induction of diabetes mellitus

Type 1 DM was induced in rats with a single intraperitoneal dose of streptozotocin (60 mg/kg, freshly dissolved in 0.1 M citrate buffer). Control animals received only the vehicle buffer.

Non-diabetic and diabetic donors

Experimental groups. Rats were divided into 2 groups: (i) control donor rats (*n* = 9) received buffer vehicle and (ii) diabetic donor rats (*n* = 11) received streptozotocin.

Electrocardiography. At 8 weeks after diabetes induction, as previously reported [11, 12], standard 12-lead electrocardiograms were recorded using subcutaneously placed needle electrodes.

Left ventricular cardiac function. After the electrocardiogram (ECG) recordings, as previously reported [11, 12], the rats were tracheotomized, intubated and artificially ventilated with ambient air. A polyethylene catheter was inserted into the left external jugular vein for fluid administration. A 2-Fr microtip pressure–volume catheter was inserted into the right carotid artery and advanced into the ascending aorta. After a 5-min stabilization period, the arterial blood pressure was recorded, and the catheter was advanced into the left ventricle under pressure control. With the use of a special pressure–volume-analysis programme (PVAN, Millar Instruments, Houston, TX, USA), heart rate, systolic blood pressure, diastolic blood pressure, mean arterial pressure, left ventricular (LV) end-systolic pressure, LV end-diastolic pressure, effective arterial elastance (Ea), maximal slope of systolic pressure increment (dP/dt_{max}) and diastolic pressure decrement (dP/dt_{min}) and time constant of the LV pressure decay (Tau-g; according to the Glantz method [13]) were calculated. LV pressure–volume relations were assessed by transiently compressing the inferior vena cava. The slope of the LV end-systolic pressure–volume relationship (ESPVR) was calculated according to the linear (E_{es}) and the parabolic curvilinear model (E_{max}) [14]. Preload recruitable stroke work, maximal elastance and the dP/dt_{max}/end-diastolic volume relation were calculated as a load-independent index of LV contractility indexes. The slope of the LV end-diastolic pressure–volume relationship was calculated as a reliable index of LV stiffness.

Biochemical analysis. Plasma glucose concentrations were measured using an ADVIA®-2400 chemistry analyser (Siemens Healthcare, Germany), and the level of whole blood haemoglobin A1c (HbA1c), also known as glycated haemoglobin, was determined immunologically on a Dimension® clinical chemistry system (Siemens) in the Central Laboratory of the Heidelberg University Clinic.

Rat model of heterotopic heart transplantation

Experimental groups. Rats were divided into 2 groups: (i) control + transplanted ($n = 9$): donor rats received buffer vehicle and (ii) diabetic + transplanted ($n = 11$): donor rats received streptozotocin. In both groups, the heart was explanted and transplanted into non-diabetic rats after 8 weeks. In each group, 2 rats were lost due to technical problems during the heart transplantation procedure.

Surgical technique of heart transplantation. Transplantations were performed in an isogenic Lewis to Lewis rat strain model. The experimental model was described elsewhere [12, 15]. Briefly, cardiac arrest was induced using the Custodiol solution (Dr Franz Köhler, Chemie GmbH, Bensheim, Germany), and then, the heart was explanted and immediately placed into the Custodiol solution (4°C).

Recipient rats were anaesthetized and then heparinized. The aorta and the pulmonary artery of the donor heart were anastomosed end to side to the abdominal aorta and the vena cava of the recipient rat, respectively. To minimize variability between surgical experiments, the duration between explantation and reperfusion was standardized to 1 h. After completion of the anastomoses, the heart was reperfused with blood *in situ* for 1 h.

Functional measurement in the graft. As previously reported [12], 1 h after transplantation, a 3-Fr latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA, USA) was introduced into the left ventricle via the apex and connected to a precision-calibrated syringe for administration and withdrawal of fluid. Additionally, a Millar micromanometer (Millar Instruments, Houston, TX, USA) was inserted in the left ventricle to determine LV systolic pressure, LV end-diastolic pressure, dP/dt_{max} and dP/dt_{min} at different LV volumes. LV volumes were calculated as the volume of saline injected into the balloon plus the volume of the empty balloon (0.02 ml). Data for a complete pressure–volume curve were obtained through incremental increases in LV volume by 0.03 ml until a volume of 0.17 ml was reached.

Histological assessment. After the functional measurements, all rats ($n = 9$–11 rats/group) were euthanized by exsanguination. Pieces of basal myocardial tissue were fixed in buffered paraformaldehyde solution (4%), embedded in paraffin, cut to 5 μm and stained with haematoxylin and eosin.

Western blotting. Myocardial protein expression of Akt, phosphorylated Akt (1:500, Abcam, Cambridge, UK) and GLUT-4 (1:100, Santa Cruz, Biotechnology, Heidelberg, Germany) was performed by Western blot by gel electrophoresis and immunoblot analysis.

Gene expression analysis

Using RT² Profiler™ PCR Array (PARN-058Z), the expression of 84 genes was profiled before and following heart transplantation (Supplementary Material, Online Table 2). These genes were selected as they have been reported to be key genes involved in PI3K/Akt signalling. Pieces of myocardial tissue were taken at apical region of the ventricles. Genes with fold changes of >2-fold at *P*-value < 0.05 were considered as significantly altered.

Statistical analysis

All data are expressed as mean ± standard deviation. Statistical analyses of data were performed using GraphPad Prism 7.02 software (GraphPad Software, Inc., CA, USA). Before statistical tests were applied, the Shapiro–Wilk normality test was used to assess normal distribution. For data with normal distribution, the 2-sample student's *t*-test was used to analyse the differences between the 2 groups. If the normality test failed, a non-parametric Mann–Whitney test was applied. In the case of graft function after transplantation, a 2-factor mixed analysis of variance (ANOVA) (independent factor: DM and dependent factor: LV volume) and Tukey's *post hoc* test were carried out for multiple comparisons. *P*-value for both factors (DM and LV volume) and interaction *P*-value are shown in the Supplementary Material, Table S1. In the case of PCR array gene expression, the *P*-value was calculated based on a Student's *t*-test of the replicate 2(-Delta Ct) values for each gene in the experimental groups. A *P*-value <0.05 was considered statistically significant.

Table 1: Haemodynamic parameters in non-diabetic and diabetic donors

	Non-diabetic	Diabetic	*P*-value
Heart rate (bpm)	383 ± 37	277 ± 36*	<0.0001
Systolic blood pressure (mmHg)	124 ± 15	104 ± 19*	0.017
Diastolic blood pressure (mmHg)	103 ± 13	81 ± 20*	0.009
Mean arterial pressure (mmHg)	110 ± 13	89 ± 20*	0.010
LV end-systolic pressure (mmHg)	114 ± 14	93 ± 18*	0.009
dP/dt_{max} (mmHg/s)	6300 ± 1210	5312 ± 973*	0.049
dP/dt_{min} (mmHg/s)	-10 379 ± 2262	-5700 ± 1815*	<0.0001
Tau (Weiss) (ms)	8.9 ± 1.2	14.5 ± 2.64*	<0.0001
Tau (Glantz) (ms)	11.0 ± 1.4	17.6 ± 3.9*	<0.0001
E_{es} (ESPVR) (mmHg/µl)	1.44 ± 0.45	0.64 ± 0.26*	<0.0001
Maximal elastance (mmHg/µl)	7.4 ± 2.6	3.2 ± 1.6*	0.0003
LV end-diastolic pressure (mmHg)	4.66 ± 0.78	5.07 ± 1.61	0.491
Slope of EDPVR (mmHg/µl)	0.034 ± 0.015	0.052 ± 0.020*	0.0497

Data are expressed as mean ± standard deviation.

*P-value <0.05 vs non-diabetic donors.

dP/dt_{max}: maximal slope of systolic pressure increment; dP/dt_{min}: maximal slope of diastolic pressure decrement; EDPVR: end-diastolic pressure–volume relationship; ESPVR: end-systolic pressure–volume relationship; E_{es} and E_{max}: the slopes of the LV end-systolic pressure–volume relationship; LV: left ventricular; Tau: time constant of the LV pressure decay.

RESULTS

Effects of Type 1 diabetes mellitus on the donor hearts

Biochemical parameters. Although the blood glucose concentration levels were identical at T0, 8 weeks after the confirmation of diabetes, significantly increased plasma glucose (18.9 ± 2.9 vs 9.5 ± 0.9 mmol/l, *P* < 0.05) and HbA1c-levels (13.6 ± 1.5 vs 7.6 ± 0.1%, *P* < 0.05) were observed in diabetic rats compared to controls.

Haemodynamic parameters. Heart rate, systolic blood pressure, diastolic blood pressure and mean arterial pressure were significantly lower in the diabetic donors when compared to the control group (Table 1). DM was associated with a significant decrease in indexes of load-dependent (decreased LV end-systolic pressure and dP/dt_{max}) and load-independent parameters (the slopes E_{es} and E_{max} of the ESPVR, preload recruitable stroke work, dP/dt_{max}/end-diastolic volume and maximal elastance) of LV contractile function and with impaired ventricular relaxation (decreased dP/dt_{min} and prolonged Tau) (Table 1 and Fig. 1). The slope of the end-diastolic pressure–volume relationship was significantly increased in the diabetic donors compared to controls, which indicates increased stiffness of the left ventricle, whereas LV end-diastolic pressure showed a tendency towards impaired values (Table 1).

ECG parameters. Decreased heart rate (239 ± 43 vs 388 ± 27 bpm, *P* < 0.05), increased QT interval (80 ± 12 vs 52 ± 3 ms, *P* < 0.05) and corrected QT intervals for heart rate (80 ± 16 vs 40 ± 3 ms, *P* < 0.05) were observed (Supplementary Material, Fig. S2). The PR interval and QRS complex duration were unchanged in the diabetic and control donors (data not shown).

Effects of Type 1 diabetes mellitus on the transplanted heart

Heart weight, heart weight to body weight ratio and rebeating time after transplantation. Lower heart weight (0.874 ± 0.053 vs 1.124 ± 0.037 g, *P* < 0.05) and higher heart weight to body weight ratios (3.940 ± 0.136 vs 2.830 ± 0.057 g/kg, *P* < 0.05) were observed in the diabetic rats compared to controls.

Heart function after transplantation. After transplantation, lower LV systolic pressure, developed pressure, dP/dt_{max}, dP/dt_{min} and prolonged Tau were observed in the diabetic group when compared with the control group, indicating decreased myocardial contractility and relaxation (Fig. 2). The mean heart rates showed a tendency towards decreased values in the diabetic rats compared to controls (109 ± 16 vs 143 ± 12 bpm, *P* > 0.05).

Myocardial histological examination after transplantation. After transplantation, histological analysis revealed a mild increase in inflammatory cells infiltration and oedema in diabetic donor hearts when compared with the controls (Fig. 3).

Myocardial expression of 84 genes involved in the phosphatidylinositol-3-kinase/Akt-signalling pathway

Diabetic donor heart versus non-diabetic donor heart. To determine the effects of DM-induced cardiac damage, 84 genes in the donor heart were surveyed (see Supplementary Material, Table S2 for genes abbreviation). A volcano plot was generated to visualize the statistical significance of gene expression changes between diabetic and non-diabetic donors (Fig. 4A) and the clustergrams coregulated genes (Fig. 4B). Among 84 genes, 15 genes were significantly down-regulated (Figs 4 and 6). These genes correspond to Akt and PI3K family members and their regulators (*Grb2*, *Hspb1* and *Prkcz*), inactivation of glycogen synthase kinase (Gsk)-3 and the accumulation of β-Catenin (*ccnd1*, *Ctnnb1*, *Nfkb1*, *Tirap* and *Tollip*), PI3K subunit p85 genes and regulation of actin organization and cell migration (*Pak1*, *Pdgfra* and *Wasl*), Bcl-2-associated death promoter phosphorylation and anti-apoptotic pathways (*Ywhah*, *Grb2* and *Rps6ka1*) and genes involved in the mTOR signalling (*Tsc2*).

Diabetic graft versus non-diabetic graft after transplantation. To determine the effects of both DM and heart transplantation, 84 genes were surveyed in the graft. Eleven genes were significantly down-regulated corresponding to Akt and PI3K family members and their regulator (*Akt2* and *Prkcz*), IGF-1-signalling pathway (*Jun* oncogene), inactivation of Gsk3 and accumulation of β-catenin (*ccnd1*, *Adar* and *Tlr4*), PI3K subunit p85 genes and regulation of actin organization and cell migration (*Pak1*, *Pdgfra*), Bcl-2-associated death promoter phosphorylation and antiapoptotic pathways (*Irs1* and *Ywhah*) and Ldha (Figs 5A, B and 6).

For further secondary validation of gene profiling, we carried out the Western blot analysis for the Akt. The obtained data were consistent to that observed by gene expression analysis.
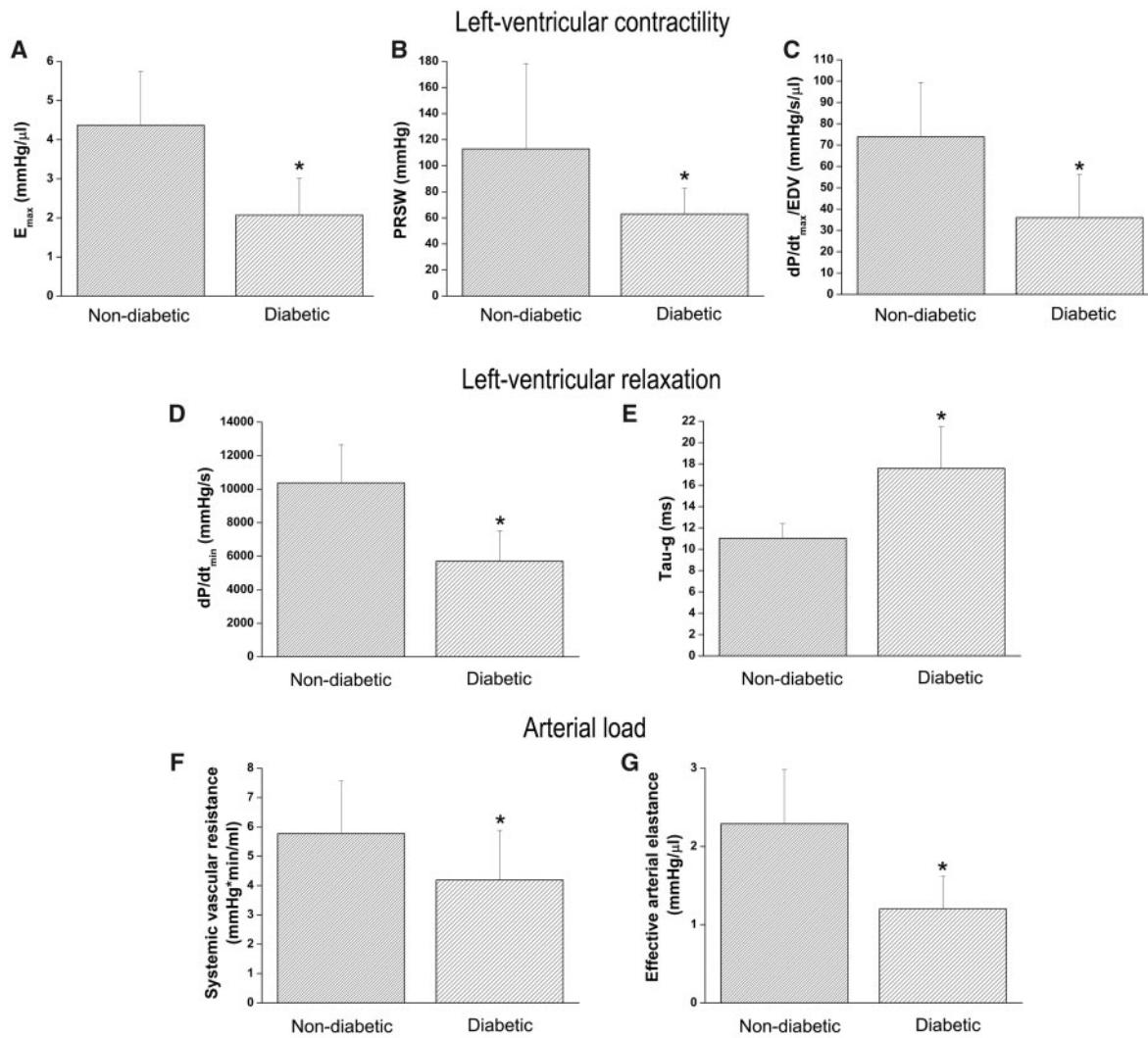
Figure 1: Effects of diabetes mellitus on left ventricular (LV) contractility, relaxation and arterial load. (**A**) Slope (E_{max}) of the LV end-systolic pressure–volume relationships, (**B**) PRSW, (**C**) dP/dt_{max}/EDV, (**D**) dP/dt_{min}, (**E**) Tau, (**F**) systemic vascular resistance and (**G**) effective arterial elastance. Data are presented as mean ± standard deviation, and $n = 9$–11 rats/group. *P-value <0.05 vs non-diabetic. dP/dt_{max}: maximal slope of systolic pressure increment; dP/dt_{min}: maximal slope of diastolic pressure decrement; EDV: end-diastolic volume; PRSW: preload recruitable stroke work; Tau: time constant of LV pressure decay.

After transplantation, the diabetic + transplanted group showed a significant decrease in the phosphorylation of Akt compared with the control + transplanted group (Fig. 5C).

After transplantation versus before transplantation. In non-diabetic hearts (Control + Htx versus Control), among the tested genes, 6 genes were significantly up-regulated, and 1 gene was down-regulated, corresponding to the inactivation of Gsk3 and the accumulation of β-catenin (*Cd14, Myd88* and *Tirap*), IGF-1 signalling (*Fos* and *jun*), Akt and PI3K family members and their regulators (*Hspb1*) and *Nfkbia* (Fig. 6).

In diabetic heart (DM + Htx versus DM), among the tested genes, 5 genes were overexpressed and 2 genes were underexpressed involved in Akt and PI3K family members and their regulators (*Mtcp1, Hspb1*), inactivation of Gsk3 and the accumulation of β-catenin (*Tlr4* and *Myd88*), IGF-1-signalling (*fos* and *jun*) and *Nfkbia*, without achieving a statistically significant difference (Fig. 6).

DISCUSSION

In the present investigation, we hypothesized that PI3K/Akt-pathway alterations may be one of the underlying pathomechanisms involved in graft dysfunction after transplantation when diabetic donors are used, as the components of PI3K/Akt signalling have been found to be disadvantageous in diabetic hearts submitted to regional myocardial IRI. To the best of our knowledge, this is the first study suggesting that the impairment of the PI3K/Akt signalling may, at least in part, be a key mechanism responsible for the increased post-transplant myocardial injury when hearts from severe diabetic donors were used.

The availability of donor hearts has always been a key limiting factor in heart transplantation. Recently, a clinical study has shown that cardiac transplantation can be safely performed with carefully selected diabetic donors [2]. However, the pathomechanisms of potential heart grafts from diabetic donors in cardiac transplantation have not been completely elucidated. Streptozotocin enters the pancreatic β-cell through GLUT-2,
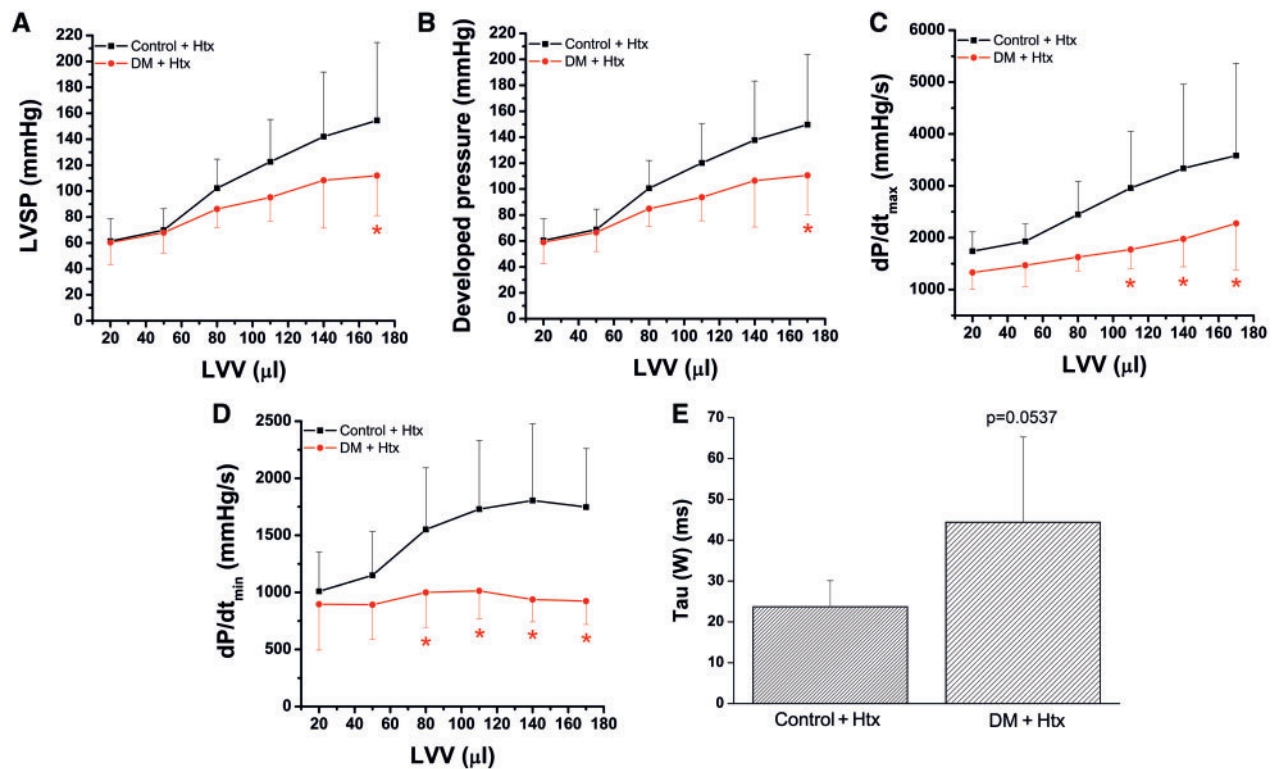
Figure 2: Effect of DM on donor heart function after transplantation. (**A**) LVSP, (**B**) developed pressure, (**C**) dP/dt_{max}, (**D**) dP/dt_{min} and (**E**) Tau at an intraventricular volume of 80 µl. Data are presented as mean ± standard deviation, and $n = 9$–11 rats/group. *P-value <0.05 vs control + Htx. DM: diabetes mellitus; dP/dt_{max}: maximal slope of systolic pressure increment; dP/dt_{min}: maximal slope of diastolic pressure decrement; Htx: heart transplantation; LVSP: left ventricular systolic pressure; LVV: left-ventricular volume.

where it causes DNA alkylation. Activation of poly(ADP-ribose)polymerase leads to subsequent cell death. In the present study, DM has been shown to have a significant impact on the haemodynamic situation as previously described [16]. It has been shown that heart rates in diabetic rats were significantly lower at 12, 24 and 48 weeks after streptozotocin administration. Blood pressure was the lowest at 12-week postinduction and returned towards control levels at 48 weeks, when compared to age-matched controls [17]. Possible explanations for this bradycardia include metabolic disorders. While hypertension is frequently observed in humans with long-standing diabetes, early hypotension in streptozotocin diabetic rats has been documented. Insulin treatment in diabetic animals reversed the hypotension, bradycardia and altered baroreflex sensitivity that was observed in 12-week diabetic rats [17]. Our investigation shows that diabetic cardiomyopathy is characterized by a decreased systolic performance accompanied by impaired LV active relaxation. Glycosuria and osmotic fluid loss may result from elevated plasma glucose and may eventually lead to hypovolaemia. Hypovolaemia is a common cause of hypotension (evidenced in the present study by decreased mean arterial pressure in diabetic animals), and it may also have an additional vascular origin through excessive systemic vasodilation (shown in our study by decreased systemic vascular resistance in diabetic donors). In line with these observations, effective arterial elastance, an index of arterial vascular load imposed by the systemic arterial system, was decreased in diabetic animals compared to controls. Altogether, in the present study, the arterial load, assessed by the systemic vascular

resistance (the steady component of arterial load), and the effective arterial elastance were decreased in the diabetic donors.

In heart transplantation, a fast recovery of myocardial function is essential for the success of cardiac transplantation, and it is an important determinant of long-term outcome. Profound haemodynamic changes occur during the early phase after transplantation. Therefore, we focused our investigations on the early phase of reperfusion. Our data demonstrated that after transplantation, an impaired myocardial contractility and altered relaxation were observed in the diabetic group compared to controls, i.e. severe DM-induced cardiac dysfunction in donors is further impaired after transplantation. Studies on animal models have demonstrated that the PI3K/Akt signalling is dysregulated in the diabetic myocardium. Its activation would protect the heart from IRI [9]. Therefore, we investigated the involvement of the PI3K/Akt pathway in the diabetic ischaemic/reperfused hearts. We profiled the expression of 84 genes relevant to PI3K/Akt signalling. We showed that the down-regulation of *ccnd1*, *ctnnb1*, *Grb2*, *Nfkb1*, *Pak1*, *Pdgfra*, *Prkcz*, *Rps6ka1*, *Tollip*, *Tsc2*, *Wasl* and *Ywhah* was related to DM, because their gene expression changes were independent from the effect of IRI. The gene expression of *Fos*, *Hspb1*, *Jun*, *Myd88* and *Nfkbia* were, however, affected only by IRI, independent of DM. The additive effect of both DM and ischaemia/reperfusion was enough to alter the gene expression of *Adar*, *Akt2*, *Irs1*, *Tlr4* and *Ldha*. Additionally, the expression of *Jun* was significantly up-regulated in the control + transplanted group compared with the control group (9-fold) but only 4-fold (without reaching the statistical significance) in the
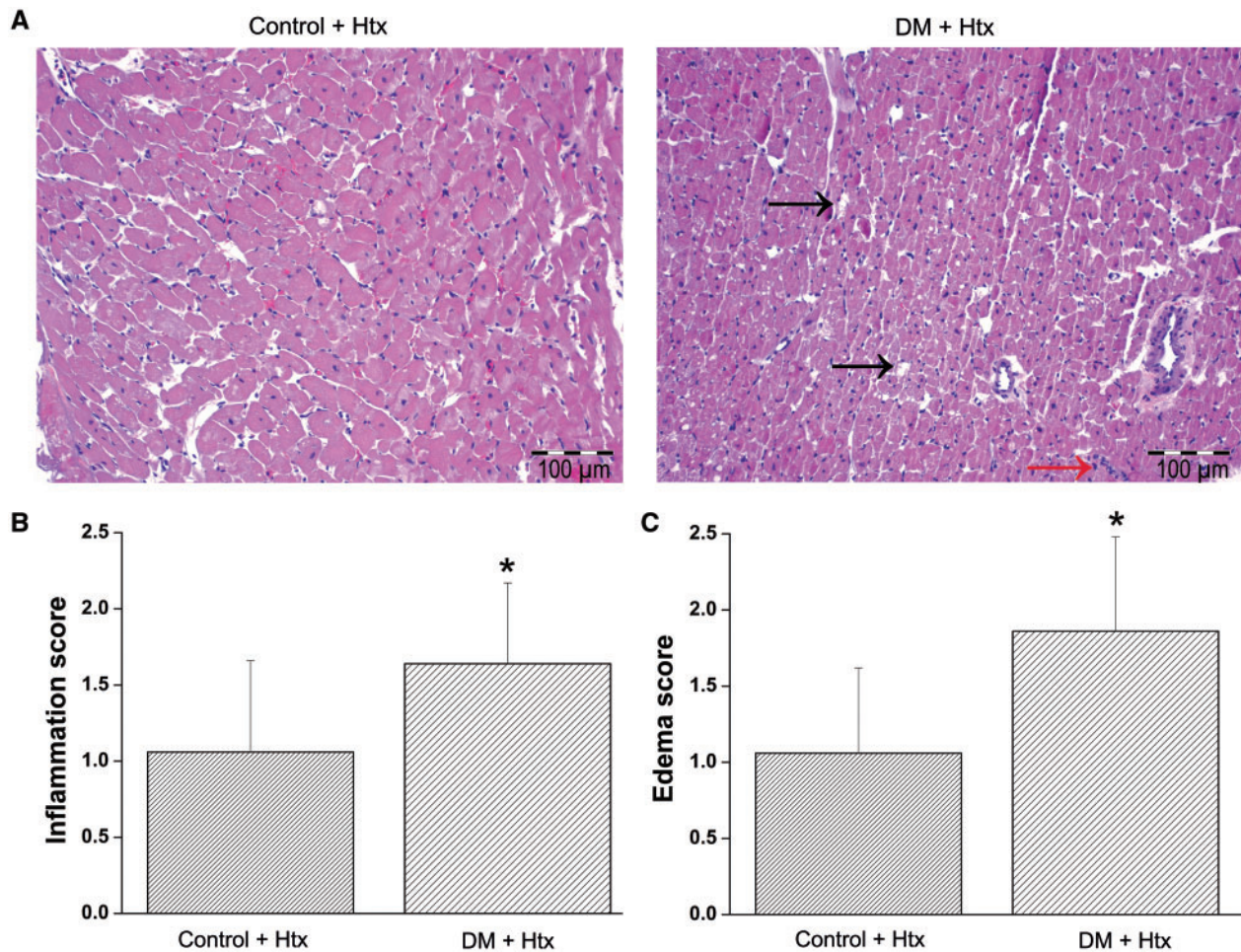
Figure 3: Myocardial histopathological examination after Htx. (**A**) Representative photomicrographs after haematoxylin and eosin staining (magnification ×100; scale bar = 100 μm). Black arrows indicate oedema and red arrow indicates site of inflammation (not all are marked). Myocardial histological score of (**B**) inflammation and (**C**) oedema. Data are presented as mean ± standard deviation, and *n* = 9–11 rats/group. *P-value <0.05 vs control + Htx. DM: diabetes mellitus; Htx: heart transplantation.

DM + transplanted group compared to the DM group. DM showed no effect on its gene expression. Our results suggest that alterations of *jun* gene expression was the effect of IRI alone. We did see a significant down-regulation of the *jun* gene expression (2-fold) in the DM + transplanted group compared to the control + transplanted group. Although at first glance, it seems that *jun* expression was only affected following IRI, and diabetes had no effect on its expression, it appears that DM had an invisible effect on the tested genes. This became evident after an additional second injury. GSK-3β has a powerful antihypertrophic effect. Its overexpression leads to attenuation of cardiac hypertrophy [18], whereas its inhibition leads to augmented hypertrophy in response to hypertrophic stimuli [19]. β-Catenin levels are increased in cardiomyocytes subjected to hypertrophic stimuli [20]. In the present study, the heart weight to body weight ratio, used as an index of myocardial mass, is increased in the diabetic animals compared to controls. Therefore, we are tempted to speculate that the decreased mRNA levels of components of the β-catenin/GSK3β-signalling (*Adar* and *Tlr4*) in the transplanted diabetic hearts could be due to a compensatory mechanism that acts to prevent the development of cardiac hypertrophy. Akt has been shown to protect against IRI in mouse hearts [9]. The loss of

cardiac insulin receptor substrates Irs-1 and Irs-2 may serve as a key mechanism for the induction of heart failure [21]. Our PCR array results revealed a down-regulation of *Irs-1* and *Akt* gene expression in the transplanted diabetic hearts. Furthermore, Irs-1 plays a critical role in the anti-apoptotic function of IGF-1 [22]. In the present study, we showed a down-regulation of the mRNA expression of genes involved in anti-apoptosis, including *Irs-1*.

From the clinical point of view, based on rapidly increasing numbers of diabetic patients plus a rising need for heart donors, our study aimed to further elucidate the feasibility of 'marginal donor' usage and to obtain further knowledge regarding the possible 'druggable' mechanisms to ameliorate graft function.

Limitations

First, our results suggest, without providing proof-of-concept, that the down-regulation of genes, including *Adar, Akt2, Irs1, Tlr4* and *Ldha*, and a decrease in phosphorylated Akt/total Akt protein expression ratio may be a possible mechanism for the increased post-transplant myocardial injury when hearts from severe diabetic donors were used. Second, one of the disadvantages with streptozotocin-induced diabetes is that the chemical can be
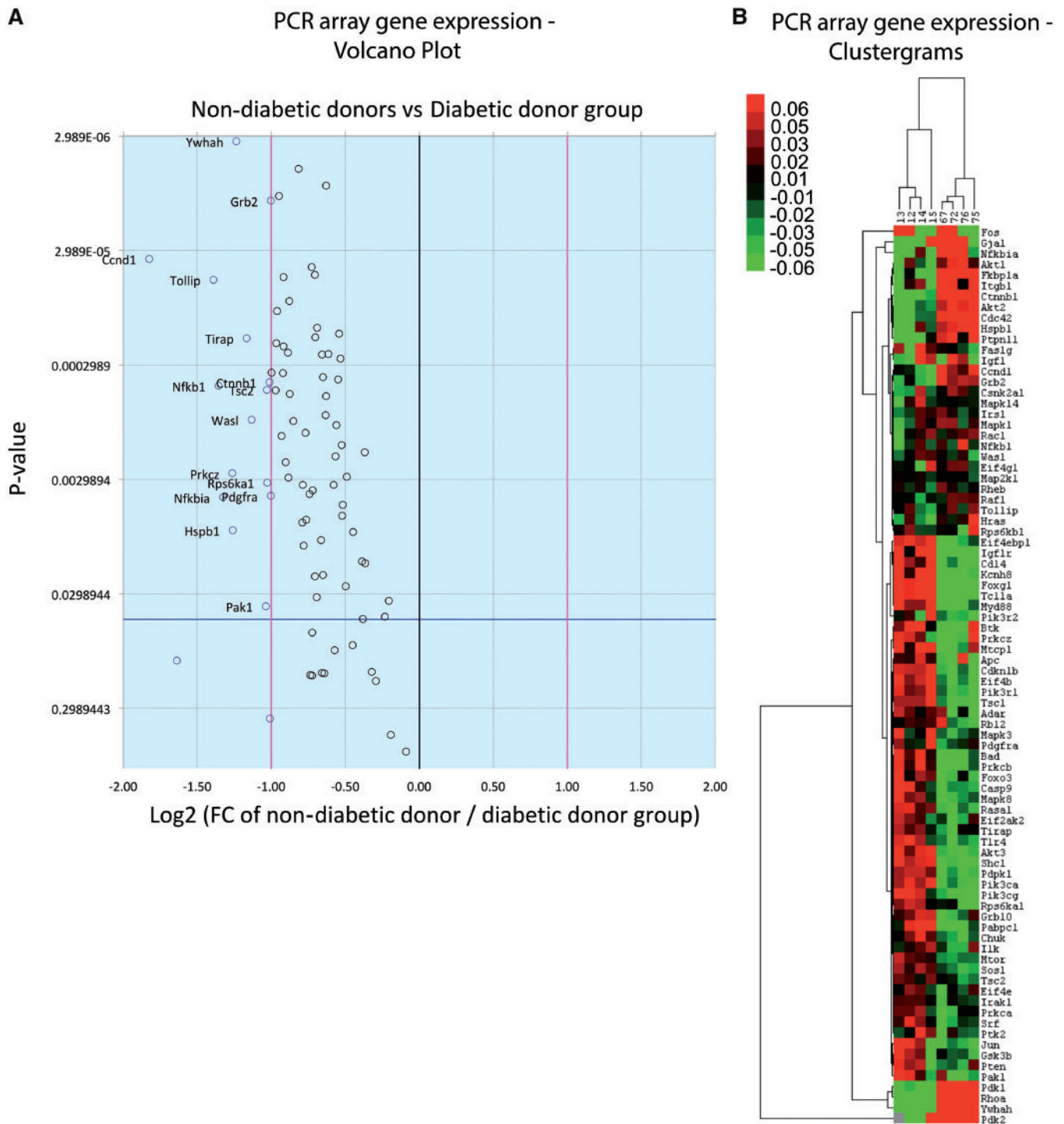
Figure 4: Effects of diabetes mellitus on myocardial expression of phosphatidylinositol-3-kinase (PI3K)/Akt-related genes. The expression of 84 genes involved in the PI3K/Akt signalling has been profiled in non-diabetic and donor hearts. (**A**) Volcano plot indicates the statistical significance of gene expression changes. The *x*-axis denotes the Log_2 of the FC for diabetic and non-diabetic donors, whereas the *y*-axis plots their *P*-value. (**B**) Clustergrams create a heat map with dendrograms to indicate which genes are coregulated. Degrees of red and green indicate relatively high expression and low expression of the corresponding gene, respectively, and black squares denote genes that are equally expressed. The *x*-axis indicates the rat's number and the *y*-axis indicates the genes. *n* = 4 rats/group. FC: fold changes.

toxic. A previous study showed that myocardial alterations after streptozotocin were not the result of direct cardiotoxic effects but a consequence of the drug-induced diabetic state [23]. Third, 8 weeks of DM duration does not mimic the changes that occur

chronically, and cardiac damage due to DM could have been be underestimated. Fourth, in a clinical scenario, the use of hearts from diabetic donors are determined after a careful selection from controlled diabetic patients, whereas in the present study,
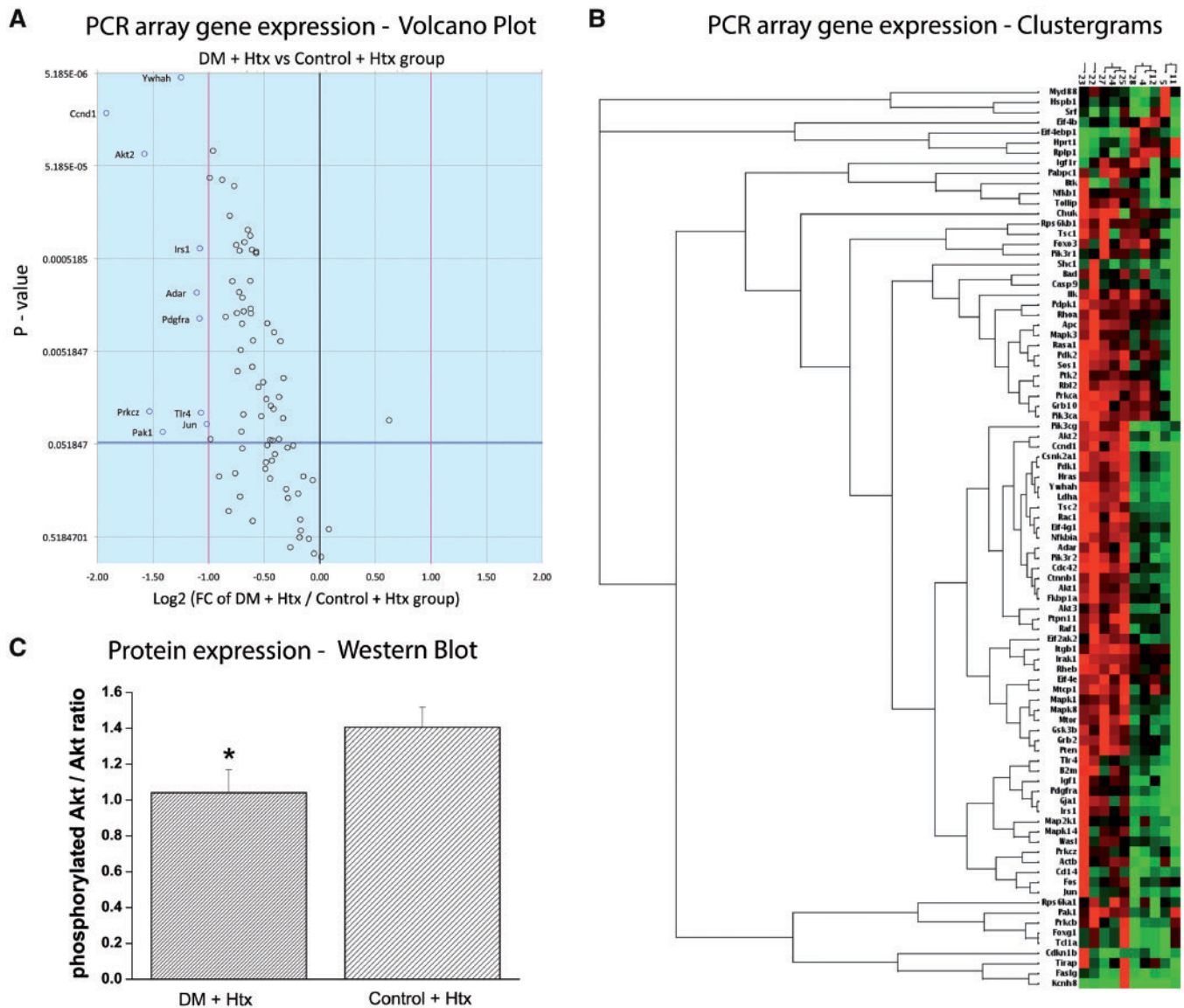
Figure 5: Myocardial expression of phosphatidylinositol-3-kinase (PI3K)/Akt-related genes and protein expression of phosphorylated Akt/total Akt ratio after Htx. (**A**, **B**) The expression of 84 genes involved in the PI3K/Akt signalling has been profiled after transplantation (Fig. 4). *n* = 5 rats/group. Immunoblot analysis for (**C**) phosphorylated Akt and total Akt protein band densities in the myocardium. Glyceraldehyde-3-phosphate-dehydrogenase, housekeeping protein, was used as a loading control and for protein normalization. Data are presented as mean ± standard deviation, and *n* = 9 rats/group. *P-value <0.05 vs control + Htx. DM: diabetes mellitus; Htx: heart transplantation.

DM was left untreated. Additionally, coronary angiography in potential diabetic cardiac donors should be considered to screen for coronary stenosis before heart transplantation. Although heart disease due to diabetes is mainly due to macrovascular damage, microvascular abnormalities (affecting small coronary vessels) also play a role in altering cardiac function and structure [24]. Finally, the possible adverse effects of immune cell activation that can be triggered by heart transplantations were not examined.

CONCLUSION

In summary, this study demonstrated that untreated DM-induced cardiac dysfunction is further impaired after heart transplantation. Alterations in the protective prosurvival PI3K/Akt pathway of the transplanted diabetic heart may be one of the mechanisms responsible.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *ICVTS* online.

ACKNOWLEDGMENTS

DM vs Control

Gene Symbol	P-Value	Fold-Regulation	Comments
Adar	0.003	-1.72	
Akt1	0.001	-1.83	
Akt2	0.000	-2.00	
Akt3	0.005	-1.43	
Apc	0.011	-1.72	
Bad	0.003	-1.49	
Btk	0.153	-1.67	B
Casp9	0.002	-1.87	
Ccnd1	0.000	-3.54	
Cd14	0.093	-1.49	
Cdc42	0.000	-1.53	
Cdkn1b	0.050	-1.30	
Chuk	0.001	-1.55	
Csnk2a1	0.001	-1.70	
Ctnnb1	0.000	-2.02	
Eif2ak2	0.000	-1.76	
Eif4b	0.047	-1.17	
Eif4e	0.000	-1.46	
Eif4ebp1	0.715	-1.06	
Eif4g1	0.003	-1.84	
Faslg	0.114	-3.11	A
Fkbp1a	0.000	-1.85	
Fos	0.367	-2.01	
Foxg1	0.009	-1.36	C
Foxo3	0.026	-1.41	
Gja1	0.032	-1.62	
Grb10	0.007	-1.70	
Grb2	0.000	-2.00	
Gsk3b	0.004	-1.67	
Hras	0.000	-1.93	
Hspb1	0.008	-2.39	
Igf1	0.065	-1.65	
Igf1r	0.509	-1.14	
Ilk	0.000	-1.58	
Irak1	0.000	-1.84	
Irs1	0.000	-1.96	
Itgb1	0.001	-1.47	
Jun	0.173	-1.23	
Kcnh8	0.155	-1.65	B
Map2k1	0.000	-1.89	
Mapk1	0.000	-1.46	
Mapk14	0.001	-1.91	
Mapk3	0.010	-1.58	
Mapk8	0.000	-1.63	
Mtcp1	0.147	-1.58	A
Mtor	0.000	-1.65	
Myd88	0.143	-1.25	
Nfkb1	0.000	-2.56	
Nfkbia	0.004	-2.51	
Pabpc1	0.034	-1.15	
Pak1	0.038	-2.05	B
Pdgfra	0.004	-2.00	
Pdk1	0.000	-1.89	
Pdk2	0.006	-1.44	
Pdpk1	0.000	-1.45	
Pik3ca	0.016	-1.31	
Pik3cg	0.084	-1.37	
Pik3r1	0.016	-1.29	
Pik3r2	0.148	-1.56	
Prkca	0.000	-1.95	
Prkcb	0.021	-1.63	
Prkcz	0.003	-2.40	B
Pten	0.002	-1.48	
Ptk2	0.007	-1.73	
Ptpn11	0.000	-1.89	
Rac1	0.001	-1.55	
Raf1	0.000	-1.61	
Rasa1	0.001	-1.44	
Rbl2	0.001	-1.80	
Rheb	0.000	-1.63	
Rhoa	0.000	-1.55	
Rps6ka1	0.003	-2.04	A
Rps6kb1	0.000	-1.95	
Shc1	0.002	-1.29	
Sos1	0.000	-1.57	
Srf	0.003	-1.40	
Tcl1a	0.009	-1.36	C
Tirap	0.000	-2.24	
Tlr4	0.004	-1.65	
Tollip	0.000	-2.62	
Tsc1	0.021	-1.57	
Tsc2	0.000	-2.04	
Wasl	0.001	-2.19	
Ywhah	0.000	-2.35	

DM + Htx vs Control + Htx

Gene Symbol	P-Value	Fold-Regulation	Comments
Adar	0.001	-2.15	C
Akt1	0.002	-1.54	C
Akt2	0.000	-2.98	C
Akt3	0.004	-1.28	C
Apc	0.002	-1.68	C
Bad	0.082	-1.40	C
Btk	0.350	-1.52	C
Casp9	0.079	-1.35	C
Ccnd1	0.000	-3.79	C
Cd14	0.058	-1.62	C
Cdc42	0.000	-1.54	C
Cdkn1b	0.546	-1.07	C
Chuk	0.338	-1.13	C
Csnk2a1	0.000	-1.84	C
Ctnnb1	0.000	-1.75	C
Eif2ak2	0.002	-1.80	C
Eif4b	0.431	1.06	C
Eif4e	0.016	-1.29	C
Eif4ebp1	0.029	1.54	C
Eif4g1	0.001	-1.65	C
Faslg	0.107	-1.69	C
Fkbp1a	0.001	-1.54	C
Fos	0.116	-1.87	C
Foxg1	0.067	-1.32	C
Foxo3	0.525	-1.13	C
Gja1	0.001	-1.73	C
Grb10	0.054	-1.39	C
Grb2	0.003	-1.33	C
Gsk3b	0.002	-1.61	C
Hras	0.000	-1.71	C
Hspb1	0.676	-1.20	C
Igf1	0.003	-1.39	C
Igf1r	0.784	-1.04	C
Ilk	0.178	-1.14	C
Irak1	0.008	-1.52	C
Irs1	0.000	-2.11	C
Itgb1	0.027	-1.26	C
Jun	0.031	-2.02	C
Kcnh8	0.273	-1.77	C
Map2k1	0.047	-1.36	C
Mapk1	0.004	-1.52	C
Mapk14	0.005	-1.64	C
Mapk3	0.011	-1.42	C
Mapk8	0.000	-1.49	C
Mtcp1	0.025	-1.61	C
Mtor	0.000	-1.65	C
Myd88	0.443	-1.13	C
Nfkb1	0.048	-1.34	C
Nfkbia	0.000	-1.57	C
Pabpc1	0.127	-1.04	C
Pak1	0.038	-2.66	C
Pdgfra	0.002	-2.12	C
Pdk1	0.000	-1.68	C
Pdk2	0.020	-1.36	C
Pdpk1	0.159	-1.23	C
Pik3ca	0.057	-1.22	C
Pik3cg	0.000	-1.99	C
Pik3r1	0.122	-1.36	C
Pik3r2	0.000	-1.60	C
Prkca	0.096	-1.41	C
Prkcb	0.046	-1.98	C
Prkcz	0.023	-2.89	C
Pten	0.000	-1.49	C
Ptk2	0.013	-1.47	C
Ptpn11	0.001	-1.62	C
Rac1	0.000	-1.53	C
Raf1	0.002	-1.54	C
Rasa1	0.017	-1.39	C
Rbl2	0.046	-1.29	C
Rheb	0.010	-1.25	C
Rhoa	0.054	-1.18	C
Rps6ka1	0.038	-1.63	C
Rps6kb1	0.026	-1.44	C
Shc1	0.116	-1.11	C
Sos1	0.003	-1.62	C
Srf	0.857	1.01	C
Tcl1a	0.067	-1.32	C
Tirap	0.192	-1.64	C
Tlr4	0.024	-2.10	C
Tollip	0.009	-1.67	C
Tsc1	0.199	-1.22	C
Tsc2	0.000	-1.95	C
Wasl	0.022	-1.34	C
Ywhah	0.000	-2.38	C

Control + Htx vs Control

Gene Symbol	P-Value	Fold-Regulation	Comments
Adar	0.175	1.15	C
Akt1	0.340	-1.07	C
Akt2	0.001	1.43	C
Akt3	0.502	1.05	C
Apc	0.277	1.08	C
Bad	0.412	-1.10	C
Btk	0.694	-1.06	C
Casp9	0.006	-1.64	C
Ccnd1	0.418	-1.09	C
Cd14	0.022	2.35	C
Cdc42	0.730	1.02	C
Cdkn1b	0.684	1.04	C
Chuk	0.561	-1.23	C
Csnk2a1	0.754	-1.01	C
Ctnnb1	0.452	-1.06	C
Eif2ak2	0.523	1.05	C
Eif4b	0.027	1.12	C
Eif4e	0.001	1.24	C
Eif4ebp1	0.346	-1.08	C
Eif4g1	0.034	1.22	C
Faslg	0.303	-1.59	C
Fkbp1a	0.042	1.14	C
Fos	0.001	40.76	C
Foxg1	0.477	1.08	C
Foxo3	0.097	1.26	C
Gja1	0.244	-1.16	C
Grb10	0.220	1.12	C
Grb2	0.068	-1.10	C
Gsk3b	0.754	-1.03	C
Hras	0.001	-1.23	C
Hspb1	0.000	3.76	C
Igf1	0.012	-1.86	C
Igf1r	0.684	1.13	C
Ilk	0.129	-1.09	C
Irak1	0.432	1.03	C
Irs1	0.189	-1.17	C
Itgb1	0.008	1.24	C
Jun	0.003	9.32	C
Kcnh8	0.583	1.19	C
Map2k1	0.162	-1.15	C
Mapk1	0.621	1.03	C
Mapk14	0.323	-1.15	C
Mapk3	0.274	-1.12	C
Mapk8	0.091	1.12	C
Mtcp1	0.018	-1.74	C
Mtor	0.217	1.07	C
Myd88	0.000	3.55	C
Nfkb1	0.185	-1.14	C
Nfkbia	0.000	2.45	C
Pabpc1	0.161	1.08	C
Pak1	0.581	-1.07	C
Pdgfra	0.592	1.09	C
Pdk1	0.059	-1.12	C
Pdk2	0.105	-1.11	C
Pdpk1	0.592	-1.02	C
Pik3ca	0.099	-1.07	C
Pik3cg	0.620	-1.04	C
Pik3r1	0.874	-1.03	C
Pik3r2	0.816	1.10	C
Prkca	0.769	1.02	C
Prkcb	0.634	-1.12	C
Prkcz	0.149	-1.36	C
Pten	0.001	1.44	C
Ptk2	0.043	-1.30	C
Ptpn11	0.060	1.18	C
Rac1	0.990	1.00	C
Raf1	0.028	1.18	C
Rasa1	0.488	1.04	C
Rbl2	0.001	-1.25	C
Rheb	0.002	1.21	C
Rhoa	0.004	1.15	C
Rps6ka1	0.006	-1.51	C
Rps6kb1	0.097	-1.15	C
Shc1	0.723	1.02	C
Sos1	0.021	-1.20	C
Srf	0.001	1.26	C
Tcl1a	0.477	1.08	C
Tirap	0.003	-2.24	C
Tlr4	0.004	-1.91	C
Tollip	0.083	-1.17	C
Tsc1	0.162	-1.21	C
Tsc2	0.037	-1.19	C
Wasl	0.170	-1.17	C
Ywhah	0.282	1.06	C

DM + Htx vs DM

Gene Symbol	P-Value	Fold-Regulation	Comments
Adar	0.251	-1.27	C
Akt1	0.742	-1.05	C
Akt2	0.662	-1.22	C
Akt3	0.873	1.01	C
Apc	0.733	-1.06	C
Bad	0.290	-1.21	C
Btk	0.979	-1.14	C
Casp9	0.027	-1.38	C
Ccnd1	0.075	-1.36	C
Cd14	0.012	1.84	C
Cdc42	0.132	-1.15	C
Cdkn1b	0.495	1.08	C
Chuk	0.625	-1.05	C
Csnk2a1	0.054	-1.28	C
Ctnnb1	0.487	-1.08	C
Eif2ak2	0.470	-1.14	C
Eif4b	0.072	1.19	C
Eif4e	0.080	1.20	C
Eif4ebp1	0.201	1.30	C
Eif4g1	0.218	1.17	C
Faslg	0.899	-1.01	C
Fkbp1a	0.133	1.16	C
Fos	0.009	37.51	C
Foxg1	0.851	-1.05	C
Foxo3	0.107	1.34	C
Gja1	0.036	-1.44	C
Grb10	0.367	1.18	C
Grb2	0.070	1.17	C
Gsk3b	0.273	-1.15	C
Hras	0.008	-1.27	C
Hspb1	0.012	6.41	C
Igf1	0.006	-1.82	C
Igf1r	0.666	1.06	C
Ilk	0.358	1.08	C
Irak1	0.592	1.06	C
Irs1	0.007	-1.47	C
Itgb1	0.048	1.24	C
Jun	0.002	4.83	C
Kcnh8	0.851	-1.05	C
Map2k1	0.707	1.04	C
Mapk1	0.171	-1.17	C
Mapk14	0.101	-1.15	C
Mapk3	0.143	-1.18	C
Mapk8	0.495	-1.05	C
Mtcp1	0.040	-2.07	C
Mtor	0.437	-1.09	C
Myd88	0.001	3.37	C
Nfkb1	0.076	1.43	C
Nfkbia	0.000	3.36	C
Pabpc1	0.461	1.03	C
Pak1	0.636	-1.63	C
Pdgfra	0.527	-1.13	C
Pdk1	0.186	-1.17	C
Pdk2	0.144	-1.22	C
Pdpk1	0.958	-1.02	C
Pik3ca	0.156	-1.18	C
Pik3cg	0.008	-1.77	C
Pik3r1	0.180	-1.28	C
Pik3r2	0.489	-1.09	C
Prkca	0.280	1.21	C
Prkcb	0.213	-1.59	C
Prkcz	0.231	-1.92	C
Pten	0.028	1.23	C
Ptk2	0.104	-1.29	C
Ptpn11	0.171	1.18	C
Rac1	0.049	-1.15	C
Raf1	0.497	1.06	C
Rasa1	0.554	-1.09	C
Rbl2	0.791	-1.04	C
Rheb	0.008	1.35	C
Rhoa	0.008	1.29	C
Rps6ka1	0.208	-1.41	C
Rps6kb1	0.822	1.01	C
Shc1	0.751	1.02	C
Sos1	0.012	-1.45	C
Srf	0.002	1.53	C
Tcl1a	0.851	-1.05	C
Tirap	0.057	-1.92	C
Tlr4	0.001	-2.84	C
Tollip	0.391	1.14	C
Tsc1	0.581	-1.10	C
Tsc2	0.025	-1.33	C
Wasl	0.159	1.20	C
Ywhah	0.310	-1.12	C

Figure 6: Fold-regulation and associated *P*-value of all tested genes. For all significantly altered genes, fold-regulation values are presented in red. See Supplementary Material, Table S2, for abbreviation of genes and further details. DM: diabetes mellitus; Htx: heart transplantation.

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