

Islet microangiopathy and augmented β -cell loss in Japanese non-obese type 2 diabetes patients who died of acute myocardial infarction

Kazuhiisa Takahashi^{1,2}, Hiroki Mizukami^{1*} , Sho Osonoi^{1,2}, Yuki Takeuchi^{1,2}, Kazuhiro Kudoh¹, Takanori Sasaki¹, Makoto Daimon², Soroku Yagihashi¹ 

¹Department of Pathology and Molecular Medicine, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori, Japan, and ²Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori, Japan

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*Correspondence

Hiroki Mizukami
Tel.: +81-172-39-5025
Fax: +81-172-39-5026
E-mail address:
hirokim@hirosaki-u.ac.jp

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ABSTRACT

Aims/Introduction: Islets have microvessels that might develop pathological alterations similar to microangiopathy in type 2 diabetes patients. It remains unclear, however, whether the changes correlate with endocrine cell deficits or whether the presence of macroangiopathy influences the islet microvasculature in Japanese type 2 diabetes patients. In this study, we characterized changes of the islet microvessels and endocrine cells in Japanese non-obese patients with type 2 diabetes who died of acute myocardial infarction (AMI).

Materials and Methods: Clinical profiles and islet pathology were examined for 35 diabetes patients who died of AMI (DM + AMI) and 13 diabetes patients who were free from AMI (DM). A total of 13 age-matched, individuals without diabetes who died of AMI and 16 individuals without diabetes who were free from AMI were also studied. Pancreata were subjected to morphometric evaluation of islets, including microvascular alterations of immunostained sections.

Results: Body mass index in DM + AMI was comparable to those in DM. Compared with DM, DM + AMI showed greater glycated hemoglobin levels, higher prevalence of renal failure, hypertension, smaller β -cell volume density and greater amyloid area. DM + AMI showed an increased microvascular area and density compared with other groups. There was a significant increase in vascular basement membrane thickness and loss of pericytes in DM and DM + AMI compared with individuals without diabetes in each group, and the extent of thickening was correlated with the amyloid area and occurrence of β -cell loss in DM + AMI.

Conclusions: Islet microangiopathy was associated with augmented β -cell loss and amyloid deposition in non-obese Japanese type 2 diabetes patients who died of AMI.

INTRODUCTION

Diabetic microangiopathy affects approximately 30% of individuals with type 2 diabetes^{1,2}. They develop under sustained hyperglycemia driven by its downstream cellular effects^{3,4}. Alterations of the microvessels include swelling of endothelial cells, thickening of basement membrane (BM) and degeneration

of pericytes. These changes elicit tissue ischemia with resultant angiogenesis.

Similarly, 20–30% of diabetes patients suffer from macroangiopathy⁵. In fact, diabetes attributes to a high prevalence of cardiovascular morbidity and mortality⁶. The combined effects of insulin resistance, lipidemia, hyperglycemia and associated metabolic abnormalities are postulated to play a role. Patients with diabetes associated microangiopathy are suggested to be at a high risk of accelerated atherosclerosis⁷, whereas recent

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studies showed that macroangiopathy is a risk factor for microangiopathy in type 2 diabetes patients^{8,9}. It is thus likely that microangiopathy and macroangiopathy reciprocally aggravate each other in type 2 diabetes. However, beneficial effects of glucose-lowering interventions on the outcome in diabetes patients with macroangiopathy remain controversial¹⁰.

Islets contain a unique portal system of microvessels branching from nutrient arterioles to draining into the exocrine pancreas^{11,12}. In an obese type 2 diabetes model of the *ob/ob* mouse, islet microvessels showed dilation and a reduction of pericyte lining^{13,14}. In Goto-Kakizaki rats, as shown by a lean type 2 diabetes model, hypercholesterolemia interacts with chronic hyperglycemia to induce islet pre-microangiopathy, which can be associated with a reduction in the β -cell mass¹⁵. In white patients with type 2 diabetes, islet microvessels showed thickened walls similar to those of microangiopathy observed in other tissues¹⁶. The findings might indicate that the vessel changes are central to the islet pathology, but the implication of islet microangiopathy in the progression of the disease and clinical outcome is largely unknown.

In the present study, we aimed to characterize specific changes of the islet microvasculature and their correlation with the β -cell deficit in Japanese non-obese patients with type 2 diabetes. Concurrently, we also addressed the question of whether the macroangiopathy represented by the presence of acute myocardial infarction (AMI) influences the islet vascular changes and endocrine cell populations.

MATERIALS AND METHODS

Human pancreas specimens

We collected pancreatic tissue samples of 16 patients without diabetes (Con), 13 without diabetes but with AMI (Con + AMI), 13 with type 2 diabetes but without AMI (DM), and 35 with type 2 diabetes who had died of AMI (DM + AMI) from the archive files from 2010 to 2018 in the Department of Pathology and Molecular Medicine, Hirosaki University Graduate School of Medicine (Table 1). All the pancreatic tissues were obtained freshly at autopsy carried out within 5 h after death. Cases with other diseases or a history of medications that might affect the data, including liver carcinoma or pancreatic ductal carcinoma, were excluded from the evaluation¹⁷⁻¹⁹. After trimming adipose tissues, the pancreas was weighed and immersed in 10% buffered formalin for 48–72 h.

The use of paraffin blocks and study design were approved by the Ethics Committee of Hirosaki University School of Medicine (date of approval: 20 October 2017, approval number #2017-121), and the study protocol conformed to the provisions of the Declaration of Helsinki.

Clinical information

Clinical records were accessed in each case to determine the main cause of death, last evaluation of bodyweight and height for calculating body mass index (BMI), duration of diabetes (years), time from the onset of AMI to death (days), glycated

hemoglobin (HbA1c) level and treatment for diabetes (Table 1). For each patient, we checked for any history of dyslipidemia, hypertension and coagulation or platelet aggregation disorders. In the present study, for characterization of the islet in classical Japanese non-obese type 2 diabetes, patients with BMI ≥ 30 kg/m² were excluded. The heart was weighed at the time of autopsy. Patients with a previous history of AMI were excluded from Con and DM. Diabetes was diagnosed if the patient had a history of hyperglycemia that fulfilled the criteria proposed by the Japan Diabetes Society²⁰. In the case of a history of valvular disease, cardiac intervention or other disorders with possible effects on the data, patients were excluded. Con and Con + AMI fulfilled the criteria of being 'normal type', in which the HbA1c level is $< 6.5\%$ and there was no family history of diabetes and its treatment. The prevalence of hypertension and dyslipidemia was determined based on clinical diagnosis and a history of any prescription using medical records. Laboratory data for blood glucose level, blood pressure and lipid level were not used because of considerable fluctuation. AMI was clinically diagnosed according to the third universal definition of the society of the American College of Cardiology²¹. The main cause of death was acute cardiac failure with decompensation in Con + AMI and DM + AMI (Table S1). The number of coronary arteries with stenosis in AMI was obtained from clinical records and autopsy records. Coronary artery stenosis was defined as maximum intraluminal stenosis of $\geq 70\%$. If the estimated glomerular filtration rate (eGFR) was < 30 mL/min/1.73 m², which was calculated using the modified Japanese coefficient (eGFR = $192 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ [if female]), diagnosis of severe renal failure was made^{22,23}. Serum creatinine level was measured on the day of admission.

Pathological evaluation

Several consecutive 4- μm thick sections were obtained from paraffin blocks and stained with conventional hematoxylin–eosin. Because the pancreatic body preserved its structural integrity most consistently, as previously described²⁴⁻²⁸, sections of two or three paraffin blocks of the pancreatic body tissues were used in the present study. Pathological evaluation throughout this study was carried out in a blinded manner by KT, HM and SO.

Morphometric analysis of islet microvasculature

Morphometric analysis of islet microvasculature was carried out using ImageJ (version 1.52; National Institutes of Health, Bethesda, MD, USA) based on the methods described by Weidner and Brissova with slight modifications^{29,30}. In brief, double immunohistochemistry was carried out using antibodies for CD31 (dilution 1:200; Dako Cytomation, Glostrup, Denmark) and chromogranin A. This staining enabled the identification of endothelial cells by the former labeled with brown chromogen and endocrine cells with red chromogen by the latter, respectively. A total of 50 islets ($\times 400$ magnification) were subjected to measurement for each patient. We evaluated all the

Table 1 | Clinical summary of investigated subjects

Group	Con	Con + AMI	DM	DM + AMI
No. cases	16	13	13	35
Age (years)	62.3 ± 13.8	68.9 ± 12.8	68.4 ± 6.6	65.2 ± 11.2
BMI (kg/m ²)	21.9 ± 3.5	23.1 ± 3.2	22.9 ± 3.0	22.9 ± 2.7
Duration of diabetes (years)			12.2 ± 10.8	10.5 ± 9.7
Days from the onset of AMI to death (days)		6.0 ± 8.9		35.0 ± 81.5
HbA1c % (NGSP)	5.5 ± 0.5	5.5 ± 0.3	6.6 ± 1.0*	7.5 ± 2.0* [†]
Dyslipidemia (%)	15 (2/13)	9 (1/11)	18 (2/11)	39 (10/26) ^{†‡}
Hypertension (%)	64 (9/14)	89 (8/9)*	55 (6/11)	80 (24/30) ^{†‡}
eGFR (mL/min/1.73 m ²)	56.6 ± 46.3	55.6 ± 31.2	61.7 ± 38.2	37.3 ± 27.2 [§]
Renal failure (%) (eGFR <30 mL/min/1.73 m ²)	27 (4/15)	31 (4/13)	19 (3/13)	48(15/31) [‡]
Smoking (%)	33 (3/9)	25 (1/4)	67 (6/9)	53 (10/19)
Treatment for diabetes (%)				
Diet			8 (1/13)	11 (4/35)
OHA for diabetes			15 (2/13)	29 (10/35)
Insulin			31 (4/13)	26 (9/35)
No treatment for diabetes			0 (0/13)	9 (3/35)
Unknown for diabetes			46 (6/13)	26 (9/35)
Treatment for hypertension (%)	39 (5/13)	73 (8/11)*	30 (3/9)	92 (24/26) ^{†‡}
Treatment for coagulation/platelet aggregation (%)	8 (1/13)	36 (4/11)*	11 (1/9)	58 (15/26) ^{†‡}
Treatment for dyslipidemia (%)	8 (1/13)	9 (1/11)	11 (1/9)	31 (8/26) ^{†‡}
Pancreas weight (g)	128.8 ± 38.6	122.2 ± 30.3	121.3 ± 21.9	109.0 ± 28.9
Heart weight (g)	347.3 ± 42.2	429.6 ± 122.6**	345.1 ± 59.4	505.2 ± 136.3 ^{‡††}
No. CA with stenosis		1.3 ± 0.6		1.9 ± 0.8 ^{††}

Values are the mean ± standard deviation. BMI, body mass index; CA, coronary artery; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; NGSP, National Glycohemoglobin Standardization Program; OHA, oral hypoglycemic agent. **p*<0.01 vs patients without diabetes and acute myocardial infarction (Con). [†]*p*<0.01 vs acute myocardial infarction (AMI). [‡]*p*<0.01 vs patients with diabetes but without AMI (DM). [§]*p*<0.05 vs Con, AMI and DM. ***p*<0.05 vs Con. ^{††}*p*<0.05 vs AMI.

microvessels located in the central and marginal portions of the islet, designated as intra- and peripheral (peri)-islet vessels, respectively, including those directly entering islets from acinar tissues (Figure 1). The vascular area was defined by the microvessel-occupying area (%), which was the sum of the CD31-positive area including their lumens relative to the whole islet area positive for chromogranin plus amyloid, as well as the stromal fibrotic area. Then, the mean islet vascular area was calculated for each patient by division of the total sum of the vascular area by the number of islets. The number and size of vessels in the islet were estimated as described by Weidner *et al.*²⁹, who defined a single, countable vessel as any CD31-positive endothelial cell or endothelial cell cluster that was clearly separated from adjacent vessels on a single slide transection. The vascular density was expressed as the total number of vessels per total islet area ($n/\mu\text{m}^2$)³⁰. The mean microvessel size (μm^2) was obtained by dividing the total vascular area by the total number of islet vessels in each patient.

Pericyte lining of islet microvessels

The pericyte lining of islet microvessels was visualized by double immunofluorescence for endothelial cells (CD31, red) and

pericytes (α -smooth muscle actin, green; dilution 1:100; Dako Cytomation). If a given vessel with >50% of which circumference was surrounded by pericyte lining, it was considered pericyte-positive. If it was not the case, the vessel was considered pericyte-negative. We calculated the rate (%) of pericyte-positive microvessels among all microvessels from 50 islets in each patient. To identify microvessels in the islets, the ratio of pericyte-positive capillaries in the exocrine tissues surrounding the islets was also evaluated. We evaluated capillaries that were not directly connected to the microvessels in the islets.

BM thickness of islet microvessels

The BM of islet microvessels was detected by green immunofluorescence for type IV collagen (dilution 1:100; Dako Cytomation). BM thickness was measured by the method of Robison *et al.*³¹ with slight modifications. In brief, 50 islets were captured at a magnification of $\times 20$, and the captured image was converted to black and white mode on ImageJ. Subsequently, the contour of vascular BM was carefully outlined, which enabled calculation of the microvascular area and perimeter. The BM thickness was obtained by the following formula:

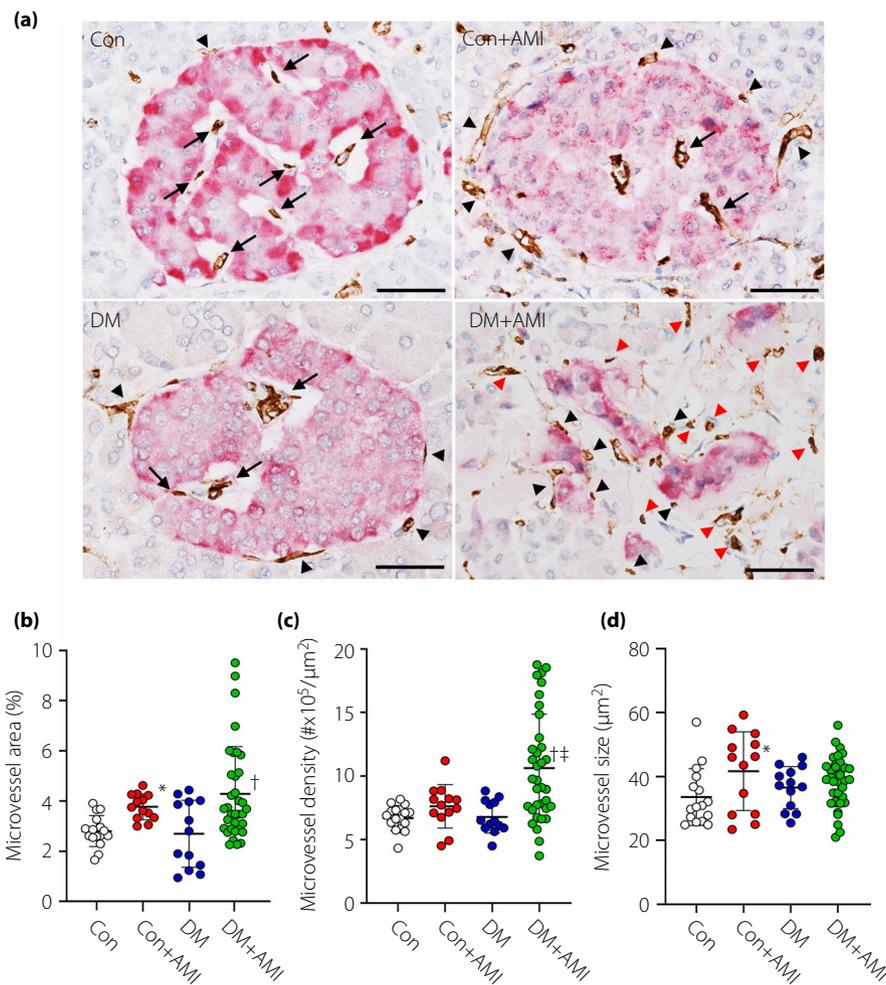


Figure 1 | (a) Alteration of islet microvasculature in the studied patients. Double immunohistochemical staining for endocrine cells with chromogranin A (red) and vascular endothelial cells with CD31 (brown) show microvessels in islets (arrows). Islet microvascular area was defined as a CD31-positive area within islets (arrows) or surrounding them (black arrowheads), which directly contacted or penetrated islets. The microvessels located in or at the border of amyloid deposits were also included as islet microvessels (red arrowheads). (b) There was a significant increase in islet microvascular area in the patients without diabetes (Con) who died of acute myocardial infarction (AMI; Con + AMI) group compared with the groups without AMI. A further increase was observed in islet microvascular area in the patients with type 2 diabetes (DM) who died of AMI (DM + AMI), which was the highest among all groups. (c) Concurrently, the DM + AMI group showed increased microvascular density compared with other groups, in which the values were comparable between Con and DM groups. (d) In contrast, microvascular size significantly increased in the Con + AMI group compared with the other groups, although there was an insignificant increase in the DM + AMI group. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ versus Con group, † $P < 0.01$ versus DM group, ‡ $P < 0.01$ Con + AMI group. Scale bar, 50 μm .

BM thickness of islet microvessels (μm) = (BM area [μm^2] / BM perimeter [μm]) / 2

The mean BM thickness obtained from all the microvessels represented the value in each patient.

Morphometric analysis of islet endocrine cells

To characterize the composition of islet endocrine cells in each case, we carried out simultaneous immunostaining of four endocrine hormones: insulin, glucagon, somatostatin and pancreatic polypeptide (PP)^{25,27}. Antibodies were purchased

from Santa Cruz Biotech., Inc. (Santa Cruz, CA, USA) for insulin, Dako for glucagon and somatostatin, and Immunobiological Lab., Ltd. (Gunma, Japan) for PP. Thereafter, the sections were lightly counterstained with hematoxylin. For determination of fractional β -, α -, δ - and PP cell volume density relative to pancreatic parenchymal area (V_{β} , V_{α} , V_{δ} and V_{PP} , respectively), pancreatic sections were imaged at $\times 40$ magnification. V_{β} , V_{α} , V_{δ} and V_{PP} were quantified on a point count basis using ImageJ^{25,27}, with ≥ 100 islets examined in each case.

Proliferation activity and apoptosis of β -cells

The evaluation of proliferation activity and apoptosis of β -cells were carried out following the previously methods for immunohistochemistry using anti-Ki67 antibody (MIB-1; Dako Cytomation) and terminal deoxynucleotidyl transferase dUTP nick-end labeling using ApopTag[®] (Millipore, Bellerica, MA, USA)^{24–27}. Cells double positive for insulin and Ki67 or apoptosis (among ~20,000 β -cells in 50 islets) were counted for each patient, and the mean value was obtained for each group.

Morphometric analysis of amyloid deposit in islets

Morphometric analysis of islets and amyloid was carried out based on the previously described protocol^{125–27}. For the determination of islet and amyloid area, endocrine cells in islets were labeled with a monoclonal antibody of chromogranin A (1:1000; Dako Cytomation) and thioflavin T staining (Wako Pure Chemicals, Osaka, Japan), respectively. Fractional islet area relative to total pancreatic parenchymal area (including endocrine and exocrine pancreas and stroma) defined as islet volume density (V_i) and amyloid volume density (V_{amy}) were measured using the point-counting method on at least 50 in each patient using ImageJ, as described previously^{24–27}.

Ultrastructure of BM around vascular channel in islet

The evaluation of transmission electron microscopy was carried out based on the previously described protocol with a small portion of the pancreatic body tissue in the pancreas³². They were examined by JEM-1230 electron microscope (Nihon Densi, Tokyo, Japan).

Statistical analysis

Data are presented as the mean \pm standard error. Statistical comparison of the mean values among all groups was carried out by analysis of variance using Tukey's test (StatView version 5.0.1; SAS Institute Inc., Cary, NC, USA). The Mann–Whitney *U*-test was carried out if the values of groups were non-parametric. A simple regression was carried out for correlation analysis. The Wilcoxon rank sum test was carried out to compare endocrine cell replication between groups due to skewed distributions of observations. Values are expressed as the mean \pm standard deviation. *P*-values of <0.05 were considered to show statistical significance.

RESULTS

Clinical profile

Clinical profiles of the study patients are summarized in Table 1. Detailed clinical data in each case are described in Tables S1–S3. BMI was comparable among all the groups. Mean HbA1c level was elevated by 14% in DM + AMI compared with DM. There was a significant increase in the prevalence of dyslipidemia in DM + AMI compared with Con + AMI and DM ($P < 0.01$ for both). The prevalence of hypertension was also higher in Con + AMI or DM + AMI compared with Con or DM, respectively ($P < 0.01$ vs Con or

DM). The mean eGFR was significantly smaller in DM + AMI than those in other groups ($P < 0.05$). When renal failure was defined as an eGFR of <30 mL/min/1.73 m², its prevalence was significantly higher in DM + AMI than in DM ($P < 0.01$). Patients free from diabetes who died of AMI showed a significantly higher prevalence of hypertension and/or coagulopathy ($P < 0.01$ for both). In contrast, diabetes patients who died of AMI showed further increases in their prevalence ($P < 0.01$ for both Con and Con + AMI vs DM + AMI). The prevalence of dyslipidemia was the highest in DM + AMI among all the groups. Heart weight was heavier in Con + AMI than in Con ($P < 0.05$), and further increased in DM + AMI ($P < 0.05$ vs Con + AMI). There was a 1.5-fold increase in the average number of stenotic coronary arteries in DM + AMI compared with Con + AMI ($P < 0.05$).

Pathological evaluation of islet microvasculature

There was a significant increase in the islet vascular area in Con + AMI compared with that in Con ($P < 0.01$; Figure 1a, b). It was also increased in DM + AMI, some of which showed a marked increase compared with that in DM ($P < 0.01$). Islet microvessel density was comparable among Con, Con + AMI and DM, whereas it was significantly increased in DM + AMI compared with Con + AMI ($P < 0.05$) and DM ($P < 0.01$; Figure 1a,c). Mean islet microvessel size was significantly greater in Con + AMI compared with Con, which was not statistically different from those in the remaining groups (Figure 1a,d).

Pericyte lining of islet microvasculature

Double immunofluorescent staining for CD31 and α -smooth muscle actin clearly delineated the microvessels with or without pericytes in islets (Figure 2a). The ratio of pericyte-positive microvessels-to-total vessels was comparable between Con and Con + AMI (Figure 2a,b). There was a significant decrease in this ratio in DM compared with Con or Con + AMI ($P < 0.01$, for both). Likewise, there was a decrease in DM + AMI compared with non-diabetes groups ($P < 0.01$, both Con and Con + AMI). There was no significant difference between DM and DM + AMI. The ratio of pericyte-positive capillaries-to-total capillaries in the exocrine tissues surrounding the islets was similar among all groups (Figure 2c). The frequency of pericyte-covered microvessels correlated with V_{β} ($r = 0.41$, $P < 0.05$; Figure 2d).

BM of islet microvessels

The BM of islets was visualized with immunofluorescence for type IV collagen (Figure 3a). BM thickness of islet microvessels in Con + AMI was similar to that in Con (Figure 3a). There was a significant increase in BM thickness in DM compared with Con ($P < 0.05$; Figure 3a,c). It was further increased in DM + AMI ($P < 0.01$, vs Con and Con + AMI; $P < 0.05$, vs DM). In the evaluation of electromicroscopy, islet microvessels were surrounded with thin layered BM in Con (Figure 3b). Corresponding to the result of immunofluorescence, BM was

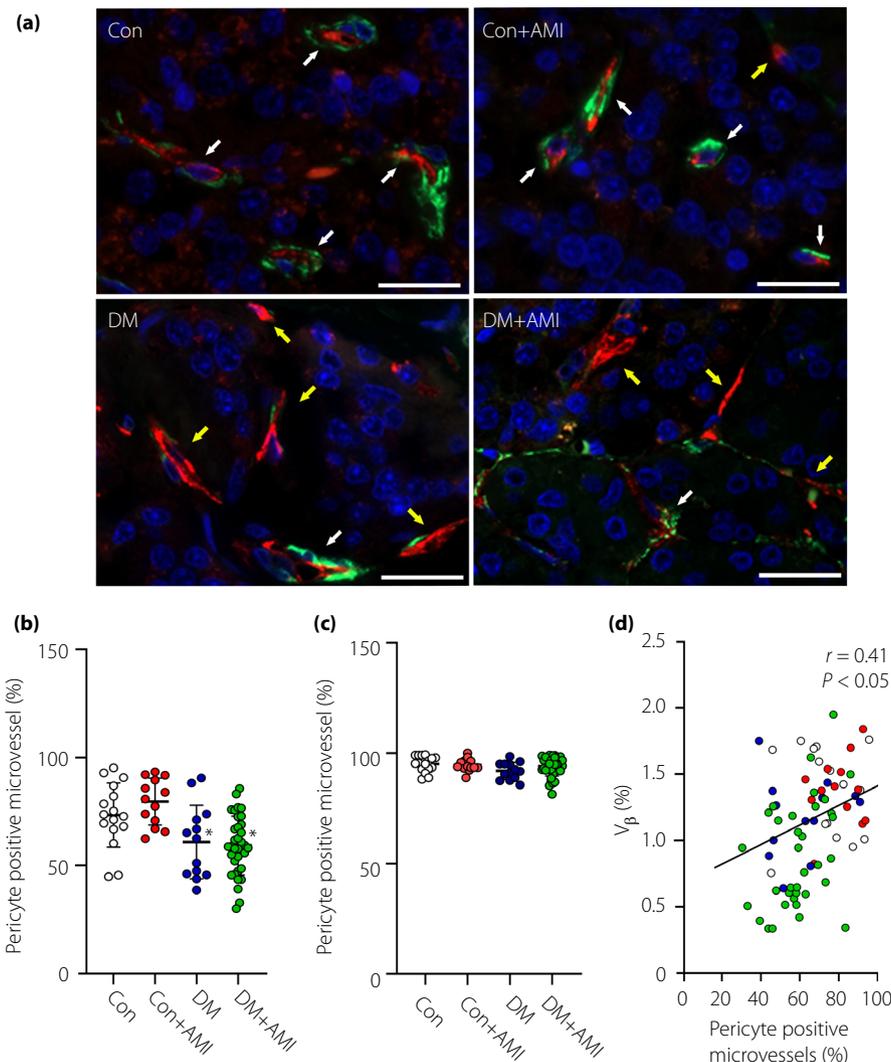


Figure 2 | (a) Pericyte lining in microvessels of islets. The pericyte lining of islet microvessels was evaluated by double immunofluorescence of vascular endothelial cells with CD31 (red) and vascular wall smooth muscle cells or pericytes with α -smooth muscle actin (green). White arrows indicate microvessels covered by pericytes, whereas yellow arrows indicate microvessels devoid of pericytes. (b) Microvessels devoid of pericytes were more frequently encountered in patients with type 2 diabetes who were free of acute myocardial infarction (DM) and patients with type 2 diabetes who died of acute myocardial infarction (DM + AMI) than in patients without diabetes and acute myocardial infarction (Con) and patients without diabetes who died of acute myocardial infarction (Con + AMI). (c) The frequency of capillaries devoid of pericytes in the exocrine tissues surrounding islets was comparable among all groups. Their population was comparable between the DM and DM + AMI groups. (d) The frequency of pericyte-positive microvessels was proportionally correlated with β -cell volume density (V_{β} ; $r = 0.41$, $P < 0.05$). Values are expressed as the mean \pm standard deviation. * $P < 0.01$ versus Con and Con + AMI groups. Scale bar, 25 μ m.

markedly thickened and multilayered in DM + AMI. BM thickness inversely correlated with V_{β} ($r = -0.48$, $P < 0.01$; Figure 3d).

Pathological evaluation of islet endocrine cells

V_i was comparable among all groups, except in DM + AMI, in which V_i was 18% smaller than that in DM ($P < 0.01$; Table 2). V_{β} was decreased in DM by 19% compared with

Con ($P < 0.05$) and further reduced by 36% in DM + AMI. The difference between DM and DM + AMI was significant ($P < 0.05$). V_{α} was increased (approximately 29%) in DM compared with Con ($P < 0.05$), but it was comparable between DM + AMI and Con. There was no significant difference in V_{δ} or V_{PP} among all the groups. There was no significant increase in the frequency of Ki67-positive cells in DM + AMI. Apoptotic cells in islets were not identified in any group.

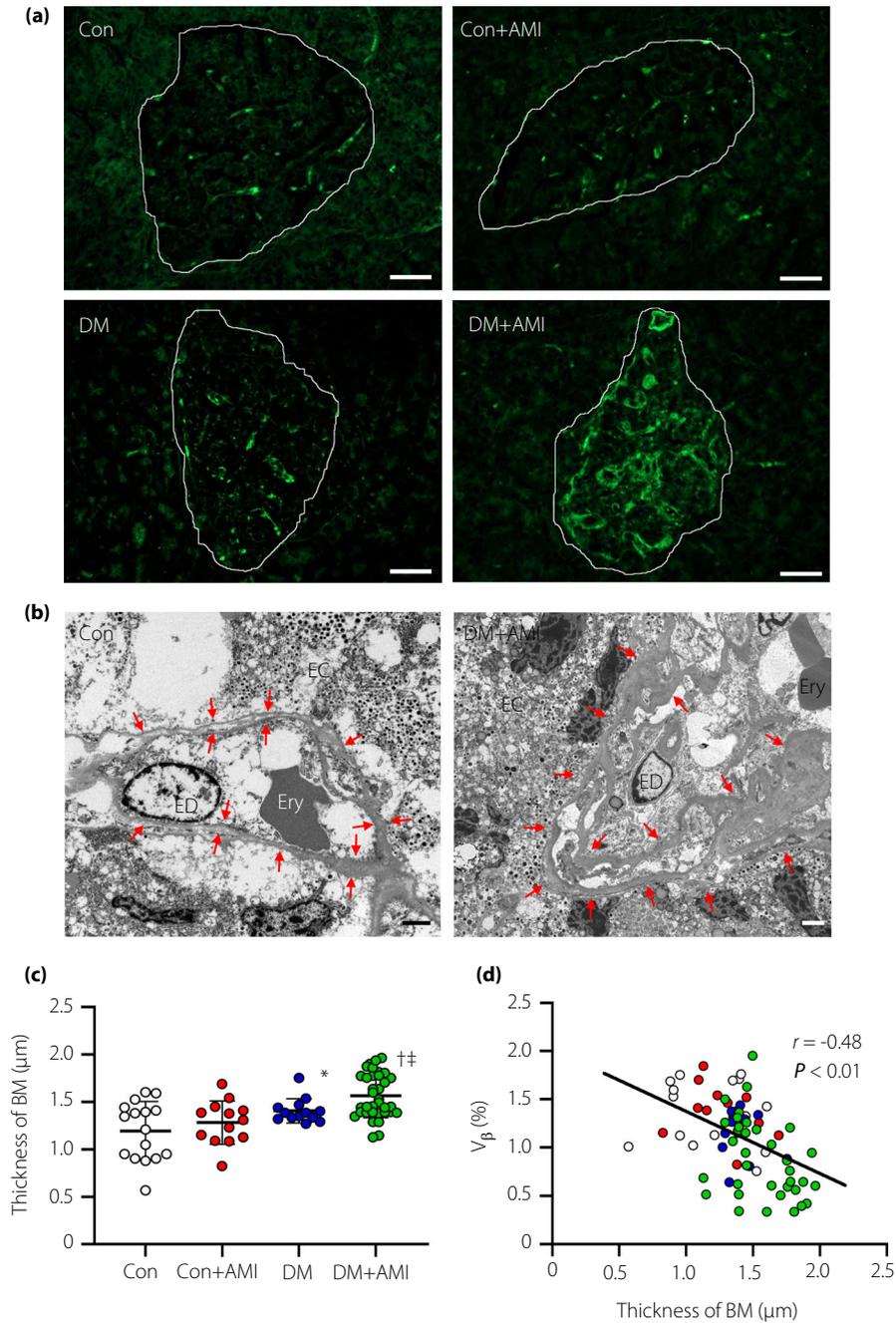


Figure 3 | Thickness of basement membrane (BM) in islets microvessels. (a) BM area delineated by immunofluorescence for type IV collagen (green) appeared to be increased in the patients with type 2 diabetes who were free of acute myocardial infarction (DM) and patients with type 2 diabetes who died of acute myocardial infarction (DM + AMI) group. The islet border is demarcated with a broken white line. (b) BM (red arrow) became markedly thickened and multilayered in DM + AMI compared with patients without diabetes and acute myocardial infarction (Con) in electron microscopy. (c) The BM of islet microvessels was significantly thicker in the DM group than in Con, and was thickest in the DM + AMI group. The thickness of BM was inversely correlated with V_{β} in the islets of the DM + AMI group ($r = -0.48$, $P < 0.01$) (d). Values are expressed as mean \pm standard deviation. * $P < 0.05$ versus Con group, $^{\dagger}P < 0.01$ versus patients without diabetes who died of acute myocardial infarction (Con + AMI) group, $^{\ddagger}P < 0.05$ versus DM group. EC, endocrine cell; ED, endothelial cell; Ery, erythrocyte. Scale bar, 50 μm (a) and 2 μm (b).

Islet amyloid deposition

On the hematoxylin–eosin-stained sections, there was no obvious amyloid deposition in Con or Con + AMI (Figure 4a), whereas it was common in islets in DM + AMI (Figure 4b). Thioflavin fluorescence confirmed a positive reaction for amyloid in this group (Figure 4c). Marked amyloid deposition was found in 54% of the cases in DM + AMI (19/35 cases; Figure 4e). V_{amy} in DM + AMI was 24-fold greater than that of DM ($0.24 \pm 0.44\%$ vs $0.01 \pm 0.03\%$, $P < 0.05$; Table 2). Electron microscopic examination showed that BM was multilayered and extended into surrounding tissues with amyloid deposition in DM + AMI (Figure 4d). BMI was not significantly correlated with V_{amy} ($r = 0.1$, $P = 0.9$; f), whereas, BM thickness was positively correlated with V_{amy} ($r = 0.36$, $P < 0.05$; Figure 4g).

DISCUSSION

In the present study, we identified a distinct clinical profile and islet pathology in Japanese patients with type 2 diabetes who died of AMI accompanied by marked structural changes in islet microvasculature, similar to those shown in microangiopathy in the kidney³³, heart³⁴, peripheral nerve³⁵ and retina³⁶. The ratio of pericyte-positive microvessels in the islets was significantly reduced in individuals with DM + AMI, which clearly differed from the capillaries in the exocrine tissues surrounding the islets. Thickness of BM was proportionally correlated with V_{amy} , which was inversely correlated with V_{β} , supporting the concept that amyloid deposition augments β -cell loss and leads to high HbA1c levels^{25,37}. It is of note that the prevalence of dyslipidemia and hypertension was greater in DM + AMI than DM despite a comparable clinical profile of age, BMI and duration of diabetes, as well as HbA1c level. This group was commonly associated with renal insufficiency. Autopsy showed a heavier heart, increased number of stenotic coronary arteries and prolonged suffering time from the onset of AMI to death.

The alterations in the islet microvasculature characterized by thickened BM, pericyte loss and vascular remodeling in DM + AMI are the hallmarks of microangiopathy in diabetes, and long-standing metabolic aberrations might underlie its development. Thickened BM obstructs drainage of amyloid-related protein, resulting in amyloid formation^{16,38}. Loss of the pericyte coverage in the islet capillaries in type 2 diabetes deteriorates islet blood supply¹⁴, in which insulin supply to target organs is reduced and islet amyloid polypeptide (IAPP) might be deposited surrounding β -cells. Furthermore, the functional defect in the islet endothelial cells can impair survival and function of the β -cell³⁹. Collectively, the presence of islet microangiopathy in DM + AMI possibly contributed to robust amyloid deposition and augmented β -cell deficit in non-obese patients with type 2 diabetes, similar to the feature repeatedly described in white, obese diabetes patients¹⁶. Experiments on human IAPP transgenic rats showed that amyloid was deposited near the capillaries that extend to interstitial tissues⁴⁰. Deposited amyloid often obliterates vessels, resulting in hypoxia with attempt of a new vessel formation in the area of amyloid deposition. It is hence likely that amyloid deposition itself might facilitate the islet microangiopathy in DM + AMI.

In the present study, the prevalence of hypertension in DM + AMI individuals was the highest among all groups. Abnormal glucose metabolism is a primary pathogenesis of type 2 diabetes and its microvascular complications. In addition, the factors related to metabolic syndromes, such as obesity, hypertension and dyslipidemia, are assumed to be the pathogenesis of microvascular complications, because complete remission is not achieved with strict glucose control alone^{9,41}. The normalization of blood pressure is an important and potentially decisive intervention for diabetic complications^{42–44}. Hypertension induces shear stress, which induces degenerative processes and, ultimately, the formation of acellular capillaries⁴⁵. Hypertension is also a known risk factor associated with islet

Table 2 | Morphometric analysis of islets

	Con	Con + AMI	DM	DM + AMI
V_i (%)	2.18 ± 0.41	2.23 ± 0.42	2.14 ± 0.57	1.74 ± 0.63* [†]
V_{α} (%)	0.65 ± 0.18	0.51 ± 0.18	0.84 ± 0.22 [‡]	0.65 ± 0.28 [†]
V_{β} (%)	1.40 ± 0.26	1.53 ± 0.31	1.13 ± 0.33 [‡]	0.90 ± 0.35 ^{†,§}
V_{δ} (%)	0.11 ± 0.05	0.16 ± 0.06	0.15 ± 0.07	0.12 ± 0.08
V_{pp} (%)	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.04 ± 0.05
V_{amy} (%)	0	0	0.01 ± 0.03	0.24 ± 0.44 [†]
Total islet density (#/mm ²)	3.52 ± 1.89	3.20 ± 0.94	5.32 ± 2.40**	4.00 ± 1.56 [†]
Small cluster islet density (#/mm ²)	2.13 ± 1.48	1.98 ± 0.57	3.44 ± 1.43 [‡]	2.47 ± 1.01 [†]
β -Cell replication rate (%)	0.17 ± 0.09	0.16 ± 0.12	0.18 ± 0.18	0.14 ± 0.12
β -Cell apoptosis rate (%)	0	0	0	0

Values are the mean ± standard deviation. V_{α} , α -cell volume density; V_{β} , β -cell volume density; V_{δ} , δ cell volume density; V_{amy} , amyloid volume density; V_i , islet volume density; V_{pp} , PP cell volume density. * $P < 0.05$ versus patients with acute myocardial infarction (AMI). [†] $P < 0.05$ versus patients with diabetes but without AMI (DM). [‡] $P < 0.05$ versus patients without diabetes and acute myocardial infarction (Con). [§] $P < 0.01$ vs AMI. ** $P < 0.01$ vs Con.

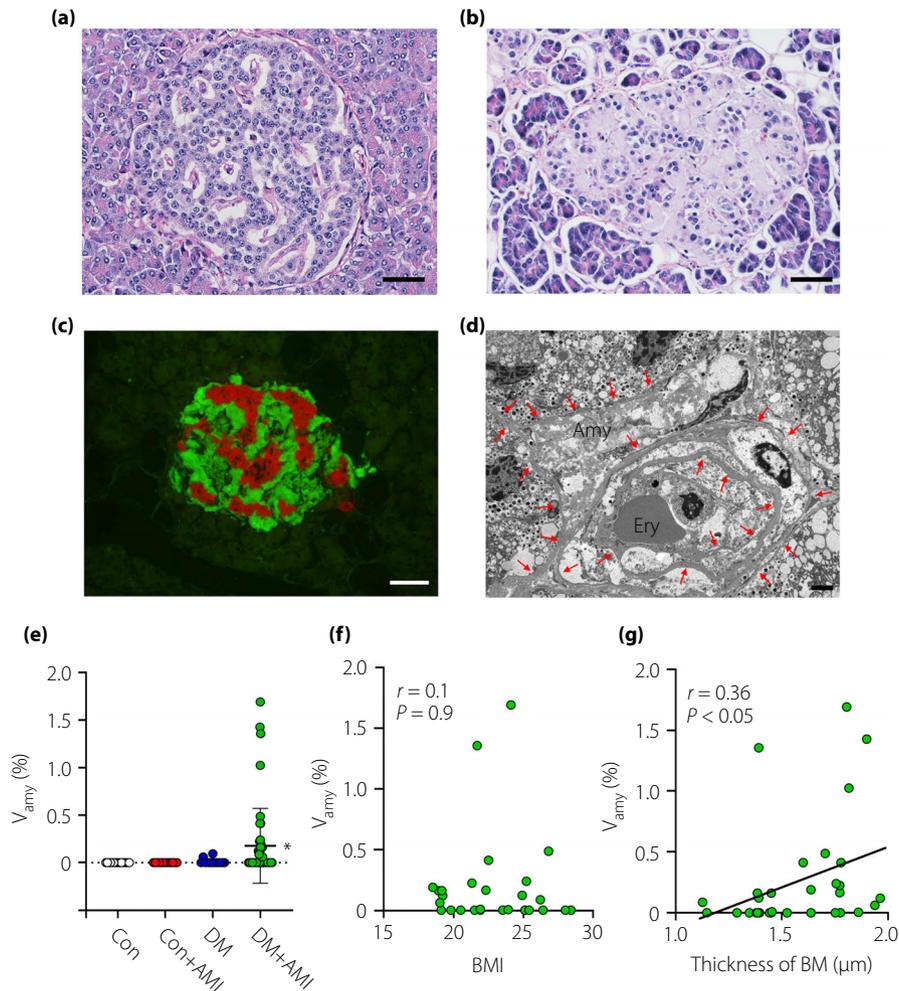


Figure 4 | Amyloid deposition of islets. Islet histology in (a) patients without diabetes and acute myocardial infarction (Con) group and marked amyloid deposition in (b) patients with type 2 diabetes who were free of acute myocardial infarction (DM) and patients with type 2 diabetes who died of acute myocardial infarction (DM + AMI). (c) Robust amyloid deposition in the DM + AMI group was clearly confirmed on the section of islet endocrine cells positive for chromogranin A (red) and amyloid positive for thioflavin T (green). (d) In electromicroscopic evaluation, multilayered basement membrane (BM; red arrow) was observed surrounding microvessels and extended into stroma adjacent to amyloid deposition (Amy) in the islet. (e) Amyloid volume density (V_{amy}) was significantly greater in the DM + AMI group than in the DM group. (f) No significant correlation between body mass index (BMI) and V_{amy} was observed. There was an inverse correlation between V_{amy} and thickness of BM ($r = 0.36$, $P < 0.05$) (g). Values are expressed as the mean \pm standard deviation. * $P < 0.05$ versus DM. Amy, amyroid; Ery, erythrocyte. Scale bar, 50 μm (a–c) and 2 μm (d).

amyloid deposition in type 2 diabetes patients⁴⁶. However, capillaries were dilated in the islets of AMI individuals, whereas the pericyte loss and BM thickness were not pronounced compared with those of the Con individuals. This might suggest that the presence of hypertension alone is not sufficient to form microangiopathy in islets. Thus, hypertension might accelerate the degeneration of microvessels, eliciting more deposition of amyloid in the islets of type 2 diabetes.

The prevalence of dyslipidemia was also higher in DM + AMI. ‘Lipotoxicity’ is one of the most widely accepted hypotheses to explain the underlying mechanisms of β -cell

dysfunction in type 2 diabetes⁴⁷. Dyslipidemia is also known to be an independent factor of diabetic microvascular and macrovascular complications^{9,48}. Although its mechanisms involved are not fully understood, oxidized low-density lipoprotein cholesterol-induced oxidative stress has been shown to mediate nerve damage in a mouse model of diabetic neuropathy⁴⁹. In the case of islets, hypercholesterolaemia interacts with hyperglycemia to evoke pre-microangiopathy in Goto-Kakizaki rats¹⁵. Thus, it is likely that dyslipidemia contributes to the formation of microangiopathic changes in the islets of non-obese Japanese type 2 diabetes patients.

The prevalence of renal insufficiency was significantly higher in DM + AMI than in DM. The measured serum IAPP levels were high in individuals with chronic kidney disease due to the impaired IAPP excretion⁵⁰. In fact, amyloid deposition in the islets was augmented in patients with end-stage renal failure⁵¹. As such, although the causal relationship between amyloid deposition and AMI remains unclear, suppressed insulin secretion and elevated serum IAPP levels might be clinical indices of the severe islet pathology of DM + AMI.

Type 2 diabetes is not a single disease entity, but rather, represents a hyperglycemic syndrome with a heterogeneous etiology⁵². There is a lower degree of insulin resistance in the pathophysiology of early type 2 diabetes in Japanese individuals with a low BMI (average 23.4) than in white individuals with a high BMI (average 27.5)^{53,54}. Japanese type 2 diabetes patients showed a decrease in basal and early-phase insulin secretion compared with white type 2 diabetes patients^{53,54}. Likewise, the islet pathology of type 2 diabetes is, therefore, expected to vary depending on genetic, ethnic or environmental factors^{24,55,56}. For example, the prevalence of islet amyloid deposition was found to exceed 80% in white type 2 diabetes patients, whereas it was just 30% in Japanese type 2 diabetes patients^{25,57}. In the present study, we clarified that amyloid deposition in the islets was apparent in type 2 diabetes patients complicated with AMI. The incidence of AMI in type 2 diabetes patients is twice as high in white patients as in Japanese patients^{58,59}. Thus, the high prevalence of AMI in white patients can partially explain the high prevalence of amyloid deposition in islets. These clinical and pathological findings suggest that the pathophysiology of Japanese type 2 diabetes patients complicated with AMI might be closer to that of white type 2 diabetes patients, despite a low BMI.

Experimental and epidemiological studies showed that AMI triggers peripheral insulin resistance, which can cause dilation of islet microvessels^{60,61}. In addition to AMI or hypertension, Takeno *et al.*⁶² disclosed that excessive ectopic fat content was relevant to muscle insulin resistance in non-obese Japanese patients who carried risk factors for vascular events, such as dyslipidemia, hypertension and hyperglycemia. Thus, such non-obese patients with type 2 diabetes possibly correspond with the group of DM + AMI in the present study. In view of the effective prevention of the progression of the islet lesion in patients with diabetes, not only the treatment for glycemic control, but also meticulous control of hypertension as well as dyslipidemia, and possibly care for renal insufficiency might be crucial.

In the present study, the cardiac weight was significantly greater in DM + AMI than Con + AMI. The cardiac weight generally reflects its hypertrophy. Therefore, it is expected that the cardiac failure was sustained longer and more severe in DM + AMI compared with Con + AMI, because the cardiac tissues might have been forced to compensate for severe ischemia caused by multiple stenotic arteries in DM + AMI. In this setting, it is unclear if excessive IAPP from islets directly

contributes to the occurrence of cardiac failure. Despa *et al.*⁶³ proposed the possibility that IAPP might directly injure the cardiac muscles. We could not find the obvious IAPP expression or amyloid formation in the infarcted heart in all the cases (data not shown).

There were several limitations to the present study. First, sufficient clinical information about the metabolic state was not available in the studied patients. In particular, information on blood insulin, C-peptide, concentrations of low-density lipoprotein, high-density lipoprotein and homeostasis model assessment of insulin resistance or homeostasis model assessment of β -cell function would have enabled us to know the presence of insulin resistance and β -cell secretory capacity in DM + AMI. Second, more detailed evaluation of the subcellular structures of islet endocrine cells and microvasculature is required for the precise understanding of endocrine and vascular relationships. Unfortunately, we could not obtain sufficient samples fresh enough for ultrastructural studies. Third, we could not obtain detailed information about the cardiac function in the examined individuals. The availability of such parameters might have provided the information on the relationships between islet pathology and heart failure. Fourth, multivariate analysis was not carried out because of a lack of a sufficient number of patients. Future multivariate analyses would be essential to identify the possible factors other than those identified in the present study that influenced the islet pathology of DM + AMI patients.

In conclusion, the presence of macroangiopathy appeared to enhance the islet vascular changes and worsen the clinical outcome in non-obese type 2 diabetes. We consider that alterations of islet microvasculature that augment β -cell deficit and amyloid deposition are structural hallmarks of an ominous outcome of non-obese type 2 diabetes patients who require intensive care for hypertension and dyslipidemia.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Detailed clinical profiles of the subjects.

Table S2 | Detailed clinical profiles of the subjects (continued).

Table S3 | Detailed clinical profiles of the subjects (continued).