Improving the use of donor organs in pancreas and islet of Langerhans transplantation

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Omslag: Rosa moyesii “Denise Hilling” (gekweekt door Thomas Hilling, 1965)
Omslag ontwerp: Pauline van der Capellen
Layout: Merlijn Hutteman

ISBN 978-94-6169-303-7

Dit proefschrift werd mede mogelijk gemaakt door:
De Jong Koster Accountants en Belastingadviseurs, Raad van Bestuur Medisch Centrum Haaglanden, Diabetes Fonds, Nederlandse Transplantatie Vereniging, Chipsoft, Astellas Pharma BV, Novartis Pharma BV, Novo Nordisk BV en Stichting Extracurriculaire Activiteiten Haagse Chirurgen.

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 1 november 2012
klokke 13.45 uur

door

Denise Eline Hilling

geboren te Rotterdam in 1981

Promotiecommissie

Promotor	Prof. dr. O.T. Terpstra
Co-promotor	Dr. P.J. Marang-van de Mheen
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Voor mijn ouders
en Roland (1983-2001)

Aan Merlijn

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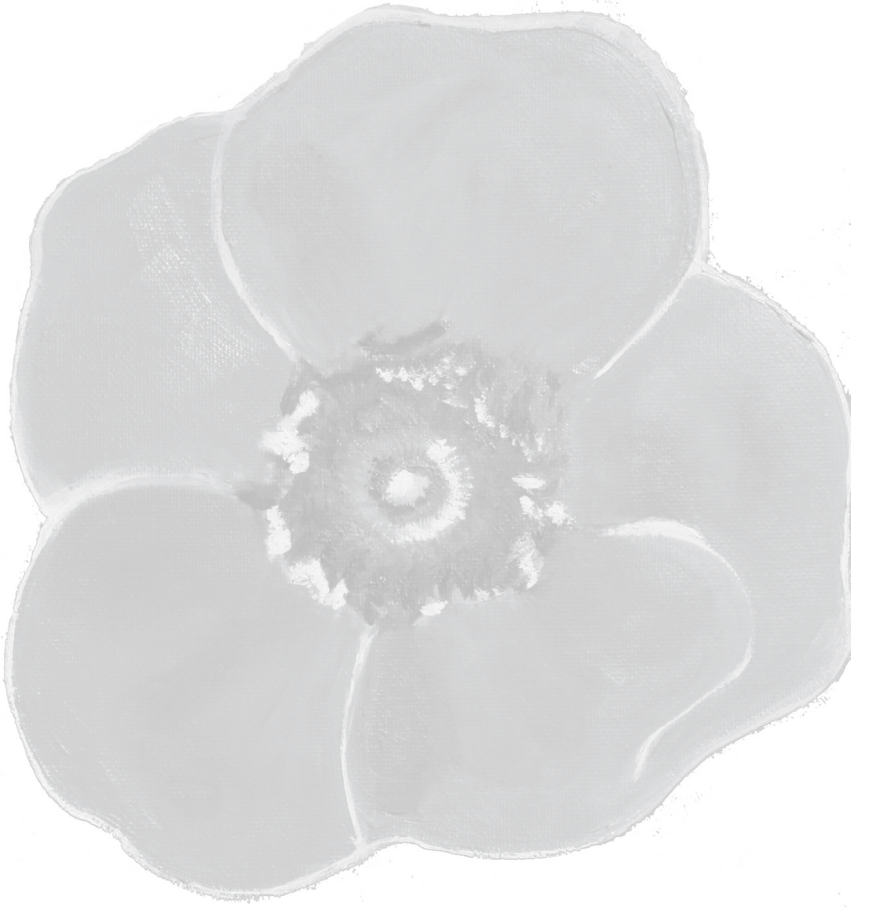
Chapter 1

Introduction



INTRODUCTION

Type 1 diabetes mellitus is caused by autoimmune destruction of insulin-producing beta cells in the islets of Langerhans of the pancreas. In patients with type 1 diabetes, insulin treatment is the only life-saving therapy. Long-term prognosis and quality of life of these patients is largely determined by the occurrence and severity of secondary diabetic complications. However, even when insulin treatment is well tolerated and carried out in a diligent way, metabolic derangements and long-term complications still occur, resulting in reduced patient survival (1, 2). Beta cell replacement, by transplantation of whole pancreas or isolated islets of Langerhans to restore endogenous insulin secretion, has emerged as a logical alternative to insulin injections. However, there is a shortage of donor pancreata relative to the needs of potential transplant recipients (3). Therefore, optimal use of the available donor organs is vital. In contribution to optimize the use of available organs, the focus of this thesis was on the improvement of pancreas graft survival in pancreas transplantation and to optimize islet isolation outcomes in islet of Langerhans transplantation. Furthermore, since porcine islet transplantation is an alternative to compensate for the shortage of human donor organs, we focused on optimizing porcine islet isolation outcome as well.

Pancreas transplantation

Transplantation of the whole pancreas is a complex procedure that can lead to good long term metabolic control and prolong survival of both nephropathic and neuropathic diabetic patients (4-7). The first clinical pancreas transplantation was performed in 1966, simultaneous with a kidney transplant in an uremic diabetic patient at the University of Minnesota (8). The success rate (long-term insulin independence) of pancreas transplantation was initially low, but increased dramatically in the 1980's. Pancreas graft and patient survival have further improved in recent years due to improved procurement and transplantation techniques, immunosuppression regimes and more emphasis on donor management and careful recipient selection (9-12).

The majority of pancreas transplantations are performed simultaneously with a kidney transplantation (simultaneous pancreas kidney transplantation: SPK), in patients with type 1 diabetes with end-stage or pre-emptive renal disease. Other possibilities are: pancreas after kidney transplantation (PAK), in which a pancreas from a deceased donor is transplanted in an insulin-dependent diabetic patient with a good functioning kidney transplant, and pancreas transplantation alone (PTA), in a type 1 diabetic patient with frequent and severe episodes of hypoglycemia, hyperglycemia or ketoacidosis but with preserved renal function. SPK transplants are performed more frequently than solitary pancreas transplants (211 SPK vs. 92 solitary pancreas transplants, in 2010 in the Eurotransplant region (3)). However, there is an increase in solitary pancreas transplantations, particularly PAK, reflecting an emphasis on living donor kidney

transplants in uremic diabetic patients to preempt the need for dialysis (13). This is also seen in the Netherlands where in 2005 a single solitary pancreas transplantation was performed, with an increase to 11 in 2010, as reported by Eurotransplant (3). The International Pancreas Transplant Registry (IPTR) maintains a database of all reported pancreas transplants worldwide. In their annual report of 2004, they reported 1 year pancreas graft survival rates of 80-85% and patient survival rates 95% for both SPK and PAK and 98% for PTA (13). In the Leiden University Medical Center, where 85% of all pancreas transplantations in the Netherlands are performed, even better graft survival rates are obtained, in particular with primary bladder-drainage followed by elective enteric conversion 6-12 months later, used in most of the patients. In these patients 1 year pancreas graft survival rate was 88% (14).

Pancreas graft and patient survival rates are influenced by several factors, e.g. procurement, transplantation technique, immunosuppression regimes and donor and recipient related factors. Many donor and recipient characteristics have been reported to influence pancreas graft survival (11, 12, 14-30). This raises the question on how the impact of donor characteristics relate to that of the recipient. In order to further improve pancreas graft and patient survival, do we have to focus on donor selection, optimize recipient condition or donor-recipient matching? No studies so far have examined the contribution of donor and recipient factors to graft survival. We therefore aimed in **chapter 2** to identify donor and recipient factors influencing pancreas graft survival, to evaluate the impact of donor and recipient factors on pancreas graft survival, and to compare their contribution in explaining graft survival differences between pancreas recipients.

The procurement technique of a pancreas graft has also been shown to influence pancreas graft and patient survival. Surgical injuries that occur during pancreas procurement may lead to complications after transplantation, impaired function of the allograft, graft loss or even death of the patient. These injuries may be so severe that the pancreas is not transplanted in order to protect the recipient. Liposis of the graft and critical vessel injuries have been reported as reasons for pancreas refusal after procurement (31). However, only few studies have addressed this issue. We therefore assessed how often pancreata were refused for transplantation during back-table inspection in our center and which type of problems were responsible for the decision not to transplant the pancreas (**chapter 3**). A better understanding of the type of problems that occur could lead to a higher awareness for injuries. In combination with training this could potentially lead to avoidance of these injuries. This would result in the use of more donor pancreata that would otherwise have been discarded because of the injuries. Furthermore the quality of the transplanted pancreata with minor injuries would improve when these are avoided. This would eventually result in better pancreas graft and patient survival.

Islet transplantation

Whole pancreas transplantation, however, is not devoid of complications, mainly secondary to surgery and immunosuppressive therapy (24). The alternative to transplantation of the pancreas is transplantation of isolated islets as a free graft. Islet transplantation is minimally invasive and has low morbidity because the islets are infused percutaneously into the hepatic portal vein. Furthermore, a pancreas graft can still be used for islet isolation and transplantation when rejected for pancreas transplantation. The first clinical islet allograft was performed in 1974 in a diabetic recipient who previous to the islet transplant received a kidney transplant (32). Since the late 1980s, the feasibility of isolating and purifying human islets from pancreatic organs of deceased donors raised hope that purified pancreatic islet cells, rather than an entire gland, could cure diabetes (33). However, a limiting factor in islet transplantation is the islet isolation yield that can be obtained from donor pancreata. In some cases, sufficient islet numbers can be obtained from a single donor, but even in the most successful studies, multiple transplantations are necessary to obtain (temporary) normalization of hyperglycemia in the recipients (34-39). Therefore, the supply of human donor pancreata as source of islets is insufficient.

In order to potentially enable the use of a single organ, several strategies were developed to maximize islet yield, e.g. by choosing better culture conditions, and improving donor and recipient selection. Many donor and recipient factors have been reported to have an influence on islet isolation yield (40-72). However, no uniformity is to be found in factors that are reported. Because of the scattered information, valuable information is potentially missed because there is insufficient power to determine the independent effect of the donor factors on islet isolation outcome in a single study. **Chapter 4** offers a review of the literature; identifying donor and recipient factors influencing islet isolation yield and provides recommendations for standardized reports of donor and recipient factors in order to provide better comparisons in the future and to improve the power by providing enough data to perform a meta-analysis.

Despite significant efforts to improve the yield of isolated islets by optimizing donor and recipient factors, isolation protocols and culture conditions, islet isolation yields in human pancreata remain unpredictable and variable. Histomorphological aspects (e.g. collagen and other matrix elements) of the pancreas are thought to play a role in these variations (73-79). When studying histological characteristics of human donor pancreata, a remarkably high number of hyperemic islets (HIs) was encountered. Similar islets have only been reported anecdotally in the literature but no mechanisms were described regarding their origin and no relevance has been determined from the perspective of islets isolation for transplantation (80-84). We therefore aimed to determine the relevance of the presence of HIs in human donor pancreata for isolation outcome and to identify donor and procurement factors associated with the occurrence of HIs (**chapter 5**).

Xenotransplantation

Xenotransplantation of porcine islets of Langerhans is another way to overcome the shortage of human donor pancreata. For various reasons, the pig is considered to be the preferred source of pancreatic xeno-islets. Pig insulin, which differs from the human type by only one amino acid, is active and well tolerated in humans. For years prior to the production of human recombinant insulin, patients were successfully treated with insulin injections extracted from swines. Transplantation of porcine islets has been proven to be successful in non-human primates as well as in humans (85-88). Moreover, pig islets can be successfully isolated and purified from adult pigs with a method that is similar to the one used for human islets (89). Advantages of using pigs as a source of islets for transplantation are, at least in theory, numerous. Besides the benefit of unlimited tissue supply, a higher quality of donor organs could be expected by planned elective organ harvesting, therefore minimizing cold ischemia and consequently improving islet yields. However, porcine islet isolation procedures have been shown to be notoriously difficult and provide unpredictable and variable islet isolation yields, even more so than in human pancreata (90-92). Because pancreata from adult pigs have resulted in large yields, a possible explanation could be related to donor age and to the relative fragility of the islets of juvenile pigs islet isolation procedures (90-94).

Furthermore, the amount of endocrine tissue present in a specific pancreas is undoubtedly an important factor in determining the islet isolation outcome. However, a high endocrine content does not ensure a high isolation yield. Despite improvement of isolation procedures, islet isolation is still associated with a considerable loss of endocrine tissue. This indicates that collagenase digestion of the pancreas is not limited to the exocrine pancreas but affects the islets as well. Because collagen is the major target in the enzymatic dissociation of the pancreas, the collagen substrate within the pancreas is one of the variables that could account for the unpredictable, highly variable islet yields. Also other matrix elements are thought to play a role (77-79, 92, 95). We have assessed the total amount and distribution of collagen within a large study population of adult and juvenile porcine pancreata and assessed the relation of these determinants to the outcome of islet isolation in adult pigs in **chapter 7**.

Another explanation for the unpredictable islet isolation outcomes could lie in morphological characteristics of porcine islets. Similar to human pancreata, we found a high number of hyperemic islets (HIs) when studying histological characteristics of porcine pancreata. We assessed the frequency of HIs in porcine pancreata compared to human pancreata. Furthermore, we studied the occurrence of HIs in relation to the outcome of islet isolation similar to the study in human pancreata (**chapter 6**). Besides the presence of HIs, we have observed morphological changes of islets after infusing the pancreas with collagenase during the isolation process. Previous studies have shown collagenase located within the islets after standard intraductal infusion

of collagenase in human and also at lower perfusion pressures in porcine pancreata (96, 97). The observed morphological changes could therefore be a result of either volume expansion of collagenase entering in the islet, leading to disruption of cell-cell contacts or be the result of the digestive effect of collagenase, subsequently leading to islet fragmentation. Both scenarios would eventually lead to lower islet isolation outcomes. In **chapter 8** we aimed to discriminate between these two hypotheses.

Finding answers to these questions will contribute to further optimization of pancreas graft survival in pancreas transplantation and improved islet isolation outcomes in islet of Langerhans transplantation, eventually leading to better use of available organs.

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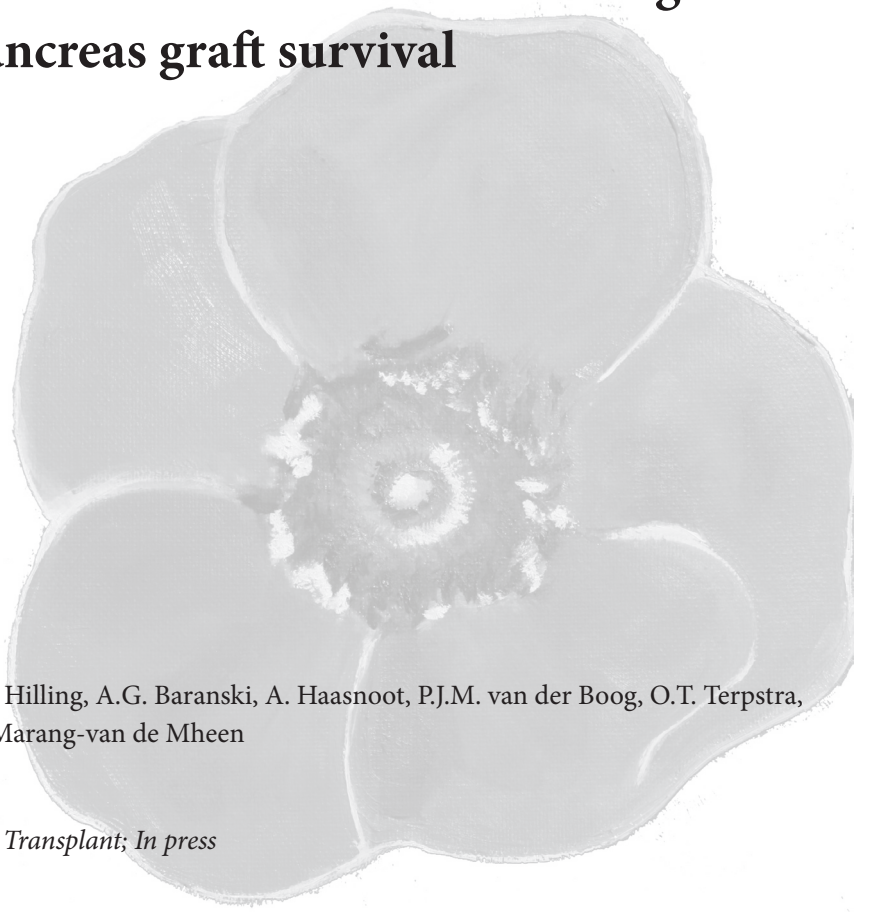
Part I

Pancreas transplantation

Chapter 2

Contribution of donor and recipient characteristics to short- and long-term pancreas graft survival



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Ann Transplant; In press

ABSTRACT

Background

Many donor and recipient factors are known to affect pancreas graft survival. However, their relative importance in explaining differences in graft survival is unknown. Purpose of this study was to retrospectively evaluate the impact of donor and recipient factors on pancreas graft survival, and compare their contribution in explaining graft survival differences.

Methods and Materials

Patient records of all 170 pancreas transplantations (158 Simultaneous Pancreas-Kidney; 12 Pancreas-after-kidney) in the period 1997-2008 were reviewed retrospectively to assess recipient factors before/during transplantation, and to assess graft survival. Eurotransplant reports were reviewed to assess donor factors.

Results

Death-censored 1-year graft survival was 88.4% and 82.3% at 3 years. Several factors significantly influenced graft survival: female recipient gender (Hazard Ratio (HR) 2.81[1.10-7.14]), enteric graft drainage (HR 2.85[1.15-7.05]), and donor-recipient match on BMI (HR 2.46[1.01-6.02]). None of the donor factors significantly affected survival. Similar results were found for 1-year survival, except for enteric graft drainage and donor-recipient BMI matching. In total, donor factors explained 3.6% and recipient factors 10.0% of the variance in graft survival. Donor factors were more important for 1-year survival (3.1%), but still less important than recipient factors which explained 6.4%.

Conclusion

Recipient factors are more important in explaining differences in pancreas graft survival than donor factors.

BACKGROUND

Pancreas transplantation is able to correct metabolic abnormalities in patients with type 1 diabetes mellitus, prevent or delay secondary complications, and in simultaneous pancreas kidney (SPK) transplants is also a treatment for diabetic nephropathy (1-3). Outcomes have improved in recent years due to improved procurement and transplantation techniques, immunosuppression regimes and more emphasis on donor management and careful recipient selection (4-7).

Many donor characteristics have been reported to influence pancreas graft survival (8-14). This led the Eurotransplant Pancreas Advisory Committee to define a pancreas donor quality score, (comparable to the SOFT score in liver transplantation (15)) based on nine clinical parameters: the "Preprocurement Pancreas Allocation Suitability Score" (P-PASS) (16). They reported that pancreata from suboptimal donors (P-PASS > 17) had a significantly higher graft failure rate within the 1st year after transplantation (17). However, this effect may be partly explained by differences in recipient factors that affect survival, which were not taken into account. Other studies have shown that recipient characteristics, surgical techniques and other transplantation features such as ischemia times have an influence on graft survival (2,4,10,18,19). This raises the question on how the impact of donor characteristics relates to that of the recipient: are both equally important? To our knowledge, no studies so far have examined the contribution of donor and recipient factors to graft survival.

Another issue that may affect survival is whether donor and recipient are properly matched. ABO blood group matching and to a lesser extent HLA matching are known to improve pancreas graft survival and have become part of routine practice (10,20-22). However, donor-recipient matching on other factors (e.g. age) could also influence pancreas graft survival, as it is shown that kidneys of older donors give better outcomes in older than in younger recipients (23). This could also be true for pancreas graft survival, but has not been examined.

The purpose of the present study was to retrospectively evaluate the impact of donor and recipient factors on 1-year and overall pancreas graft survival, and to compare their contribution in explaining graft survival differences between pancreas recipients.

METHODS AND MATERIALS

Patients

Between January 1997 and September 2008 a total of 170 pancreas transplantations (158 SPK and 12 Pancreas After Kidney (PAK) transplantations) were performed at the Leiden University Medical Center in the Netherlands, with the number of transplantations

increasing from 9 per year in 1997 to 22 per year in 2007. All patients were insulin dependent diabetes mellitus type I. Patients undergoing SPK transplantation also had kidney insufficiency due to end-stage diabetic nephropathy. PAK recipients had previously received a kidney transplant (1 patient) or lost the pancreas graft after a previous SPK transplantation (11 patients). Pancreas Transplantation Alone was not performed in this period.

Donors

All donor pancreata were procured from multi-organ donations after brain death (DBD). Abdominal organs were mobilised and flushed "in situ" via the abdominal aorta with either cold University of Wisconsin (UW) or Histidine Tryptophane Ketoglutarate (HTK) organ preservation solution. Subsequently, the pancreata were procured "en bloc" with the spleen and stapled loop of the duodenum. In case of SPK transplantation, the kidney was procured with the ureter, renal vein and renal artery. Directly after procurement, the pancreata were packed and stored according to Eurotransplant guidelines and transported to our center (24,25).

Technical aspects

The procedure of SPK transplantation has been described previously (26,27). In short: a midline incision was made with both organs placed intraperitoneally. The kidney was placed in the left iliac fossa, with the renal vessels anastomosed end-to-side to the common or external iliac vessels. The pancreas was placed in the right iliac fossa, and the portal vein of the pancreas allograft was anastomosed end-to-side to the recipient's inferior vena cava or to the common or external right iliac vein. In most cases, the superior mesenteric and splenic arteries were reconstructed using a donor iliac artery Y graft. If the iliac artery could not be used (e.g. due to atherosclerosis), the brachiocephalic trunk or aortic arch of the donor were used for the arterial reconstruction. In some cases, no vascular reconstruction was performed due to anatomical abnormalities of the arterial vascularization of the pancreas allograft. Therefore, the pancreas graft was procured with the celiac trunk and the superior mesenteric artery together on the aorta patch. Arterial anastomosis in all pancreas grafts were performed end-to-side with one of the right common or external iliac arteries of the recipient. PAK transplantation was performed in a similar fashion. Pancreas transplants were either enteric (ED, n = 31) or bladder drained (BD, n = 139), with BD patients undergoing elective pancreas conversion 6 – 12 months after transplantation, as described previously (26,27).

Perioperative management

Prophylactic intravenous antibiotics were given for 24 hr perioperatively, consisting of benzylpenicillin 1x106 U four times per day, gentamycin 1.5 mg/kg once per

day, metrodinazol 500 mg three times per day and ceftazolin 1000 mg three times per day. Until the end of 2007, the immunosuppression regime consisted of prednisone, tacrolimus/cyclosporine and mycophenolate mofetil as maintenance immunosuppression and antithymocyt globulin (ATG) or daclizumab as induction treatment. Since the end of 2007, patients received a steroid-free regime with tacrolimus and mycophenolate mofetil as maintenance therapy and campath (preoperatively and the first postoperative day 15 mg subcutaneously) as induction treatment. Episodes of acute rejection are treated with solumedrol. Steroid-resistant rejections are subsequently treated with ATG.

Definitions and methods

Eurotransplant donor reports were reviewed retrospectively to assess donor characteristics included in the P-PASS score as well as other characteristics known to affect survival. Donor P-PASS scores were calculated as described by Vinkers et al. (16) from the following characteristics: age, Body Mass Index (BMI), intensive care unit (ICU) stay, cardiac arrest, last sodium, last amylase or lipase blood levels before procurement, and vasopressor dosage before procurement. Both the P-PASS score and the included individual characteristics were assessed. In addition, we collected data on the following donor factors known to affect graft survival: gender, ABO blood group, Human leukocyte antigen (HLA) type, Cytomegalovirus (CMV) infection, cause of death, hypotensive periods before procurement (Systolic Blood Pressure < 90 mm Hg and/or Diastolic Blood Pressure < 60 mm Hg) smoking and preservation fluid (7,10-12,21,22,28-32). Furthermore, patient records were reviewed retrospectively to assess graft survival, and the following recipient characteristics given their reported impact on graft survival (2,4,10,11,18,19,21,22,26,31-33):

- Preoperative recipient characteristics: age at transplantation, gender, BMI, ABO blood group, HLA type, duration and type of diabetes, duration and modality of dialysis, time on waiting list, preoperative anticoagulant therapy, positive anti-CMV antibody, last systolic and diastolic blood pressure before transplantation and last total cholesterol in blood before transplantation.
- Other (operative) factors: type of transplant (SPK or PAK), primary drainage (bladder or enteric), warm and cold ischemia time and postoperative anticoagulant therapy in addition to fraxiparine (GlaxoSmithKline inc, London, United Kingdom) 0.3 ml once per day, which was given to all patients postoperatively as prophylaxis.

All of these characteristics were assessed for each transplant, shortly before transplantation. In this way, recipient characteristics of patients receiving multiple transplants were not counted twice. Follow-up of graft survival was based on the last visit of the patient to the hospital or the outpatient clinic (or date of death in case of deceased patients). Mean duration of follow-up was 3.1 years, range [0 – 11 years].

Graft loss was defined as removal of the graft or return to exogenous insulin therapy. Patients who deceased with a functioning graft were censored at the time of death.

Statistical analysis

We calculated 1-year and overall survival rates for pancreas graft survival using the Kaplan-Meier method. Cox proportional hazard analysis was used to assess which donor and recipient factors significantly affected 1-year and overall pancreas graft survival. First, univariate analysis was performed for each of the following variables:

1. Donor factors: P-PASS score (> 17 versus <17), age, body mass index (BMI), length of ICU stay, last sodium blood level before procurement, last amylase blood level before procurement, last lipase blood level before procurement, cardiac arrest (yes/no), vasopressin use before procurement (yes/no), gender, cause of death (CVA or other), hypotensive periods (yes/no), smoking (yes/no), and preservation fluid (UW versus other)
2. Recipient factors: age at transplantation, gender, BMI, duration of diabetes, type of diabetes (type 1 or type 2), duration of dialysis, dialysis modality (hemo dialysis versus peritoneal dialysis), time on waiting list, pre- and postoperative anticoagulant therapy (yes/no), last systolic and diastolic blood pressure before transplantation, last total cholesterol before transplantation, type of drainage (bladder or enteric), type of transplant (SPK or PAK), warm and cold ischemia time.
3. Donor-recipient matching: age, gender, BMI, ABO blood group (yes/no, no meaning ABO compatible but non-identical), HLA type (yes/no), positive anti-CMV antibody (yes/no). For age and BMI, we assessed whether donor and recipient matched (yes/no) for either age group (<30, 30-40, >40 years) or BMI group (<20, 20-25, >25). These categories were chosen since these were used in the P-PASS score. For HLA, we assessed whether donor and recipient matched (yes/no) for HLA group (<5, >5 loci).

The adjusted R2 (% variance explained by the model) (34) was calculated for each variable and used as a measure of the importance of each variable in explaining the variance in graft survival.

Since the effect on graft survival in univariate analysis may be confounded by other factors, a multivariate analysis was performed, including only variables significantly influencing graft survival in univariate analysis. To assess the relative importance of donor factors versus recipient factors versus donor-recipient matching, we included the variables in separate blocks of donor factors, versus recipient factors versus donor-recipient matching. In case that none of the factors in a particular block showed a significant effect on graft survival in univariate analysis, we included the factor explaining the highest percentage of variance. The adjusted R2 (% variance explained by the model) was calculated for each block and used as a measure of the

importance of each block in explaining the variance in graft survival. In this way, we were able to compare the contribution of donor factors, relative to recipient factors and donor-recipient matching.

A P value of less than 0.05 was considered statistically significant in all analyses.

RESULTS

Donor and recipient characteristics of the 170 pancreas transplantations performed during the period 1997-2008 are listed in Table 1. In accordance with Eurotransplant

Table 1. Characteristics of 170 pancreas transplantations in the Leiden University Medical Center (1997-2008)

	SPK transplants (n=158)	PAK transplants (n=12)	All transplants (n=170)
Donor characteristic		**Mean ± SD or N (%)**	
P-PASS score			
< 17	81 (51.3%)	7 (58.3%)	88 (51.8%)
17+	40 (25.3%)	5 (41.7%)	45 (26.5%)
missing	37 (23.4%)	0 (0.0%)	37 (21.8%)
Age (years) [1]	32.8 ± 12.1	30.6 ± 14.5	32.7 ± 12.2
Body mass index (kg/m2) [1]	23.1 ± 3.2	24.0 ± 2.4	23.1 ± 3.2
ICU stay (days) [a, 1]	2.5 ± 2.6	2.7 ± 4.1	2.5 ± 2.7
Last sodium blood level before procurement (mEq/l) [1]	144.7 ± 7.3	146.7 ± 6.5	144.9 ± 7.2
Last amylase blood level before procurement (U/l) [b, 1]	147.7 ± 168.1	178.8 ± 180.8	150.0 ± 168.7
Last lipase blood level before procurement (U/l) [c, 1]	50.4 ± 66.4	46.3 ± 50.0	49.7 ± 63.6
Cardiac arrest [d, 1]	15 (9.6%)	3 (25.0%)	18 (10.7%)
Vasopressin use before procurement [1]	125 (79.1%)	9 (75.0%)	134 (78.8%)
Male gender	76 (48.1%)	5 (41.7%)	81 (47.6%)
Smoking [f]	58 (39.5%)	5 (45.5%)	63 (39.9%)
Cytomegalovirus infection	67 (42.2%)	1 (8.5%)	68 (40.0%)
Cause of death			
CVA	86 (54.4%)	6 (50.0%)	92 (54.1%)
Other	72 (45.6%)	6 (50.0%)	78 (45.9%)
Hypotension [2]	50 (31.6%)	5 (41.7%)	55 (32.4%)
Hypotension duration (min) [d]	8.0 ± 20.6	10.8 ± 14.4	8.2 ± 20.2
Preservation fluid			
UW	149 (94.3%)	12 (100%)	161 (94.7%)
Other	9 (5.7%)	0 (0%)	9 (5.3%)
Recipient characteristic			
Age (years)	41.5 ± 7.4	43.4 ± 4.6	41.6 ± 7.3
Male gender	92 (58.2%)	4 (33.3%)	96 (56.5%)
Body mass index (kg/m^2)	23.5 ± 3.1	23.8 ± 2.2	23.6 ± 3.1
Duration of Diabetes (years) [f]	29.2 ± 7.3	32.6 ± 5.6	29.4 ± 7.3
Dialysis preoperative [g]	100 (63.3%)	0 (0%)	100 (58.8%)
Duration of dialysis (months) [g]	1.2 ± 1.4	0.0 ± 0.0	1.2 ± 1.4
Modality of dialysis [g]			
Haemodialysis	35 (35.0%)	0 (0%)	35 (35.0%)
Peritoneal dialysis	65 (65.0%)	0 (0%)	65 (65.0%)
Time on waiting list (months)	15.9 ± 8.3	16.9 ± 13.3	16.0 ± 8.7
Positive anti-CMV antibody	63 (39.9%)	4 (33.3%)	67 (39.4%)

Preoperative anticoagulant therapy	44 (27.8%)	7 (58.3%)	51 (30.0%)
Last Systolic Blood Pressure preoperative (mmHg)	150.6 ± 24.9	149.3 ± 12.3	150.5 ± 24.2
Last Diastolic Blood Pressure preoperative (mmHg)	84.8 ± 12.2	83.7 ± 8.1	84.8 ± 11.9
Last Total cholesterol blood level preoperative (mmol/l) [h]	4.6 ± 1.2	4.7 ± 1.1	4.6 ± 1.2
Warm ischemia time pancreas (minutes) [j]	29.2 ± 7.7	28.9 ± 6.0	29.2 ± 7.6
Cold ischemia time pancreas (hours) [k]	12.9 ± 3.3	11.0 ± 2.8	12.8 ± 3.3
Drainage			
Enteric	28 (17.7%)	3 (25.0%)	31 (18.2%)
Bladder	130 (82.3%)	9 (75.0%)	139 (81.8%)
Postoperative anticoagulant therapy [3]	29 (18.4%)	7 (58.3%)	36 (21.2%)
Donor – recipient matching			
Matching on age (<30, 30-40, >40 years)	53 (33.5%)	3 (25.0%)	56 (32.9%)
Matching on gender (male, female)	115 (72.8%)	11 (91.7%)	126 (74.1%)
Matching on BMI (<20, 20-25, >25 kg/m^2)	77 (48.7%)	8 (66.7%)	85 (50.0%)
ABO blood group mismatch (ABO compatible, but non-identical) [h, 4]	6 (3.8%)	1 (8.3%)	7 (4.1%)
Donor-recipient HLA type mismatch (> 5 loci)	81 (51.3%)	5 (41.7%)	86 (50.6%)

[a] Data missing for 35 donors (35 SPK), [b] Data missing for 6 donors (6 SPK), [c] Data missing for 125 donors (120 SPK, 5 PAK), [d] Data missing for 2 donors (2 SPK), [e] Data missing for 12 donors (11 SPK, 1 PAK), [f] Data missing for 1 donor (1 PAK), [g] Data missing for 2 donors (1 SPK, 1 PAK), [h] Data missing for 1 donor (1 SPK), [j] Data missing for 3 donors (3 SPK), [k] Data missing for 7 donors (6 SPK, 1 PAK)

[1] Characteristics of the P-PASS: Preprocurement Pancreas Allocation Suitability Score
[2] Hypotension: last measured blood pressure before transplantation, Systolic Blood Pressure (SBP) <90 mm Hg and/or Diastolic Blood Pressure (DBP) <60 mm Hg
[3] Started independently of preoperative anticoagulant therapy
[4] Mismatches were 5 donor O, recipient B; 1 donor A, recipient AB; 1 donor B, recipient AB

regulations for pancreas allocation, donor age did not exceed 50 years and donor BMI did not exceed 30 kg/m2. Most grafts were matched on gender (74.1%) but not so much on age (32.9%) and BMI (50.0%). Death censored graft survival was 88.4% at 1 year, 82.3% at 3 years and 80.9% at 5 years. In total, 31 (18.2%) of the pancreas grafts were lost at some point during follow-up. Graft loss was due to thrombosis (n = 17), rejection (n = 5) or to an unknown cause (but patient returning to insulin dependence) (n = 9), comparable to other studies (35). 71% of the graft loss due to thrombosis were lost within 2 weeks, 82% were lost after 1 year and 100% after 2,5 years. For rejection, 20% was lost within 2 weeks, the remaining 80% was lost between 1,5 and 7 years after transplantation. 56% of the grafts lost due to an unknown cause was lost after 1 year, 100% was lost after 2,5 years.

Univariate analysis showed that several factors significantly increased the probability of graft loss and thus reduced graft survival: female gender, recipient total cholesterol, enteric graft drainage, and donor-recipient match on BMI (Table 2). In multivariate analysis, only enteric graft drainage and donor-recipient match on BMI remained as independent predictors of graft survival (Table 3). Because no donor

factors were found to significantly influence graft survival in univariate analysis, the last donor serum amylase before procurement was added as a variable in the multivariate analysis since this factor explained the highest percentage of variance (Table 4). Similar results were then shown, with female gender as an additional variable significantly reducing pancreas graft survival. Taken together, this model explained 11.6% of 1-year graft survival and 15.5% of overall graft survival.

When we excluded the PAK transplants from our analysis, similar results were found, except that enteric graft drainage was no longer a significant predictor for pancreas graft survival (even though results were in the same direction). Further exploration of the results regarding donor-recipient BMI match showed that pancreas graft survival was better in recipients with higher BMI than the donor, compared with recipients receiving a graft from a donor with similar BMI (BMI match). Graft survival in recipients with lower BMI than the donor was similar as in recipients with matching donor-BMI (data not shown).

The included donor characteristics explained 3.1% of the variance in 1-year graft survival and 3.6% of overall survival. Recipient characteristics were more important and explained 6.4% of the variance in 1-year survival and 10.0% of overall survival. Donor-recipient matching explained 2.6% of the variance in 1-year and 2.6% of overall survival. These results suggest that donor characteristics are approximately equally important for short-term and long-term graft survival, but that recipient factors remain most important in explaining the variance in graft survival.

DISCUSSION

The present study has shown that both donor and recipient characteristics as well as donor-recipient matching influence graft survival. Pancreas graft survival was reduced in female patients, who receive a graft from a donor with a similar BMI, with enteric graft drainage. While donor factors were equally important in explaining differences in short- and long-term pancreas graft survival, recipient factors remain most important and explain the largest proportion of the variance in both 1-year and overall survival.

In the Netherlands, the Leiden University Medical Center is the largest center performing pancreas transplantations. In 2007, 87% of all pancreas transplantations in the Netherlands were performed in our center (36). Even though all pancreas transplantations performed in our centre during the period 1997-2008 were included in the present study, thereby including all eligible patients, our results might (in theory) be influenced by selection. If pancreata from suboptimal donors (P-PASS > 17) were accepted only for the best, most optimal recipients, this may only slightly reduce survival rates, given the importance of recipient factors. Such selection would underestimate the effect of the P-PASS score on pancreas graft survival as the reduction in survival would have been larger when these pancreata were accepted randomly

Table 2. Univariate analysis of the impact of donor and recipient factors on 1-year and overall pancreas graft survival (Leiden University Medical Center, 1997-2008).

	1-year follow-up			Overall follow-up		
	Hazard Ratio	**95% CI**	**% variance**	**Hazard Ratio**	**95% CI**	**% variance**
Donor						
P-PASS > 17 vs < 17 a	1.86	0.72 – 4.81	2.14	1.59	0.67 – 3.77	1.11
missing vs < 17 a	0.49	0.11 – 2.27	2.14	0.78	0.28 – 2.15	1.11
Age (years)	1.04	1.00 – 1.08	1.97	1.02	0.99 – 1.05	0.80
Body mass index (kg/m2)	1.00	0.87 – 1.15	0.00	0.98	0.87 – 1.10	0.07
ICU stay (days)	1.11	0.98 – 1.26	1.64	1.08	0.95 – 1.23	0.89
Last sodium blood level before procurement (mEq/l)	0.97	0.91 – 1.04	0.47	0.98	0.93 – 1.03	0.35
Last amylase blood level before procurement (U/l)	0.99	0.99 – 1.00	3.06	1.00	0.99 – 1.00	3.88
Last lipase blood level before procurement (U/l)	1.00	0.99 – 1.01	0.99	1.00	0.99 – 1.01	0.51
Cardiac arrest (yes vs no)		Model could not be fitted			Model could not be fitted	
Vasopressin use before procurement (yes vs no)	0.99	0.33 – 2.97	0.00	0.82	0.33 – 2.04	0.10
Gender (female vs male)	1.62	0.64 – 4.10	0.62	1.53	0.70 – 3.34	0.68
Smoking (yes vs no)	1.78	0.69 – 4.61	0.88	1.48	1.66 – 3.30	0.57
Cause of death (CVA vs other)	1.91	0.73 – 5.03	1.07	1.29	0.60 – 2.77	0.25
Hypotensive period (yes vs no)	0.74	0.27 – 2.04	0.21	0.65	0.27 – 1.53	0.62
Preservation fluid (UW vs other)	0.43	0.10 – 1.88	0.59	0.57	0.14 – 2.42	0.29
Recipient						
Age (years)	1.00	0.94 – 1.07	0.01	1.00	0.95 – 1.05	0.01
Gender (female vs male)	**2.88**	**1.09 – 7.58**	**2.91**	**3.30**	**1.45 – 7.55**	**5.13**
Body Mass Index (kg/m2)	0.92	0.79 – 1.07	0.70	0.93	0.82 – 1.06	0.70
Duration of diabetes prior to transplantation (years)	1.03	0.97 – 1.10	0.67	1.00	0.95 – 1.05	0.01
Preoperative dialysis (yes vs no)	1.36	0.51 – 3.62	0.23	1.41	0.61 – 3.25	0.40
Duration of dialysis prior to transplantation (years)	1.00	0.71 – 1.40	0.00	1.08	0.84 – 1.40	0.20
Dialysis modality (hemo vs peritoneal dialysis)	0.16	0.02 – 1.23	4.98	0.65	0.23 – 1.82	0.72
Time on waiting list (months)	0.16	0.02 – 1.23	4.98	0.65	0.23 – 1.82	0.72
Preoperative anticoagulant therapy (yes vs no)	0.44	0.13 – 1.51	1.18	0.68	0.28 – 1.69	0.43
Systolic Blood Pressure (mmHg)	1.00	0.98 – 1.02	0.03	1.00	0.99 – 1.02	0.04
Diastolic Blood Pressure (mmHg)	1.00	0.96 – 1.04	0.00	1.00	0.97 – 1.04	0.02

Total cholesterol (mmol/l)	1.40	0.98 – 1.99	1.91	**1.41**	**1.05 – 1.91**	**2.80**
Type of drainage of the pancreas (ED vs BD)	**2.88**	**1.13 – 7.33**	**2.52**	**2.60**	**1.12 – 6.03**	**2.50**
Type of transplant (PAK vs SPK)	1.53	0.35 – 6.60	0.17	1.20	0.28 – 5.09	0.03
Warm ischemia time (minutes)	1.01	0.95 – 1.07	0.01	1.00	0.96 – 1.05	0.00
Cold ischemia time (hours)	0.88	0.75 – 1.04	1.56	0.94	0.83 – 1.07	0.53
Postoperative anticoagulant therapy (yes vs no)b	0.99	0.33 – 3.00	0.00	0.73	0.25 – 2.12	0.21
Match Donor - Recipient						
ABO blood group match (yes vs no)		Model could not be fitted		1.22	0.17 – 8.99	0.02
HLA match (>5 vs < 5 loci)	1.36	0.55 – 3.39	0.26	1.48	0.68 – 3.18	0.59
CMV infection match (yes vs no)	1.14	0.45 – 2.91	0.05	1.00	0.46 – 2.16	0.00
Age (<30, 30-40, >40 years; match vs no match)	1.88	0.76 – 4.62	1.07	1.22	0.56 – 2.66	0.14
Gender (male, female; match vs no match)	1.30	0.43 – 3.91	0.13	1.69	0.64 – 4.46	0.72
BMI (<20, 20-25, >25 kg/m2; match vs no match)	**3.97**	**1.32 – 11.97**	**4.28**	**3.12**	**1.32 – 7.38**	**4.43**

Hazard Ratio's in bold indicate significant differences
[a] P-PASS: Preprocurement Pancreas Allocation Suitability Score. This score is based on 9 donor characteristics: age, Body Mass Index, ICU stay, cardiac arrest, last sodium and amylase or lipase before procurement, vasopressor dosage before procurement (14).
[b] Started postoperatively independently of preoperative anticoagulant u

Table 3. Multivariate analysis of the impact of donor and recipient factors on 1-year and overall pancreas graft survival (Leiden University Medical Center, 1997-2008). Factors with a significant effect in the univariate analysis were included.

	1-year follow-up			Overall follow-up		
	Hazard Ratio	**95% CI**	**% variance**	**Hazard Ratio**	**95% CI**	**% variance**
Donor						
Recipient						
Gender (female vs male)	2.44	0.92 – 6.47		2.36	0.98 – 5.66	
Type of drainage of the pancreas (ED vs BD)	**2.65**	**1.04 – 6.80**		**2.96**	**1.23 – 7.14**	
Total cholesterol (mmol/l)	-	-		1.32	0.95 – 1.83	
			5.2%			8.4%
Match Donor - Recipient						
BMI (<20, 20-25, >25 kg/m2; match vs no match)	**3.64**	**1.20 – 11.04**		**2.94**	**1.22 – 7.04**	
			3.7%			3.8%
Total % variance explained			8.7%			11.9%

Hazard Ratio's in bold indicate significant differences

Table 4. Multivariate analysis of the impact of donor and recipient factors on 1-year and overall pancreas graft survival (Leiden University Medical Center, 1997-2008). Factors with a significant effect in the univariate analysis were included, as well as donor factor explaining most of the variance.

	1-year follow-up			Overall follow-up		
	Hazard Ratio	**95% CI**	**% variance**	**Hazard Ratio**	**95% CI**	**% variance**
Donor						
Last amylase blood level before procurement (U/l)	1.00	0.99 – 1.00		1.00	0.99 – 1.00	
			3.1%			3.6%
Recipient						
Gender (female vs male)	**2.89**	**1.00 – 8.34**		**2.81**	**1.10 – 7.14**	
Type of drainage of the pancreas (ED vs BD)	2.57	0.98 – 6.77		**2.85**	**1.15 – 7.05**	
Total cholesterol (mmol/l)	-	-		1.28	0.91 – 1.80	
			6.4%			10.0%
Match Donor - Recipient						
BMI (<20, 20-25, >25 kg/m2; match vs no match)	3.00	0.98 – 9.23		**2.46**	**1.01 – 6.02**	
			2.6%			2.6%
Total % variance explained			11.6%			15.5%

Hazard Ratio's in bold indicate significant differences

and thus also for less optimal recipients. However, given Eurotransplant allocation procedures, the surgeon decides whether quality of the graft is acceptable, after which it offered to the first patient on the waiting list. It therefore seems unlikely that our results were influenced to a great extent by such selection.

The donor and recipient characteristics found to influence pancreas graft survival in the present study have also been found in other studies (2,9,17,26). With respect to operative factors, it was found that our routinely used two-step approach of primary BD followed by elective ED after 6-12 months, with the aim to prevent short-term disadvantages of enteric drained grafts and long-term (urological) complications of related to bladder drainage, resulted in better graft survival consistent with previously shown results (26).

Matching donor and recipients on age has been shown to influence kidney graft survival (23,37). However, we did not find this for pancreas transplantation in our study. Donor-recipient matching on BMI on the other hand, was shown to increase graft loss, which to our knowledge has not been described before. Pancreas graft survival was shown to be better in recipients with higher BMI than the donor, compared to patients who received a graft from a donor with a similar BMI. Mean recipient BMI was 23.6 and only 6 recipients had a BMI higher than 30. A possible explanation may be that both recipients with high BMI and recipients with a very low BMI have worse outcomes than recipients with an average BMI, similar to the effects of BMI on cardiovascular mortality found in the general population (38-40). Graft survival in these patients is reduced particularly if these patients receive a graft from a donor with a similarly high or low (matched) BMI. These results should be tested and explained in further research.

Our method of quantifying the impact of donor versus recipient factors has not been shown before. Recipient factors were shown to be more important for graft survival than donor factors. The advantage of this method is that besides the assessment of which factors significantly influence pancreas graft survival, their importance in terms of their contribution to graft survival can also be established. Optimizing recipient factors thus seem more important for long-term survival than optimizing donor factors. This seems logical when considering that pancreas donors are highly selected, prior to procurement and transplantation. Because of this selection, the variation in donor factors (e.g. age) is much smaller than in recipient factors and would thus have a smaller effect in explaining differences in pancreas graft survival. Recipients on the other hand are selected to a smaller extent, in particular in more recent years in which pancreas transplantation is also offered to more high-risk patients (e.g. older patients with comorbidity) so that they differ far more in various characteristics that may influence survival. Further research may lead to improvement of this model by including other factors, which may result in a higher explained variance in survival.

In conclusion, even though both donor factors and donor-recipient matching explain part of the differences in short-term and long-term pancreas graft survival, recipient factors remain most important and explain the largest proportion of the variance in both 1-year and overall survival. Hence, emphasis should be placed in optimizing these recipient factors to improve graft survival after pancreas transplantation. Surgeons may thus choose to first optimize recipients factors, e.g. by treating comorbidity or cholesterol levels before transplanting the patient, to obtain better graft survival after transplantation.

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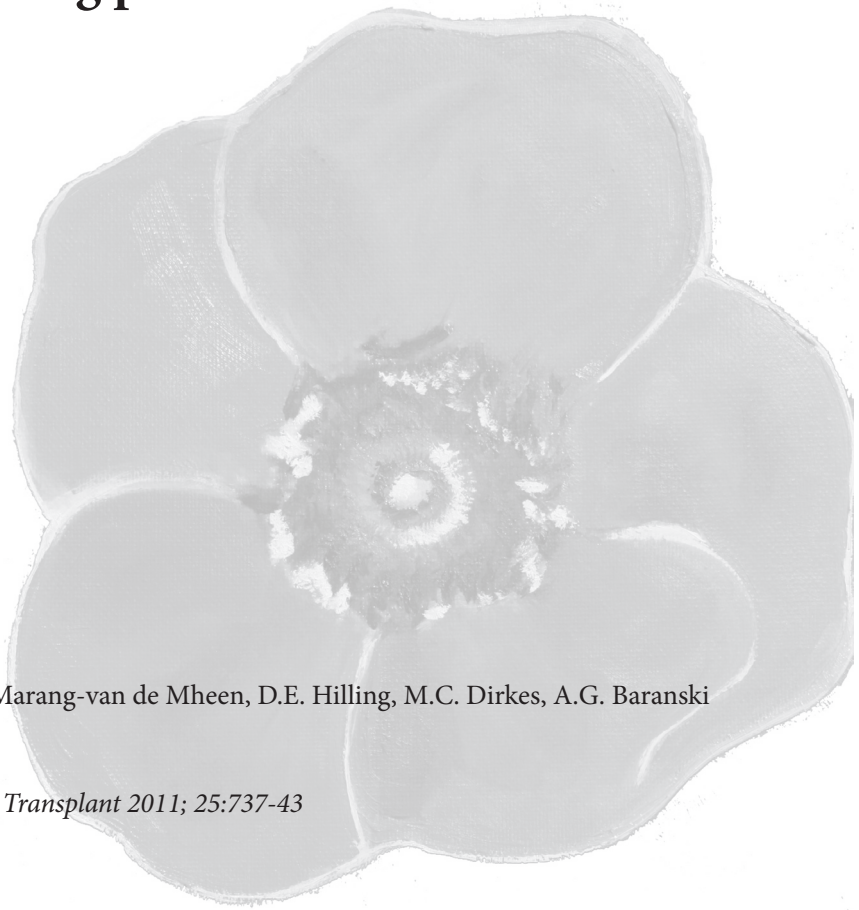
Chapter 3

Surgical injuries of pancreatic allografts during procurement



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Clin Transplant 2011; 25:737-43

ABSTRACT

Background

Quality of most procured pancreata is considered acceptable or good by surgeons, but remains difficult to ascertain. Little is known on how often pancreata are refused for transplantation during back-table inspection. Purpose of this study was to determine the frequency and type of problems responsible for refusal during back-table inspection, and to identify possible risk factors.

Methods and Materials

All 134 pancreata accepted and procured for whole-organ transplantation and transported to the Leiden University Medical Center in the period February 2002 until May 2008 were included. These were retrospectively analyzed on: donor characteristics, procurement characteristics and (non-)critical problems.

Results

A total of 111 (82.8%) pancreata were transplanted while 23 (17.2%) were refused for transplantation during back-table inspection, regardless of procurement region (χ^2 = 0.16 p = 0.93). Fourteen pancreata (13.4%) were refused solely due to surgical injuries. In refused pancreata, on average 2.7 critical problems per pancreas were found and 0.6 non-critical problems (versus 0.3 in transplanted pancreata, t = 1.83 p = 0.08). Chances of refusal increased in pancreata from older donors (Odds Ratio 1.08 [1.02 – 1.14]) procured in centers not performing pancreas transplantations (Odds Ratio 7.95 [2.43 – 25.97]).

Conclusions

We conclude that pancreatic allografts are frequently refused during back-table inspection, partly due to surgical injuries suggesting that quality of procurement may be improved.

INTRODUCTION

Since the first pancreas transplantation in 1966 (1), this procedure has developed into an acceptable treatment for diabetes type I. In the period 2002-2008, on average 21 pancreas transplantations are performed annually in the Netherlands of which 18 Simultaneous Pancreas Kidney (SPK) transplantations (2). Most (85%) of the pancreas transplantations in this period are carried out in the Leiden University Medical Center. The surgical procurement technique of this fragile organ is essential for good graft outcomes, but may be challenging for local procurement teams.

Pancreas procurement may be cancelled for reasons such as abnormal arterial vascularization between the liver and the pancreas making it impossible to successfully split and transplant both organs, problems relating to the organ itself (e.g. fibrosis) or neoplasms in the donor discovered during the organ donation procedure (3). Surgical injuries that occur during pancreas procurement may lead to complications after transplantation, impaired function of the allograft, graft loss or even death of the patient. These injuries may be so severe that the pancreas is not transplanted in order to protect the recipient. Proper procurement and constant training of surgeons are therefore very important to maintain high quality of abdominal organ procurement.

In the Netherlands, a pancreas is offered to the first patient on the national waiting list. If the pancreas is refused by the first center, then it is refused for all patients in that center (so regardless of any recipient risk factors) and is consequently offered to the next patient on the waiting list. Once accepted and transported to a center, a pancreas is only refused during back-table inspection if it is considered too dangerous for the patient to transplant the pancreas given for instance severe injuries that are encountered. Other recipient factors do not play a role anymore at this stage.

Little is known on how often pancreata are refused during back-table inspection. A recent report from Germany shows that vascular lesions were observed in three of the 18 (16.7%) pancreatic grafts, which could be transplanted after back-table repair procedures, but also suggests that procurement may be improved by better surgical training and standardization in procurement techniques (4). Schultz et al. (5) showed that 8% of the pancreatic grafts procured by teams that were not part of the pancreas transplant team, were discarded for transplantation during back-table preparation. Liposis of the graft and critical vessel situations (e.g. severe atherosclerosis) were reported as the main reasons for pancreas refusal. In the Netherlands, information on the type of problems encountered during back-table inspection is always returned to the procurement center on the standard Pancreas Quality Form for each pancreas procurement as feedback to the procurement team. However, this does not give procurement teams information on whether this was just a problem for this particular case, or that this type of problems occur more in their center than in others, because data on the most frequently encountered problems on a national level are not available.

Purpose of the present study therefore was to retrospectively evaluate all accepted pancreata transported to our center for transplantation in the period February 2002 until May 2008, to determine how often pancreata were refused for transplantation during back-table inspection and which type of problems were responsible for the decision not to transplant the pancreas. Furthermore, we aimed to determine whether donor characteristics, injuries or other factors in the procurement process may increase or decrease the probability of pancreas refusal. These findings may be used in training programs of organ procurement surgeons to avoid injuries and thereby improve the quality of procured pancreata.

MATERIALS AND METHODS

Technical aspects

All pancreata accepted, procured and transported for transplantation to the Leiden University Medical Center in the period February 2002 until May 2008 were included. Allografts primarily destined for islet transplantation were excluded. All allografts were procured in one of the contributing centers within the Eurotransplant zone and procured using standard procurement techniques (3, 6). According to the Dutch pancreas procurement protocol in that period, all abdominal organs are first mobilized, the common bile duct is ligated close to the pancreas head and transected. After organ perfusion, the duodenum is sterilized before it is closed with 50-80 ml povidone-iodine water solution together with Amphotericin B given through the nasogastric tube with the aim to decontaminate the duodenum content (3). After closure of the duodenum, the stomach, small bowel and colon are completely dissected and placed outside the abdomen. Then, liver and pancreas are separated starting with further dissection of the hepatoduodenal ligament. The gastroduodenal artery is transected and the pancreatic distal stump is tagged with a suture. The length of the portal vein and level of transection must be agreed upon by the procurement team, but is usually 2-3 cm above the pancreas head. Next, the celiac axis with the common hepatic artery is dissected along the superior edge of the pancreas head until the celiac trunk. The splenic artery is transected close to its origin and tagged with a suture to facilitate later identification. The spleen is always procured with the pancreas. To finish the pancreas procurement, the superior mesenteric artery (SMA) is transected carefully with a small aorta patch (3). As viability of the pancreatic allograft depends on restoration of the blood flow through the superior mesenteric and splenic artery, the procured vessels (mostly iliac arteries and veins) must have sufficient length to allow this mandatory reconstruction. In case of abnormal anatomical arterial vascularization of the pancreas (occurring in about 17% of the cases) when the dorsal pancreatic artery arises from the celiac trunk or common hepatic artery, the celiac trunk and the SMA on the aorta

patch were procured with the pancreas to ensure its best arterial vascularization (3). In all other cases, the dorsal pancreatic artery is not seen during organ procurement, so that the celiac trunk is procured with the liver. In our series, a simultaneous intestine-pancreas procurement did not occur. A right aberrant hepatic artery was never considered a contraindication for pancreas procurement.

In the Leiden University Medical Center, all organs are inspected by the transplant surgeon prior to taken the recipient to the operating room. All problems (or none if no problems were encountered) are reported on the Pancreas Quality Form, which is routinely used in the Netherlands and always faxed to the procurement center as feedback on the procurement. This form distinguishes between arterial problems, venous problems, duodenal problems, quality of parenchyma and other problems.

Data and definitions

For all pancreata, donor characteristics (age, gender, Body Mass Index (BMI)), preservation solution, pancreas anatomy and quality of procured organ, as assessed by the surgeon performing organ procurement, were obtained from the Eurotransplant Pancreas report. Furthermore, data were collected on type of problems reported by the pancreas transplant surgeon on the Pancreas Quality Form. Procurement centers were categorized into 3 regions: Netherlands West (Leiden, Rotterdam, Amsterdam, Utrecht), Netherlands East (Maastricht, Nijmegen, Groningen), and International (all pancreata procured outside the Netherlands). Furthermore, procurement centers were grouped based on whether or not they also performed pancreas transplantations (yes/no). Centers were categorized as not performing pancreas transplantations if they had not performed any pancreas transplantation in the entire period 2002-2008. Data on the number of pancreas transplantations per year per procurement center were obtained from Eurotransplant.

Problems reported on the Pancreas Quality Form were retrospectively categorized into critical and non-critical problems. Problems were considered critical if they were so severe that even when encountered alone, this was sufficient reason to refuse the pancreas for transplantation. Non-critical problems in itself are not responsible for pancreas refusal, but added to other problems may lead to refusal of the pancreas for transplantation. With respect to the type of problems, we distinguished between arterial injuries (head, neck, body or pancreas tail), venous injuries (portal, mesenteric superior or splenic vein), pancreas parenchyma injuries, duodenal and other problems, consistent with the categories on the Pancreas Quality Form. Atherosclerosis was considered severe if vascular reconstruction between the "tool-kit" and the pancreas was impossible, thereby increasing the risk on thrombosis.

Statistical analysis

We first estimated the frequency of pancreas refusal by the type of problem. Consequently, the frequency of refusal for transplantation was compared between procurement regions using chi-square tests, to assess whether some regions could improve more than others. Transplanted and refused pancreata were then compared on donor characteristics (age, gender, BMI), preservation solution, procurement region, procurement center performing pancreas transplantations (yes/no), average number of pancreas transplantations per year in procurement center, pancreas quality as assessed by the procurement surgeon, as well as on the number and type of critical and non-critical problems. Chi-square tests were used for categorical variables and *t*-tests for continuous variables. Variables that significantly differed between transplanted and refused pancreata were consequently entered in multivariate logistic regression analyses to assess whether these had an independent effect on the probability of refusal when adjusted for the other variables.

RESULTS

Of the 134 pancreata transported to our center, 111 (82.8%) were transplanted while 23 (17.2%) were refused during back-table inspection, regardless of procurement region ($\chi^2 = 0.16$ p = 0.93) (Table 1). The probability of refusal did not depend on whether the pancreas was procured in our own region (West of the Netherlands) or in another region (respectively 19% versus 16.3%, $\chi^2 = 0.15$ p = 0.70). In the 23 pancreata refused for transplantation, 63 critical problems occurred, ranging between one and five per pancreas. Fourteen pancreata (13.4%) were refused solely due to critical surgical injuries without any other critical problems. An example of a pancreas with one critical injury was a pancreas in which the parenchyma of the pancreas tail was completely destroyed. Within all regions, pancreata refused for transplantation more often were procured in centers not performing pancreas transplantations, or showed a trend towards significance (data not shown).

Nearly one-third of the pancreata refused for transplantation had severe atherosclerosis as a critical problem thereby increasing the risk on pancreas thrombosis,

Table 1. Pancreatic allografts by region of organ recovery: number of organs transplanted and refused for transplantation at back-table inspection (Leiden University Medical Center, February 2002 – May 2008)

Region of organ recovery	Transplanted Number (%)	Refused Number (%)	Total Number
Netherlands East	56 (83.6%)	11 (16.4%)	67
Netherlands West	34 (81.0%)	8 (19.0%)	42
International	21 (84.0%)	4 (16.0%)	25
Total	111 (82.8%)	23 (17.2%)	134

such that reconstruction became impossible (Table 2). Most critical injuries in the pancreata refused for transplantation concerned severe injuries of the pancreas parenchyma, superior mesenteric or splenic vein, and splenic or dorsal pancreatic artery such that reconstruction and transplantation became impossible (Table 2). In addition, 14 non-critical problems occurred in these pancreata, ranging from 0 to 3 per pancreas. In comparison, 33 non-critical problems occurred in transplanted pancreata, which did not differ from the pancreata refused for transplantation (on average 0.3 versus 0.6 in rejected pancreata, $t = 1.83$ $p = 0.08$). Most frequently occurring non-critical problems were portal vein injuries (or too short but with possibilities for reconstruction) or other problems like an open choledochal duct because of not ligating the common bile duct or severe atherosclerosis (which increases the risk on pancreas thrombosis) but with possibilities for reconstruction (Table 2).

The procurement surgeon also makes an assessment of the quality of the pancreas after procurement, reported in the Eurotransplant Pancreas report, which can be rated as poor, acceptable or good. Of the 23 pancreas allografts refused for transplantation,

Table 2. Frequency of critical and non-critical problems encountered in pancreatic allografts during back-table inspection (Leiden University Medical Center, February 2002 – May 2008)

		Pancreas transplanted (n=111)	**Pancreas refused** (n=23)
Critical problems			
Average number per pancreas (SD)		-	2.7 ± 1.6
Injuries			
Severe injuries pancreas parenchyma		-	17 (73.9%)
Arterial	– head, neck, body pancreas	-	1 (4.3%)
	– tail pancreas	-	8 (34.8%)
Venous	– Portal vein	-	7 (30.4%)
	– Splenic vein	-	9 (39.1%)
	– Mesenteric superior vein	-	7 (30.4%)
Other problems			
Duodenal problems (e.g. open duodenum)		-	6 (26.1%)
Severe artherosclerosis, reconstruction impossible		-	7 (30.4%)
Non-critical problems			
Average number per pancreas (SD)		0.3 ± 0.5	0.6 ± 0.8
Injuries			
Minor injuries pancreas parenchyma		6 (5.4%)	1 (4.3%)
Arterial	– head, neck, body pancreas	2 (1.8%)	1 (4.3%)
	– tail pancreas	4 (3.6%)	1 (4.3%)
Venous	– Portal vein (e.g. too short)	9 (8.1%)	0 (0%)
	– Splenic vein	0 (0%)	1 (4.3%)
	– Mesenteric superior vein	0 (0%)	1 (4.3%)
Other problems			
Duodenal problems (e.g. no povidone iodine)		8 (7.2%)	2 (8.7%)
Other (artherosclerosis but reconstruction possible, open ductus choledochus)		9 (8.1%)	9 (39.1%)

Values are mean ± SD

20 (87.0%) were assessed as a good-quality organ by the procurement surgeon (Table 3). Quality was not reported for the other three pancreas allografts. Part of the reason for the missing quality assessment may be that the procurement surgeon was not sure about the quality but thought that the pancreas may be potentially usable and needed to be examined on the back-table by someone more experienced. Of the 111 pancreas allografts that were transplanted, 31 (27.9%) had missing quality assessment, 2 (1.8%) were assessed as acceptable quality and 78 (70.3%) as good quality pancreas by the procurement surgeon.

All pancreas allografts were procured from deceased heart-beating donors. Pancreata refused for transplantation during back-table inspection on average were procured from older donors, with higher BMI, more often procured during office hours and by centers with significantly less experience in pancreas transplantation, compared with transplanted pancreata (Table 3). However, when looking at donor BMI $\geq$ 25, a risk factor for surgical complications and technical failure in pancreas recipients (7), the difference between refused and transplanted pancreas allografts was no longer statistically significant (Table 3). A higher percentage of male donors and on average more non-critical problems in refused pancreata showed a trend towards significance (Table 3). Because part of these differences may be caused by differences in some of the other variables, these variables were entered in a multivariate regression analysis. Only pancreata from older donors and procurement by centers not performing pancreas transplantation, were independent risk factors for pancreas refusal (Table 4). The probability of refusal increased by 8% per year increase in age of the donor, and was increased 8-fold for procurement teams from centers not performing pancreas transplantations.

DISCUSSION

This study has shown that pancreatic allografts are frequently refused during back-table inspection, partly because of surgical injuries. Most critical problems concerned severe injuries of pancreas parenchyma, superior mesenteric or splenic vein, and splenic or dorsal pancreatic artery such that reconstruction and transplantation became impossible, or severe atherosclerosis. Donor age and procurement by centers not performing pancreas transplantations were both found to significantly increase the probability of pancreas refusal. Quality of procurement may thus be improved by constant (compulsory) training of procurement surgeons by surgeons who perform pancreas transplantations, showing which type of injuries occur frequently, how to prevent these, and how to procure organs with severe atherosclerosis.

The frequency of refusal (17.2%) is higher than the 8% reported by Schultz et al. (5). They reported liposis of the graft and critical vessel situations as the main reasons for pancreas refusal, whereas parenchyma injuries and severe atherosclerosis were the

Table 3. Differences between pancreatic allografts transplanted and refused for transplantation at back-table inspection (Leiden University Medical Center, February 2002 – May 2008)

	Pancreas transplanted (n=111)	**Pancreas refused** (n=23)	**Test of difference**
Donor characteristics			
Age (years)	31.7 ± 12.6	39.5 ± 8.7	t=3.56 p<0.01
Age ≥ 35 years	48.6%	73.9%	X^2=4.88 p=0.03
Male gender	49.5%	69.6%	X^2=3.06 p=0.08
Body Mass Index (kg/m²)	23.0 ± 3.0	24.3 ± 2.0	t=2.72 p<0.01
BMI ≥ 25	24.3%	30.4%	X^2=0.38 p=0.54
Cause of death			
Brain bleeding	50.5%	65.2%	X^2=1.67 p=0.20
Trauma	36.0%	26.1%	X^2=0.84 p=0.36
Procurement			
UW preservation fluid	94.6%	91.3%	X^2=1.54 p=0.67
Good organ quality, assessed by procurement surgeon	70.3%	87.0%	X^2=2.80 p=0.25
Procurement time during the day[a]	27.0%	47.8%	X^2=3.88 p<0.05
Procurement center performing PTx	66.7%	21.7%	X^2=15.89 p<0.01
Average number of PTx per year in procurement center	4.7 ± 5.9	0.5 ± 1.0	t=-7.06 p<0.01
Number of non-critical problems	0.3 ± 0.5	0.6 ± 0.8	t=1.83 p=0.08

Values are mean ± SD

[a] Procurement between 8.00 and 18.00

Table 4. Determinants of pancreatic allografts being refused for transplantation (Leiden University Medical Center, February 2002 – May 2008)

	Odds Ratio [95% Confidence Interval]
Donor age (years)	**1.08 [1.02 – 1.14]**
Male donor	2.67 [0.85 – 8.43]
Donor Body Mass Index (kg/m²)	1.07 [0.86 – 1.33]
Procurement time (day[a] versus night)	2.45 [0.81 – 7.47]
Procurement center performing PTx (no versus yes)	7.95 [2.43 – 25.97]
Number of non-critical problems	2.18 [0.96 – 4.93]
Model fit: Nagelkerke R-square=0.391	

Odds Ratio's in bold indicate significant differences

[a] Procurement between 8.00 and 18.00

most frequent critical problems in our study, besides severe injuries of (pancreatic) vessels. One of the explanations may be a more strict selection of pancreatic grafts in our center. If this were true, transplanted pancreata from our center may be expected to have better graft survival rates. Schultz et al. (5) reported a 83% one-yr graft survival rate which is comparable to the rates reported by the International Pancreas Transplant Registry (IPTR) over the period 2000-2004 (8, 9). We have shown previously that pancreata transplanted in our center seem to have better graft survival

rates than the IPTR, in particular with primary bladder-drainage followed by elective enteric conversion 6-12 months later, used in most of the patients (10). All pancreas recipients in this study were insulin-dependent diabetes mellitus type I with end-stage diabetic nephropathy, and the rate of post-operative complications was comparable to that in other studies. Complications like enteric or bladder leaks, possibly related to procurement techniques, occurred as frequently as reported in other studies (10). Selection may thus explain part of the difference with the study by Schultz et al. However, given the large number of 63 critical problems found in 23 refused pancreata (on average 2.7 per pancreas), this does not seem to be the entire explanation. Another explanation may be that the study by Schultz et al concerned an earlier period (1994-2003) when it may have been customary that pancreata were procured by teams with experience in pancreas transplantation, or that solely by chance they were offered more organs procured in centers experienced in pancreas transplantation. Another option relating to this difference in time period is that the population of donors has become more marginal over time (11). Since we did not have more detailed data, this could not be further explored.

Donor age was found to increase the chances of pancreas refusal. One of the explanations is that it is a true age effect, e.g. reflecting increased atherosclerosis at older ages. Another option may be that procurement is more difficult in older donors, for because of the fattening of the pancreas. It is known that acceptable outcomes can be achieved with pancreatic grafts from older donors but that graft survival is reduced on average (11). If it is true that procurement is more difficult in older donors, it is likely that experienced procurement surgeons are performing organ procurement in older donors. Selection of experienced procurement surgeons may then interact with donor age, but would underestimate chances of refusal for pancreata procured from older donors rather than that it would increase refusal rates. Another explanation would be that transplant surgeons use more stringent criteria to accept an organ from an older donor, requiring the organ to be more 'perfect' than from a younger donor given that they know that graft survival is reduced on average (11). This hypothesis seems likely, but is difficult to test.

Higher chances of refusal were also found for procurement centers without experience in pancreas transplantation in the entire period. This makes sense because a pancreas transplantation surgeon may be more aware of potential consequences of procurement for pancreas transplantation, given that he has faced these problems and knows what is possible and what is not. On the other hand, it may seem contrary to results from previous studies, which have shown that early outcomes after SPK transplantation are not influenced by the surgical team (from the transplant center versus another center) (12). However, no information was given on the experience of the 'other center', which may have performed pancreas transplantations in recent years, whereas the reference category in our study concerned procurement centers

without any pancreas transplantations in recent years. Furthermore, given that the pancreata were transplanted, it may be assumed that the organs were well procured in the study by Fellmer et al. (12), whereas our study focused on pancreas refusal due to procurement problems. To our knowledge, this is the first study showing that chances of refusal are higher when pancreata are procured in centers without experience in pancreas transplantation.

Experience of the individual performing the procurement would be an important variable in this context, but unfortunately no data were available on the level of training of the procurement surgeon. In the Netherlands, as in other countries, trainees may be sent to procure pancreas allografts, supervised by a more experienced procurement surgeon. However, the latter surgeon may be more experienced but not necessarily in pancreas transplantation. It is therefore not clear whether procurement surgeons are experienced enough to always perform a well-procured pancreas, even in difficult cases. The decision to refuse the pancreas is thus made based on organ quality only. Experience or name of the procurement surgeon is not considered since even the most experienced surgeon may make a mistake or overlook something, and excellent quality procurement may be performed by relatively inexperienced surgeons.

It is important to note in this context that there is a difference between a pancreas with unrecognized damage, which is potentially dangerous if the injury goes unnoticed and expensive, and a pancreas that is considered potentially usable but needs to be examined during back-table inspection by a more experienced pancreas transplant surgeon. It seems more appropriate to let a more experienced pancreas transplant surgeon examine a graft from, e.g., an older donor, than to accept the opinion of a less experienced procurement surgeon that it is not transplantable. A recommendation may therefore be to add the option 'potentially usable, requires further examination' to the Eurotransplant Pancreas report in the assessment of organ quality, along with a specification of which part of the pancreas requires further examination.

Pancreata procured during office hours at first seem to be the best procured organs with the lowest chances for refusal, because these teams should be fresh. However, even though non-significant, our data seem to suggest the contrary. One of the explanations may be that procurement surgeons during the day are more junior, since the senior surgeons have other daytime commitments, suggesting procurement by less-experienced surgeons during the day. No data were available to test this hypothesis of seniority of retrieval teams, but it would give support to the evidence presented above that less experience – both in pancreas transplantation and procurement – results in higher refusal rates. Further research is needed to support or refute this hypothesis.

These results have important implications for current practice in pancreas procurement. Quality of pancreas procurement may be improved by reducing refusal rates, which can be achieved by more extensive and recurrent training of pancreas

procurement surgeons. Surgeons with experience in pancreas transplantation may be excellent teachers in such a training program. Another possibility to reduce pancreas refusal may be to leave pancreas procurement to those centers also performing pancreas transplantations, but this seems hard (if not impossible) to implement in practice. It seems better to complement training with annual feedback to each center on the extent to which procured organs could be transplanted compared to other centers, which may lead to further improvement if rates are lower than expected. Given the crucial importance and lack of organs, it is vital that all procured organs can be used and do not have to be discarded because of injuries inflicted in the procurement.

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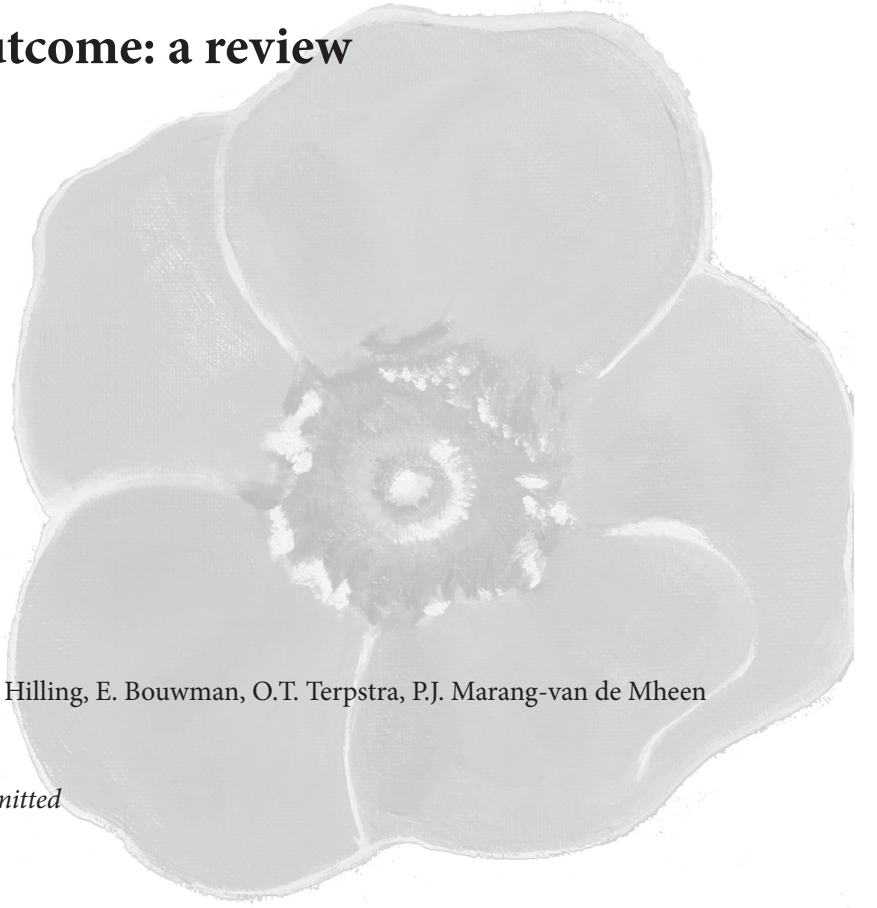
Part II

Human islet transplantation

Chapter 4

Effects of donor, pancreas and isolation-related variables on human islet isolation outcome: a review



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Submitted

ABSTRACT

Introduction

Different factors have been reported to influence islet isolation outcome, but vary between studies and are hampered by small study samples per study. The purpose of this study was to perform a systematic review to assess the impact of donor, pancreas and isolation-related variables on successful human islet isolation outcome.

Methods and Materials

Pubmed, Embase and Web of Science were searched electronically in April 2009. All studies reporting on donor, pancreas and isolation-related factors relating to pre-purification, post-purification islet isolation yield and proportion of successful islet isolations were selected. 74 retrospective studies had sufficient data and were included in the analyses.

Results

Higher pre-and post-purification islets yields and a higher proportion of successful islet isolations were obtained when pancreata were preserved with TLM, rather than UW in donors with shorter cold ischemia times (one hour longer cold ischemia time resulted in an average decline of pre-purification, post-purification yields and proportion of successful isolations of 59IEQ/g, 54 IEQ/g and 21%, respectively). Higher pre-purification yields and higher percentage successful islet isolations were found in younger donors with higher BMI. Lower yields were found in donation after brain death (DBD donors) compared to donation after cardiac death (DCD donors). Higher post-purification yields were found for isolation with Serva collagenase.

Conclusion

This review identified donor, pancreas and isolation-related factors that influence islet isolation yield. Standardized reports of these factors in all future studies may improve the power, identify additional factors and thereby contribute to improving islets isolation yield.

INTRODUCTION

Transplantation of islets of Langerhans can improve metabolic control and quality of life in patients with longstanding type 1 diabetes. Despite improvement and standardization of isolation procedures, the outcome of human islet isolation remains unpredictable and highly variable. Furthermore, generally more than one islet preparation is required per recipient to achieve insulin independence after transplantation (1-6).

Previous studies have reported donor and other factors associated with higher success rates in terms of attaining adequate islet numbers for transplantation (7-13). However, different factors have been identified and large-scale trials in humans demonstrating the influence of a set of donor factors are lacking. Because previous studies are relatively small, factors could be missed. Therefore, different factors could be identified when studying larger numbers of donors and the question remains which factors independently affect islet isolation outcome when corrected for the effect of other variables.

Because there is a shortage of donor pancreata relative to the needs of potential transplant recipients, optimal use of the available donor organs is vital. We carried out a systematic review of the literature on human studies reporting on donor, pancreas and isolation-related factors and their influence on isolation outcome. In this way we can identify factors that have an independent effect on islet isolation outcome.

METHODS AND MATERIALS

Study selection

PubMed, Embase and Web of Science were searched to retrieve articles in English on human islet isolation from 1966 onwards.

The following search string was used:
("Islets of Langerhans Transplantation"[Mesh] OR (("Islets of Langerhans"[Mesh] OR "islets"[all fields]) AND ("transplantation"[MeSH Terms] OR "transplantation"[All Fields])))
AND
("isolation"[all fields] OR "Cell Separation"[Mesh] OR "Separation"[all fields] OR "Tissue and Organ Harvesting"[Mesh] OR "Harvesting"[all fields] OR "Tissue and Organ Procurement"[Mesh] OR "Organ Preservation Solutions"[Mesh] OR "Solution"[all fields] OR "Solutions"[all fields] OR "tissue donors"[MeSH Terms] OR "donor"[All Fields] OR "donors"[All Fields])
AND

(yield[All Fields] OR yields[All Fields] OR "isolation outcome"[All Fields] OR "isolation outcomes"[All Fields] OR "isolation result"[All Fields] OR "isolation results"[All Fields] OR harvest[All Fields] OR profit[All Fields] OR profits[All Fields] OR earnings[All Fields] OR earning[All Fields] OR output[All Fields] OR "success rate"[All Fields] OR "success rates"[All Fields] OR "recovery"[All Fields])

The search resulted (by April 2009) in 412 Pubmed, 60 Embase and 228 Web of Science titles, constituting a total of 702 titles. Two independent reviewers (DEH and PJMvdM) examined titles and read relevant abstracts to decide if the full-text articles should be obtained. Cases of disagreement were resolved by discussing the title and abstract. Full-text articles (n = 141) were examined and selected based on the following criteria: (1) Reporting on either donor, pancreas or isolation-related variables and their relation to islet isolation outcome, (2) Reporting isolation outcome in IE/g pancreas pre- or post-purification (3) sufficient specification of "successful" and "unsuccessful" islet isolation outcome as used in that study, (4) sufficient specification of donor organs used for islet isolation procedures with respect to selection characteristics.

Exclusion criteria were: (1) Histologically obtained pancreas variables and their relation to islet isolation outcome (2) Animal donor, pancreas and isolation-related variables and their relation to islet isolation outcome

Literature references were checked to minimize the risk of missing relevant studies. For duplicate papers reporting on the same study, we selected the article that reported the most complete and detailed data. This resulted in a total of 74 studies, eligible for further analysis (7-12, 14-81).

Data extraction

Data were extracted independently by DEH and PJMvdM by means of a predefined form. The following topics were included based on data availability in at least 50% of the studies:

General variables: year of index admission, country of study, number of pancreata in the study

- Donor pancreas variables: age, body mass index (BMI), last serum glucose before procurement, donation after brain death (DBD donors)/donation after cardiac death (DCD donors)
- Pancreas variables: pancreas weight, cold ischemia time (CIT), method of preservation
- Isolation variables: method of purification (continuous vs discontinuous and Ficoll vs other), brand of collagenase
- Study results: islet isolation outcomes in terms of pre-purification isolation yield, post-purification isolation yield, proportion of successful islet isolations (according to the definitions in the particular study).

Statistical analysis

Since the number of pancreata varied considerably between studies we weighted all isolation outcomes by the number of pancreata per study in all analyses. We studied the previously listed variables with respect to their relation with 3 outcomes: pre-purification isolation yield, post-purification isolation yield, and proportion of successful islet isolations.

We first performed univariate analysis, relating each variable to each of the 3 outcomes. However, since the effect of some factors on isolation outcome may be confounded by others, a multivariate analysis was performed, including only the variables that had a significant effect on isolation outcome in the univariate analysis. In this way, the independent effect of each of the variables on the 3 outcomes was assessed. The analysis with the outcome proportion of successful islet isolations was adjusted for differences between studies in the criteria used to define successful by including the criterium as a variable in the multivariate analysis.

RESULTS

A total of 74 studies met our inclusion criteria, all retrospective studies. When studies compared different groups in relation to isolation outcome (e.g. TLM vs UW), these were included as separate groups, giving a total of 132 groups that were finally compared in the analysis.

When studies addressed both pre- and post-purification isolation yield and/or proportion of successful isolations, we included the studies in the analyses of each outcome.

Pre-purification isolation yield

Thirty-nine studies (7, 9-12, 14, 20, 21, 27, 31, 32, 34, 37-45, 48-57, 59, 62-64, 71, 75, 76, 80), 70 groups in total, reported characteristics influencing pre-purification isolation yield. Univariate analysis showed several factors to significantly affect pre-purification isolation outcome (Table 1): higher yields were obtained in studies with younger donors, with higher BMI, without a last glucose or a low last glucose reported, with relatively few DBD donors, short cold ischemia time and preservation with TLM rather than UW. These effects remained in multivariate analysis (Table 2), suggesting that each of these factors independently influenced pre-purification yield. For example, from donors who are one year older, on average a 64IEQ/g lower pre-purification yield was obtained. Furthermore, when cold ischemia time was 1 hour longer, on average a 59IE/g lower pre-purification yield was obtained, independently from other factors.

Less than 50% of the included studies reported data on pancreas weight and isolation specific characteristics so these were excluded from the analysis.

Post-purification isolation yield

Fifty-nine studies (7, 9-12, 14-20, 23-27, 30, 31, 33-37, 39-47, 50-56, 59-63, 65-70, 72-79, 81), 106 groups in total, reported characteristics related to post-purification isolation yield. Univariate analysis showed several factors to significantly affect post-purification isolation outcome (Table 1): higher yields were obtained in studies without a last glucose or a low last glucose reported, with relatively few DBD donors, short cold ischemia time, preservation with TLM rather than UW, purification with Ficoll and isolation with Serva collagenase. In multivariate analysis (Table 2), these effects remained as independent significant effects influencing post-purification isolation yield, except for last glucose before procurement and purifcation with Ficoll. For example, when cold ischemia time was 1 hour longer, on average a 54IEQ/g lower post-purification yield was obtained, independently from other factors.

In contrast with pre-purification yield, age, BMI and last glucose before procurement are no independent predictors of post-purification yield.

Less than 50% of the included studies reported data on pancreas weight and isolation specific characteristics so these were excluded from the analysis.

Proportion of successful isolations

Thirty-one studies (7-12, 22, 23, 28-30, 32, 34, 36, 38, 44, 48, 51, 52, 57, 58, 60, 66, 68, 69, 73-75, 78-80), 57 groups in total, reported characteristics related to the proportion of successful isolations. In univariate analysis (Table 1) higher yields were obtained in studies with younger donors, with higher BMI, without a last glucose or a low last glucose reported, with relatively few DBD donors, short cold ischemia time, higher pancreas weight and preservation with TLM rather than UW. In multivariate analysis (Table 2) these effects remained as independent predictors of a high percentage of successful isolations, except that higher percentage successful isolations were found in studies that did reported the last glucose before procurement. Furthermore, the percentage DBD donors had no independent significant influence on the percentage of successful islet isolations.

For example, from donors who are one year older on average a 1% lower percentage of successful isolations was obtained. Furthermore, when cold ischemia time was 1 hour longer, on average a 21% percentage of successful isolations was obtained, independently from other factors.

In contrast with pre-purification yield, percentage DBD donors is not and pancreas weight is an independent predictor as well as age, BMI and last glucose before procurement in contrast with post-purification yield.

In total, data of 2198, 4122 and 2769 pancreata were available for uni- and multivariate analysis of pre- and post-purification yield and proportion of successful islet isolations, respectively. However, in univariate analysis, 12.5% to 33.7% of the pancreata were

excluded in at least 1 analysis due to missing data. In multivariate analysis this was even higher (79.4-89.7%) since studies had to report on all of the variables included in the analysis, to have their pancreata included.

DISCUSSION

The present study has shown that donor, pancreas and isolation-related factors have an influence on both pre- and post-purification islet isolation outcome, as well as on proportion of successful islet isolations.

Higher islets yields and a higher proportion of successful islet isolations were obtained when pancreata were preserved with TLM, compared to UW. This is in accordance with Agrawal et al (13). In their meta-analysis, significantly higher yields were found in pancreata preserved with TLM compared to UW. However, in their study, they found an equal rate of successful islet isolations in both groups. A possible explanation for this difference with our study could lie in the fact that in our multivariate analysis, the influence of TLM is corrected by other factors that have an influence on islet isolation yield.

Higher BMI and shorter cold ischemia times were also associated with higher islet isolation outcome pre- and post-purification as well as with a higher percentage of successful isolations when looking at cold ischemia time. Since larger islets are usually encountered in patients with higher BMI to obtain the higher insulin demand and longer cold ischemia times result in more damage to the islets, these results seem to have face validity and have been well reported in previous studies (11, 12, 19, 52, 58, 60, 82).

Our study showed lower isolations yields and proportion of successful isolations in studies with a higher percentage of DBD donors. This is remarkable since in previous studies, generally, higher yields were found in DBD donors compared to DCD donors. However, successful islet isolations from DCD donors have also been reported previously (3, 48, 81, 83). In our multivariate analysis, studies with a large percentage of DBD donors had significantly lower yields in pre-, post-purification isolation outcome and also a lower proportion of successful isolations, when adjusted for the effects of other variables. Part of the explanation could be that in previous studies there was insufficient power to correct for other variables. In the studies that did correct for other factors, the results could be prone to the effect of the other, potentially underreported, factors in the models. This last explanation could also have an effect on our results as well. Furthermore, results of different studies can not be easily compared without correcting for certain factors like age, since an age difference of 1 year has an influence on pre-purification islet yield of 64IEQ/g.

This study is a first attempt to look at the effect of donor, pancreas and isolation-related factors on isolation outcome. When reports of these variables in future

Table 1. Factors influencing pre- and post-purification isolation yield and proportion of successful islet iolations

Univariate analysis

	Pre-purification isolation yield		Post-purification isolation yield		Proportion of successful islet isolations	
	B	95% CI	B	95% CI	B	95% CI
Donor						
Age (yrs)	-74.83	-85.97; -63.68	NS		-1.08	-1.36; -0.81
Body Mass Index (kg/m2)	59.78	45.03; 74.53	NS		1.84	1.44; 2.24
Last serum glucose before procurement (mmol/l)	-13.14	-18.10; -8.19	-2.70	-4.29; -1.11	-0.10	-0.19; -0.02
Last serum glucose before procurement reported (yes/no)	-509.35	-630.48; -388.22	-173.10	-279.78; -66.43	-4.70	-8.02; -1.39
Percentage DBD donors	-24.32	-28.12; -20.53	-15.59	-20.82; -10.36	-0.38	-0.54; -0.23
Pancreas						
Cold ischemia time (hours)	-46.83	-66.67; -26.99	-72.27	-89.69; -54.84	-1.63	-2.35; -0.91
Pancreas weight (g)	*		*		1.23	1.08; 1.39
Preservation fluid (TLM vs UW)	713.83	544.23; 883.43	596.84	463.11; 730.58	14.82	8.59; 21.05
Isolation						
Brand of collagenase (Serva vs other)	*		1688.03	1529.14; 1846.92	*	
Brand of collagenase (Sigma vs other)	*		-1122.67	-1253.74; -991.58	*	
Purification (continuous vs discontinuous)	*		NS		*	
Purification (Ficoll vs other)	*		104.74	4.93; 204.55	*	

* Data available in less than 50% of the studies

Table 2. Factors influencing pre- and post-purification isolation yield and proportion of successful islet iolations

Multivariate analysis

	Pre-purification isolation yield		Post-purification isolation yield		Proportion of successful islet isolations	
	B	95% CI	B	95% CI	B	95% CI
Donor						
Age (yrs)	-64.07	-86.50; -41.64	*		-1.04	-1.22; -0.87
Body Mass Index (kg/m2)	151.62	110.63; 192.61	*		8.02	7.26; 8.77
Last serum glucose before procurement reported (yes/no)	-1929.34	-2180.30; -1678.38	NS		57.61	55.66; 59.55
Percentage DBD donors	-32.35	-37.93; -26.78	-11.31	-15.37; -7.25	Constant in model	
Pancreas						
Cold ischemia time (hours)	-58.74	-94.37; -23.11	-54.36	-74.55; -34.17	-21.08	-21.91; -20.25
Pancreas weight (g)	*		*		1.81	1.68; 1.94
Criterium successful isolation (IEQ)	*		*		-0.01	-0.01; -0.01
Preservation fluid (TLM vs UW)	788.36	564.52; 1012.20	237.05	56.12; 417.98	32.88	31.33; 34.43
Isolation						
Brand of collagenase (Serva vs other)	*		1290.56	1112.85; 1468.26	*	
Brand of collagenase (Sigma vs other)	*		-650.88	-943.12; -358.65	*	
Purification (continuous vs discontinuous)	*		*		*	
Purification (Ficoll vs other)	*		NS		*	

* Data available in less than 50% of the studies or not significant in univariate analysis

studies would be standardized we could possibly identify other factors and make more accurate estimation of the independent effect of these factors. To illustrate the necessity of these standardized reports, we have looked at the missing variables in our analysis. In univariate analysis 66.3-87.5% of the available pancreata were analyzed on the effect on pre- and post-purfication yield or percentage of successful isolation. In multivariate analysis this percentage was only 10.3-20.6%, due to missing data on at least 1 of the variables included. This indicates that the studies differ to such a great extent in the variables that they report, even when we selected only those variables that were reported in most studies.

Standardized reporting of the factors in all studies in the future on a minimal set of variables would also lead to a better fit of the model used in any meta-analysis. In the current analysis on post-purification yield 19% of the variance in islet isolation outcome could be explained by the included variables. In pre-purification islet yield and proportion of successful isolations, this percentage was better, but still only 50% of the variance could be explained. This suggests that besides the reported variables other factors also influence isolation outcome.

In conclusion, this study identified donor, pancreas and isolation relating factors that influence islet isolation yield. However, standardized reports of these factors are lacking, and are needed to get more reliable evidence. To improve the power and provide better comparisons in future research, standardized reporting of these factors are recommended.

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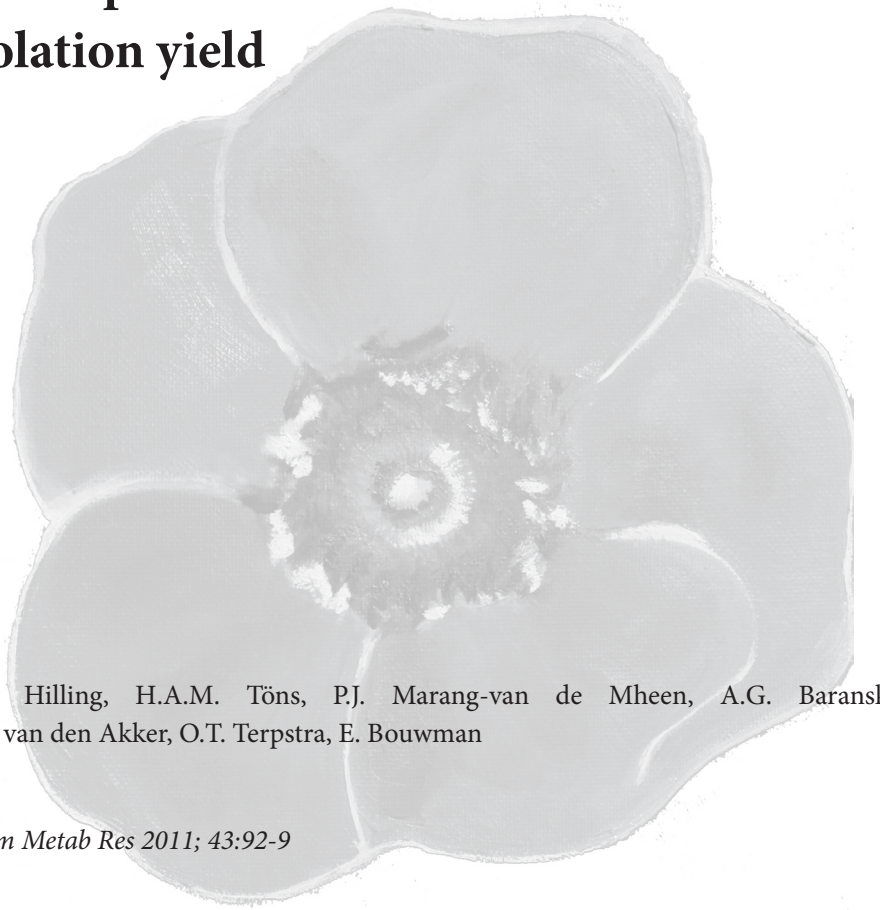
Chapter 5

Presence of hyperemic islets in human donor-pancreata results in reduced islet isolation yield



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Horm Metab Res 2011; 43:92-9

ABSTRACT

Background

When studying histological characteristics of human donor-pancreata, a remarkably high number of hyperemic islets (HIs) was encountered. The abnormalities in these HIs ranged from single/multiple dilated vessels to hemorrhages extending into the exocrine tissue. We aimed to determine the relevance of the presence of HIs in human donor-pancreata for isolation outcome and to identify donor and procurement factors associated with the occurrence of HIs.

Methods and Materials

The presence of HIs was scored semi-quantitatively (HI-,HI+) in 102 human donor-pancreata. Islet isolation was performed in 40 cases. Donor and procurement factors were retrospectively analyzed in 94 donors.

Results

HIs were found in 54.6% of all donor-pancreata. However, only 4.5% of all islets in the affected pancreata was hyperemic. The affected pancreata contained slightly more endocrine tissue, but produced significantly lower yields. When corrected for other factors known to influence isolation outcome, the presence of HIs and endocrine content were the only factors significantly influencing isolation outcome. Prolonged ICU stay and pre-procurement hypertension were associated with the presence of HIs.

Conclusions

This study is a first indication that the presence of HIs in human donor-pancreata are associated with reduced isolation outcomes and suggest an impact of the procurement procedure and pre-procurement hemodynamic status of the donor on the islet quality. It is tempting to speculate that this contributes to the generally experienced difficulties in obtaining sufficient amounts of human islets.

INTRODUCTION

Transplantation of islets of Langerhans can improve metabolic control and quality of life in patients with longstanding type 1 diabetes (1-4). Sufficient islet numbers can be obtained from a single donor, but generally more than one islet preparation per recipient is required to obtain insulin independence (1, 3, 5-7). An important factor in determining the islet isolation outcome is the amount of endocrine tissue present in a specific pancreas. However, a high endocrine content does not ensure a high isolation yield. Other factors, such as collagen and other matrix elements are thought to play a role (8-15). When studying histological characteristics of the human pancreas in relation to islet isolation, a remarkably high number of hyperemic islets (HIs) was encountered, a phenomenon that, besides our previous report in pigs (16), has not been described in detail before. The abnormalities observed in these HIs ranged from a single dilated vessel through multiple widely dilated vessels to hemorrhages extending into the surrounding exocrine tissue. In some cases, the endocrine tissue was reduced to a small rim of cells, with only a few scattered cells left, which may consequently affect isolation yield.

The aim of the present study was to assess the frequency and different types of HIs in human donor-pancreata. Furthermore, we studied the occurrence of HIs in relation to the amount of endocrine tissue in situ and the relation to islet isolation outcome. In addition we assessed donor and procurement factors to identify possible factors associated with the presence of hyperemic islets.

MATERIALS AND METHODS

Organ Procurement and Pancreatic Sampling

A total of 102 human pancreata were obtained through Eurotransplant. The organs were obtained from 66 multi-organ donations after brain death (DBD) and 36 multi-organ donations after cardiac death (DCD), for which consent for islet isolation and transplantation related research was given by relatives. The majority of the obtained organs was unsuitable for whole organ transplantation. However, all organs were procured and handled in the same way as the organs that were procured for whole organ transplantation. Organs were flushed in situ via the abdominal aorta with either cold University of Wisconsin (UW) or Histidine Tryptophane Ketoglutarate (HTK) organ preservation solution and removed “en bloc” with the spleen and stapled loop of the duodenum.

After dissection, the pancreata were stored in cold (4°C) UW or HTK (with the exception of 2 pancreata which were stored in another preservation solution) and transported according to Eurotransplant regulations, on ice, to the laboratory for

further processing. Mean (± SD) cold ischemia time (CIT, the interval between the aortic cross-clamp and initiation of the digestion procedure in the laboratory) was 8.0 ± 3.2 h. The pancreata were dissected free of spleen, duodenum, surrounding fat and vessels in the laboratory. Before the isolation procedure, biopsies of head, neck and body of the pancreas were taken, immersed in Formalin (Klinipath, Duiven, The Netherlands) fixative for 24-48 h and subsequently cleared and stored in ethanol 70%.

Histology

From each pancreas, paraffin-embedded sections of 5 μm were stained using the Aldehyde Fuchsine-Halmi (AF) technique: Rehydration, 90 s 2.5% potassium permanganate, 2' distilled water, 2' 1% oxalic acid, 10' running water, 1' distilled water, 15' AF, 3' 90% ethanol (2 times), 1' 70% ethanol, 1' 50% ethanol, 5' aquadest, 10-60 s Halmi, 10 seconds 0.2% acetic acid, 2' 100% ethanol (2 times), 5' xylene (2 times). Sections were embedded in malinol (Chroma-Gesellschaft, Köngen, Norway).

Microscopic evaluation

Islet sizes were determined in each pancreas using a calibrated grid and grouped into 8 categories: >50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400 and >400 μm diameter. As is generally practiced in the endocrine quantification in islet isolation procedures, islets smaller than 50 μm were not included because of their neglectable contribution to the total endocrine content. For determination of the endocrine content and the islet numbers, all islets in a tissue area of 1 cm^2 per slide were assessed. The endocrine area density was determined by summation of all calculated (πr^2) islet areas and expressed as percentage of the total area. According to the principle of Delesse (area density = volume density) and the assumption that islets are spherical, the determined endocrine area represents the endocrine content.

Islets described as hyperemic in this study ranged from islets showing only a single slightly dilated vessel to hemorrhages extending into the surrounding exocrine tissue (Fig.1). The presence of HIs was scored semi-quantitatively (HI-, HI+). HI- pancreata contained none and HI+ pancreata at least one hyperemic islet.

To assess the influence of the severity of the hyperemia, the HI+ pancreata were subdivided into HI+ and HI++ pancreata, also on a semi-quantitative basis. HI++ pancreata were more severely affected than the HI+ pancreata. Because of the enormous diversity in the occurrence of hyperemic islets both the amount of affected islets and the severity of hyperemia in the tissue sample were considered. For example, a tissue sample with 2 severely affected islets (with only a rim of islet cells left, as shown in Fig 1F) was scored as ++, while another tissue sample with 3 only slightly affected islets (with 1 dilated vessel, as shown in Fig 1B) was scored as +.

The pancreata were independently allocated to the different categories by 2 observers. The observers were blinded for the isolation outcome when allocating

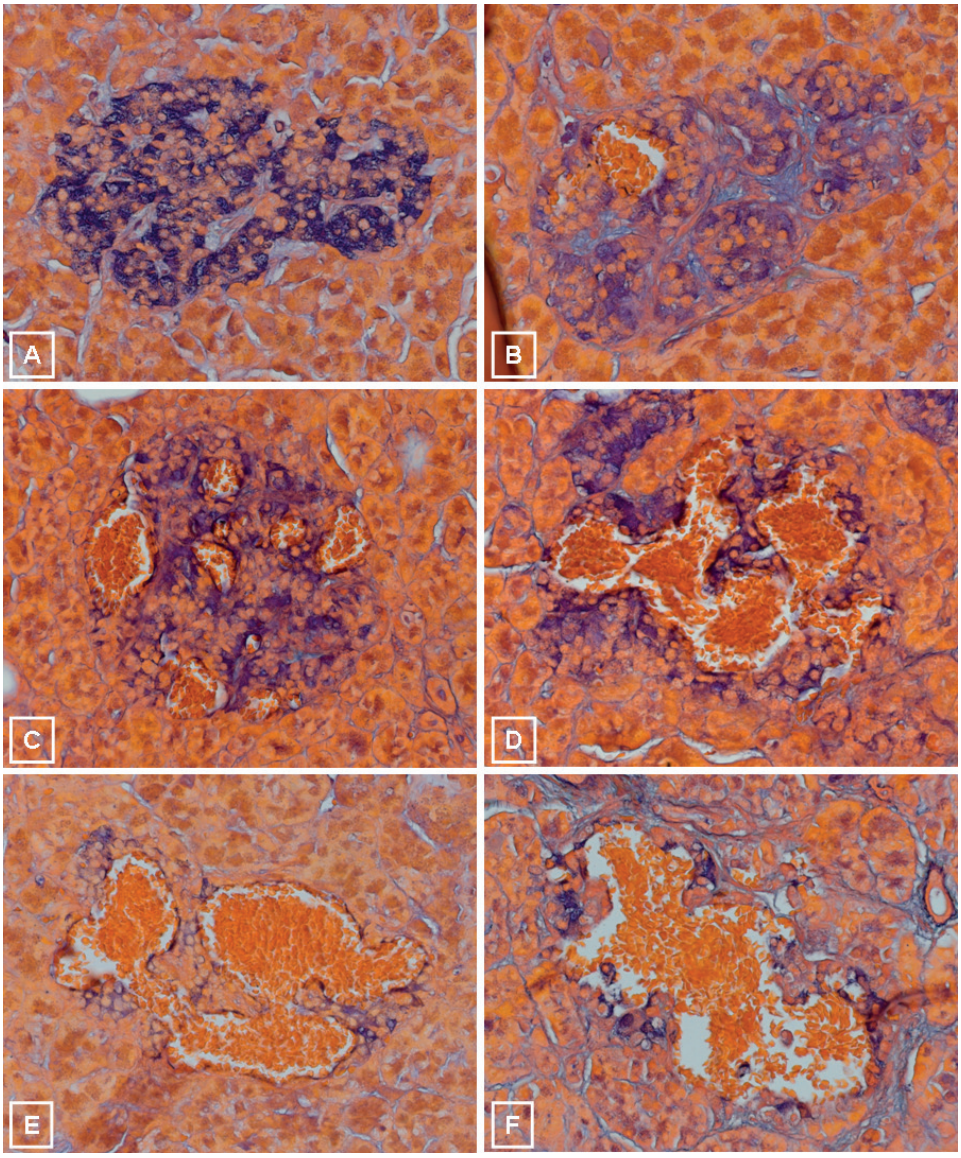

Figure 1: Histology of pancreata (AF staining) showing different stages of hyperemic islets.
(A) Normal islet. (B) Islet with a single dilated vessel. (C) Islet with multiple dilated vessels. (D) Islet with multiple, more severely dilated vessels. (E,F) Swollen islet with vastly dilated vessels and only a rim of islet cells left.

the pancreata to the different categories. Any differences between observers in the allocation of the pancreata, were resolved by discussion.

Donor and Procurement factors

To identify donor and procurement factors associated with the presence of HIs, we compared donor and procurement factors in HI+ and HI- pancreata. From the 102

pancreata, 8 (5 HI+ and 3 HI-) were excluded due to incomplete data, leaving 94 pancreata (52 HI+ and 42 HI-) for analysis.

Variables of interest were identified based on the literature (17-21). Donor variables included: age, sex, height, weight, body mass index (BMI = weight / height^2), ABO blood group, vascular co-morbidity (cardiac, cerebral and/or peripheral vascular event or disease), DBD/DCD donors, presence and duration of cardiac arrest and hypotensive periods, length of stay on the intensive care unit (ICU), cause of death, use of vasopressors and steroids, as well as routine biochemical blood screen (levels before organ removal) and pre-procurement hemodynamic measurements. Procurement variables included: CIT, procurement team and preservation solution.

Average beta cell/endocrine content ratio per islet

To assess possible swelling of the islets, the average beta cell/endocrine content ratio per islet was compared in HI+ and HI- pancreata. This was done in a smaller sample of 40 randomly selected pancreata (20 HI+ and 20 HI-).

In paraffin-embedded sections of 5 μm, beta cells were stained with a polyclonal peroxidase-labeled rabbit anti-human insulin antibody (Santa Cruz Biotechnology, Heidelberg, Germany) at a dilution of 1:100 overnight and visualised with 3,3'-diaminobenzidine (DAB) dissolved in Tris/HCl (0.05 M) with 15 μl H_2O_2. An average of 75 islets per tissue sample was assessed. To quantify the beta cell content (Fig. 2A), the brown stained area was quantified using the Zeiss KS400 image analysis system (Zeiss-Vision, Germany). Only islets larger than 50 μm in diameter were included. To quantify the endocrine content, every islet used in the assessment of the beta cell content was surrounded by a drawn line, manually delineated with a pen tablet using ImageJ (freeware image processing tool) (Fig. 2B). In human islets, beta cells represent 50-60% of the endocrine content. Because alpha, beta and delta cells have been shown to appear scattered throughout the islet (22, 23), the area inside the surrounding line was considered to represent the endocrine content (Fig 2C). This area was also quantified using the Zeiss KS400 image analysis system. To quantify a possible swelling of the islets, the ratio between the beta cell and endocrine content was calculated. The greater the swelling of the islets due to vasodilatation or edema, the lower the beta cell/endocrine content ratio would be.

Islet isolation

Islet isolation was performed in a smaller sample of 40 pancreata. These were different pancreata than those used in the determination of the beta cell/endocrine content ratio. Islet isolations were performed by a modification of the automated method previously described by Ricordi et al. (24). In short: the body and tail of the pancreas were used in the islet isolation procedure. The main pancreatic duct was identified, cannulated and perfused for 10 min (5 min at 80 mmHg and 5 min at 180 mmHg) with

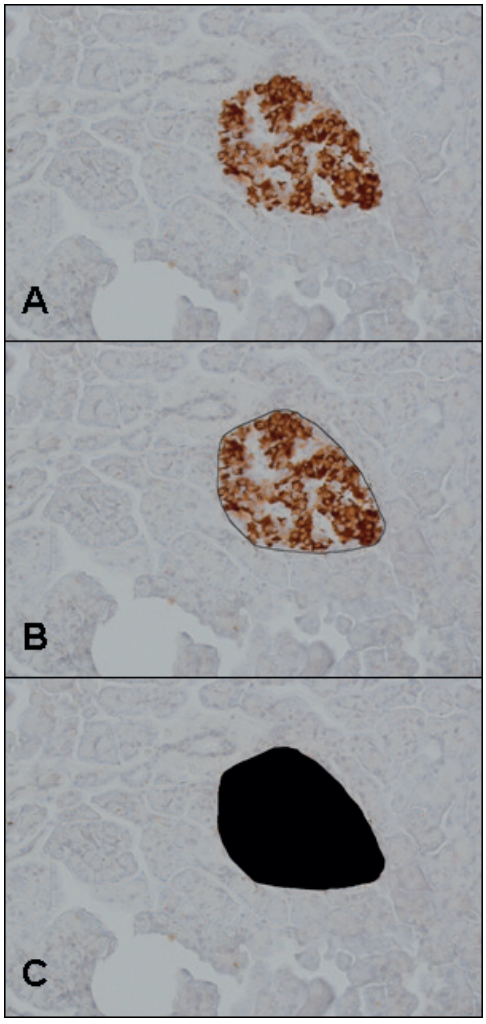

Figure 2: Calculation of the beta cell/endocrine content ratio
The brown stained (beta cell) area was quantified using an image analysis system (A). To quantify the endocrine content, every islet used in the assessment of the beta cell content, was surrounded by a manually drawn line (B). The area inside the surrounding line was considered to represent the endocrine content (C). To quantify the swelling of the islets, the ratio between the beta cell and endocrine content was calculated: area A / area C.

a chilled solution of Collagenase NP (Serva Electrophoresis, Heidelberg, Germany), Neutral Protease NP (25U, Serva Electrophoresis, Heidelberg, Germany) and (Aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF-HCl, 20 mg/ml, Serva Electrophoresis, Heidelberg, Germany) in Hanks Balanced Salt Solution (HBSS). The distended tissue was cut into 5 or 6 pieces and placed in the Ricordi chamber, in a closed recirculating system and heated to 37°C to activate the enzyme blend. During the course of enzymatic and mechanical dissociation of the gland, samples were taken and evaluated in real-time using Dithizone (DTZ, diphenylthiocarbazone, 13 mM DTZ dissolved in 91.2% ethanol supplemented with 1.2% NH_4OH, Sigma, Steinheim, Germany). After the appearance of islets unembedded in acinar tissue, the circulation circuit was cooled and the digest collected. The digested tissue was placed in a beaker on ice and islet samples of 50 µl were stained with an equal volume of DTZ solution,

freshly prepared by the addition of 5% DTZ to HBSS. Islet numbers, expressed as islet equivalents (IE, representing islets of 150 μm diameter), were determined in duplicate.

Data analysis

Statistical analysis was performed using SPSS 16.0 statistical software. HI- and HI+ pancreata were compared on endocrine content, number of islets, isolation outcome and the average beta cell/endocrine content ratio per islet using student *t*-tests. HI-, HI+ and HI++ pancreata were compared to assess the influence of the severity of hyperemia on the above outcomes using one-way analysis of variance. Post hoc Bonferroni's multiple comparisons tests were performed to determine which groups differed from each other.

To assess whether the relation between the presence of HIs and isolation outcome was confounded by other factors known to influence isolation outcome (17-21, 25), multivariate analyses was performed using linear regression with isolation outcome as dependent variable. Independent variables included: the presence of HIs, endocrine content, donor age, sex, BMI, cause of death, DBD/DCD donors, ICU stay, cardiac arrest, use of vasopressors, hypotensive periods (last measured blood pressure before procurement, SBP < 90 mm Hg and/or DPB < 60 mm Hg), pre-procurement hypertension, local/distant procurement, CIT and preservation solution.

To identify possible relevant variables in the occurrence of HIs, HI+ and HI- pancreata were compared on differences in donor and procurement factors using student *t*-tests for continuous variables and χ^2 analyses for categorical variables. In case of expected count less than 5, the Fisher exact test was used. Multivariate analysis was consequently performed using binary logistic regression with presence of HIs as the dependent variable. As independent variables were included: age, sex, BMI, cause of death, vascular co-morbidity, ICU stay, use of vasopressors, use of steroids, hypotensive periods, blood creatinine, amylase, glucose and sodium levels, pre-procurement hypertension, duration of cardiac arrest, DBD/DCD donors, local/distant procurement team, CIT and preservation solution.

All p-values < 0.05 were considered to be statistically significant.

RESULTS

Presence of HIs

Histological analysis showed that 57 of the 102 pancreata (54.6%) contained at least one hyperemic islet and were scored as HI+. However, only an average of 4.5% of all assessed islets in the HI+ pancreata was hyperemic. Hence, 45 pancreata (45.4%) contained no HIs and were scored as HI-.

We assessed the presence of HIs in different parts of the pancreata. There were no differences in the occurrence of HIs in the head, neck or body of the pancreata (results not shown).

Donor and Procurement factors in relation to HIs

Differences in donor and procurement factors between the HI- and HI + groups are listed in Table 1, 2. ICU stay was significantly longer in the HI+ group compared to the HI- group. Furthermore, pre-procurement hypertension (last measured blood pressure before procurement, Systolic Blood Pressure (SBP) > 140 mmHg and/or Diastolic Blood Pressure (DBP) > 90 mm Hg) was more frequently found in the HI+ than in the HI- group. We also found a trend ($p = 0.07$) towards longer cold ischemia times in the HI+ group.

Multivariate analyses confirmed that length of ICU stay (Odds ratio (OR) 1.32, 95% Confidence Interval (CI) [1.02 – 1.71]) and the presence of pre-procurement hypertension (OR 3.43 [1.07 – 11.03]) significantly increased the probability of HIs. Last sodium level before organ procurement was found to significantly reduce the probability of HIs (OR 0.85 [0.76 – 0.95]).

Endocrine content in relation to HIs

The mean endocrine content in the HI+ pancreata was 0.66%. This was significantly higher than in the HI- group where this was 0.55% ($p = 0.04$) (Fig. 3A).

To assess the influence of the severity of the hyperemia on the endocrine content, the HI+ pancreata ($n = 57$) were subdivided into 31 HI+ and 26 HI++ pancreata (Fig. 3B). The mean endocrine content was 0.55% in the HI-, 0.58% in the HI+ and 0.75% in the HI++ group. One way analysis of variance showed that the mean endocrine content differed between the groups ($p = 0.004$). Bonferroni's test revealed that endocrine content was significantly higher in the HI++ group compared to the HI- ($p = 0.004$) and the HI+ group ($p = 0.03$).

The mean number of islets (in 1 cm^2 tissue) was 72 in the HI- and 78 in the HI+ group. We found no significant differences in numbers of islets between these groups ($p = 0.19$).

These results suggest that the higher endocrine content in the affected pancreata is a result of swelling of the islets, due to vasodilatation, edema or both.

In a smaller sample of 20 HI+ and 20 HI- randomly selected pancreata, we observed that the mean beta cell/endocrine content ratio in the HI- group was 0.67%, which is significantly higher ($p = 0.02$) than the 0.55% shown for the HI+ pancreata. This supports our hypothesis that the higher endocrine content in the most affected pancreata is the result of islet expansion.

Table 1. Differences in donor characteristics between HI+ and HI- pancreata

Donor characteristic	HI + (n = 52)	HI - (n = 42)	Test of difference
Age (y) (± SD)	47.4 ± 13.0	46.9 ± 14.3	t = -0.181, p = 0.86
Sex			X^2 = 0.416, p = 0.52
Male	27 (51.9%)	19 (45.2%)	
Female	25 (48.1%)	23 (54.8%)	
ABO Blood Group			X^2 = 0.614, p = 0.94
O	18 (34.6%)	14 (33.3%)	
A	25 (48.1%)	19 (45.2%)	
B	6 (11.5%)	5 (11.9%)	
AB	3 (5.8%)	4 (9.5%)	
Body mass index (kg/m^2)	25.2 ± 5.3	24.4 ± 4.3	t = -0.823, p = 0.41
Cause of Death			X^2 = 0.314, p = 0.86
Cerebrovascular accident	30 (57.7%)	26 (61.9%)	
Trauma	11 (21.2%)	9 (21.4%)	
Other	11 (21.2%)	7 (16.7%)	
DBD/DCD donors			X^2 = 0.105, p = 0.75
DBD	33 (63.5%)	28 (66.7%)	
DCD	19 (36.5%)	14 (33.3%)	
ICU stay (days)	3.4 ± 3.5	2.2 ± 2.1	t = -2.033, p = 0.05
Cardiac arrest			X^2 = 0.024, p = 0.88
Yes / No	19 (36.5%) / 33 (63.5%)	16 (38.1%) / 26 (61.9%)	
Cardiac arrest duration (min)	5.5 ± 10.7	8.1 ± 13.8	t = 1.004, p = 0.32
Hypotension [a]			X^2 = 0.001, p = 0.97
Yes / No	20 (38.5%) / 32 (61.5%)	16 (38.1%) / 26 (61.9%)	
Hypotension duration (min)	6.5 ± 13.2	9.0 ± 20.4	t = 0.738, p = 0.46
Hypertension [b]			X^2 = 3.753, p = 0.05
Yes / No	29 (55.8%) / 23 (44.2%)	15 (35.7%) / 27 (64.3%)	
Vascular comorbidity [c]			X^2 = 0.171, p = 0.68
Yes / No	22 (42.3%) / 30 (57.7%)	16 (38.1%) / 26 (61.9%)	
Sodium (mmol/l) [d]	145 ± 7	147 ± 7	t = 1.470, p = 0.15
Creatinine (mg/dl) [d]	77.9 ± 37.5	72.5 ± 27.0	t = -0.786, p = 0.43
Amylase (U/l) [d]	240 ± 349	206 ± 161	t = -0.595, p = 0.55
Glucose (mmol/l) [d]	8.1 ± 2.8	8.5 ± 3.7	t = 0.623, p = 0.54
Hyperglycemia [e]			X^2 = 0.211, p = 0.65
Yes / No	26 (50.0%) / 26 (50.0%)	23 (54.8%) / 19 (45.2%)	
Vasopressors			X^2 = 0.004, p = 0.95
Yes / No	35 (67.3%) / 17 (32.7%)	28 (66.7%) / 14 (33.3%)	
Vasopressor (µg/kg/min)			
Noradrenaline [1]	0.32 ± 0.74	0.27 ± 0.40	t = -0.301, p = 0.77
Dobutamine [2]	5.60 ± 3.15	5.37 ± 4.92	t = -0.083, p = 0.94
Dopamine [3]	7.10 ± 5.42	5.87 ± 2.95	t = -0.612, p = 0.55
Steroids			X^2 = 0.369, p = 0.54
Yes / No	12 (23.1%) / 40 (76.9%)	12 (28.6%) / 30 (71.4%)	

DBD: Donation after brain death, DCD: Donation after cardiac death, ICU: Intensive care unit

[a] Hypotension: last measured blood pressure before procurement, SBP < 90 mm Hg and/or DPB < 60 mm Hg
[b] Hypertension: last measured blood pressure before procurement, SBP > 140 mm Hg and/or DBP > 90 mm Hg
[c] Vascular co-morbidity: cerebral, cardiac and/or peripheral vascular event or disease
[d] Levels before organ procurement
[e] Hyperglycemia: last serum glucose level before organ procurement > 10.0 mmol/l
[1] Means were calculated for 51 donors who received Noradrenalin before procurement
[2] Means were calculated for 8 donors who received Dobutamine before procurement
[3] Means were calculated for 20 donors who received Dopamine before procurement

Table 2. Differences in pancreas characteristics between HI+ and HI- pancreata

	HI + (n = 52)	HI - (n = 42)	Test of difference
Pancreas characteristic			
Cold ischemia time (h) (± SD)	8.5 ± 3.4	7.3 ± 2.5	t = -1.871, p = 0.07
Procurement team			X^2 = 1.098, p = 0.30
Local	18 (34.6%)	19 (45.2%)	
Distant	34 (65.4%)	23 (54.8%)	
Preservation solution [1]			X^2 = 0.128, p = 0.72
UW	34 (66.7%)	26 (70.3%)	
HTK	17 (33.3%)	11 (29.7%)	

UW: University of Wisconsin Solution, HTK: Histidine Tryptophane Ketoglutarate
[1] Data missing for 1 HI+ and 5 HI- donors. Percentages calculated for 51 and 37 donors, respectively.

Islet isolation yield in relation to HIs

In a different series of 40 pancreata (20 HI- and 20 HI+), we assessed whether the presence of HIs might influence isolation outcome. In spite of their histologically assessed higher endocrine content, the mean isolation yield was significantly lower in the HI+ pancreata compared to the HI- group (2634 IE/g and 4069 IE/g, respectively p = 0.05).

However, the HI+ pancreata contained significantly higher endocrine content. When this higher endocrine content was taken into account and the isolation results were expressed as the ratio of yield and content, the results were even more pronounced and illustrated a severely compromised endocrine quality (p = 0.003) (Fig.4A).

We also assessed the influence of the severity of hyperemia on isolation outcome and subdivided the HI+ group (n = 20) into 13 HI+ and 7 HI++ pancreata (Fig. 4B). One way analysis of variance showed that the mean yield/endocrine content ratio differed between the groups (p = 0.008). Bonferroni's test revealed that the yield/endocrine content ratio was significantly lower in the HI++ group compared to the HI- group (p = 0.02). Furthermore, a trend towards a lower yield/endocrine content ratio was found in the HI++ group compared to the HI+ group (p = 0.06).

Furthermore, we assessed whether the reduced isolation outcome in the HI+ groups was confounded by other factors know to influence isolation outcome. We corrected for endocrine content, donor age, sex, BMI, cause of death, DBD/DCD donors, ICU stay, cardiac arrest, use of vasopressors, use of steroids, hypotensive periods, local/distant procurement, CIT, preservation solution. The presence of HIs (B -1648.9, p = 0.01) and the endocrine content (B 6809.4, p < 0.001) were the only factors significantly influencing isolation outcome.

Etiology of HIs

To gain more insight in the etiology of HIs and validate whether other differences existed between HI groups, an experienced pathologist assessed a random selection of 10 HI+ and HI- pancreata. The exocrine tissue and the nonhyperemic islets appeared to be visually normal in both groups. The pathologist found no macrophages or iron

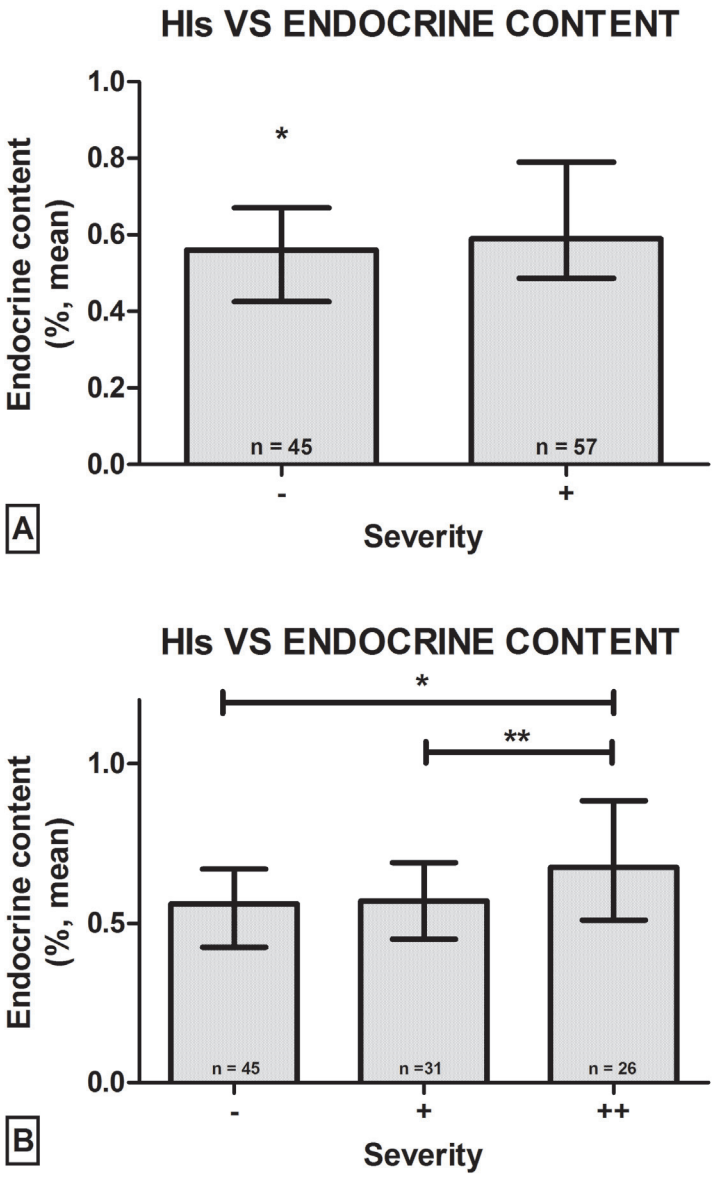

Figure 3: The endocrine content in relation to hyperemic islets.
The bars represent the mean endocrine content per hyperemic islet category. The error bars represent the standard deviation.
(A) The mean endocrine content in 45 HI- and 57 HI+ pancreata was 0.55, and 0.66%, respectively. Significantly higher endocrine content was found in the HI+ group, * p = 0.04
(B) The influence of the severity of the hyperemia, by subdividing 57 HI+ pancreata into 31 HI+ and 26 HI++ pancreata, in relation to the endocrine content. Significant higher endocrine content was found in the HI++ group compared to the HI- and HI+ group, * p = 0.004, ** p = 0.03.

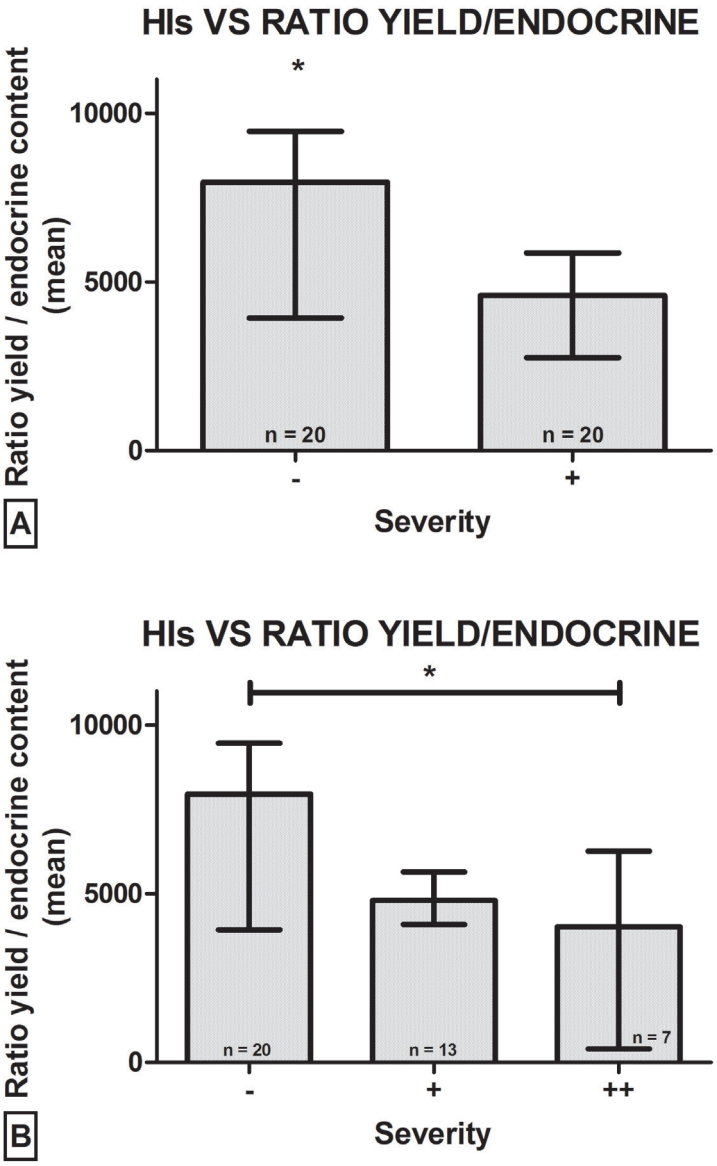

Figure 4: Yield/endocrine content ratio in relation to hyperemic islets.
The bars represent the mean yield/endocrine content ratio per hyperemic islet category. The error bars represent the standard deviation.
(A) The mean yield/endocrine content ratio in 20 HI- and 20 HI+ pancreata. A significantly lower ratio was found in the HI+ group, * p = 0.003.
(B) The influence of the severity of the hyperemia, by subdividing 20 HI+ pancreata into 13 HI+ and 7 HI++ pancreata, in relation to the yield/endocrine content ratio. A significantly lower yield / endocrine content ratio was found in the HI++ group compared to the HI- group, * p = 0.02.

deposits and no evidence for an ongoing chronic process in the affected pancreata. Beside the presence of HIs, no differences were seen between the HI- and HI+ pancreata.

To assess whether HIs also occurred in normal pancreatic tissue, we examined a series of 30 "normal" pancreatic tissue samples excised at elective surgical procedures (e.g. after pancreatoduodenectomy for pancreatic carcinoma) at the Academic Medical Centre, Amsterdam, The Netherlands. Areas with pancreatic pathology were identified by an experienced pathologist and were left out of the assessment. Only areas with "normal" pancreatic tissue were used in our assessment. At least 1 HI was found in 43.3% of these pancreata. Apparently, the presence of HIs is not limited to donor organs, but they can also be found in pancreatic tissue excised in elective surgery, suggesting that besides the pre-procurement hemodynamic status, the handling of the pancreas during surgery could also be a contributing factor in the development of HIs.

DISCUSSION

Human islet isolations produce unpredictable, highly variable islet yields. We previously reported HIs as a possible explanatory factor in the varying porcine islet yields (16). When we studied histological characteristics of the human pancreas in relation to islet isolation, we found a similar remarkably high number of HIs. Similar islets have only been reported anecdotally in the literature but no mechanisms were described regarding their origin and no relevance has been determined from the perspective of the isolation of islets for transplantation (26-30). Slight hyperemia in islets has been reported in normal metabolic situations when higher insulin release is demanded (30, 31). However, like in our pig study, the phenomena encountered in the present study are far more dramatic and are most likely not related to the normal glucose metabolism.

In the present study, HIs were found in more than half of the pancreata. However, the HI+ category consisted of pancreata with at least 1 HI. This could potentially lead to an overestimation of the HI- group, when a hyperemic islet was missed by an observer or when an islet appeared just outside the sectioning plane. However, because 2 observers independently assessed the tissue samples and since we have found no differences in the presence of HIs between different parts of the pancreata, it is unlikely that our results were influenced by this. Because no macrophages or lytic erythrocytes were seen and no evidence for an ongoing chronic process was found, it is most likely that the HIs arose shortly before or during the procurement of the pancreas. Since length of ICU stay and pre-procurement hypertension were shown to increase the probability of HIs, it can be speculated that a rise of blood pressure just before procurement and hemodynamic instability associated with prolonged ICU stay, are responsible for the formation of HIs. This in line with our porcine study where we

reported a rise in blood pressure, induced by the slaughtering process, as a possible causative moment for the presence of hyperemic islets in pigs. It is not clear how our findings with respect to the last sodium level fit in this explanation. Hence, since the mean sodium level of both the HI positive and HI negative groups are around the upper limit of normal values and the mean values only differed by 2 points, this finding might not be of clinical relevance. However, HIs were also found in 43.3% of "normal" pancreatic tissue samples obtained from elective surgical procedures. Since we have very little donor specifications of these tissue samples, we cannot make any statement about whether these influenced the presence of HIs, but apparently the presence of HIs is not limited to donor organs. However, these "elective patients" were most likely not admitted to the ICU before undergoing surgery and were probably hemodynamically more stable than the pancreas donors, especially when compared to DBD donors. Therefore, it seems likely that, besides the pre-procurement hemodynamic status, the handling of the pancreas during surgery could also be a contributing factor in the development of HIs.

We found no microscopic hemorrhages in the exocrine pancreas, which makes up 98-99% of the organ, indicating that the islet vasculature is probably very sensitive for blood pressure rises and hemodynamic instability. This vulnerability may be reflected in its unique structure: blood vessels from the surrounding exocrine tissue abruptly increase in diameter upon entering the islet, have a thinner wall and are extensively fenestrated inside the islet, as reported in a rat study (32).

We have also investigated the possible relevance of the presence of HIs from the perspective of islet isolation. A higher presence and severity of HIs was accompanied by a small increase in the endocrine content. This increase, however, is not paralleled by a similar increase in islet numbers, and could therefore be caused by expansion of the individual islets. Additionally, we found a lower beta cell/endocrine content ratio in the most affected pancreata, indicating that islets in these pancreata are more swollen, probably through vasodilatation, edema or both. In spite of the slightly higher endocrine content of the HI+ pancreata, substantial lower yields were found. When we corrected for the amount of endocrine tissue, even more pronounced lower yields were found in the affected groups. Furthermore, when corrected for other factors that are known to influence isolation outcome, the presence of HIs and endocrine content were the only factors significantly influencing isolation outcome. This is remarkable since even in the affected pancreata only a few percent of all islets were histologically abnormal. Most likely, the substantial increase in islet volume, or the reduced isolation outcome is not caused by this small fraction itself. A possible explanation could be that the entire islet population is compromised and that the abnormal islets are just an indicator of the actual damage of the islets. It can be speculated that besides the reduced isolation outcome, this phenomenon could also provide a possible explanation for the variable, unexplained loss of islets during culture and after transplantation. When

islets are obtained from donor-pancreata containing HIs, these islets would appear to be "normal" when in fact, the entire islet population of these pancreata is affected. Therefore, these islets are more likely to fail in culture or have impaired function when transplanted. In whole organ transplantation this would probably be less of a problem because of the intact integrity of the exocrine pancreas, providing external support for the islets and a possibility for recovery and angiogenesis. However, further research would be necessary to support this hypothesis, Furthermore, in this study, we have assessed the relation between the presence of HIs and pre-purification yield. To establish the importance of HIs for islet transplantation, further research, involving post-purification yield, purity, viability etc. would be necessary.

In conclusion, these data are a first indication that the presence of hyperemic islets negatively influence isolation outcome and suggest an impact of the procurement procedure and pre-procurement hemodynamic status of the donor on the islet quality. It is tempting to speculate that this contributes to the generally experienced difficulties in obtaining sufficient amounts of human islets.

ACKNOWLEDGMENTS

The authors would like to thank Dr. I.M. Bajema MD, PhD for the histological assessment of the differences between most and not affected tissue samples, Dr. V. Terpstra MD, PhD (Academic Medical Center, Amsterdam, the Netherlands) for the pancreatic tissue samples of elective surgical procedures and K.G. van der Ham for the images of the hyperemic islets.

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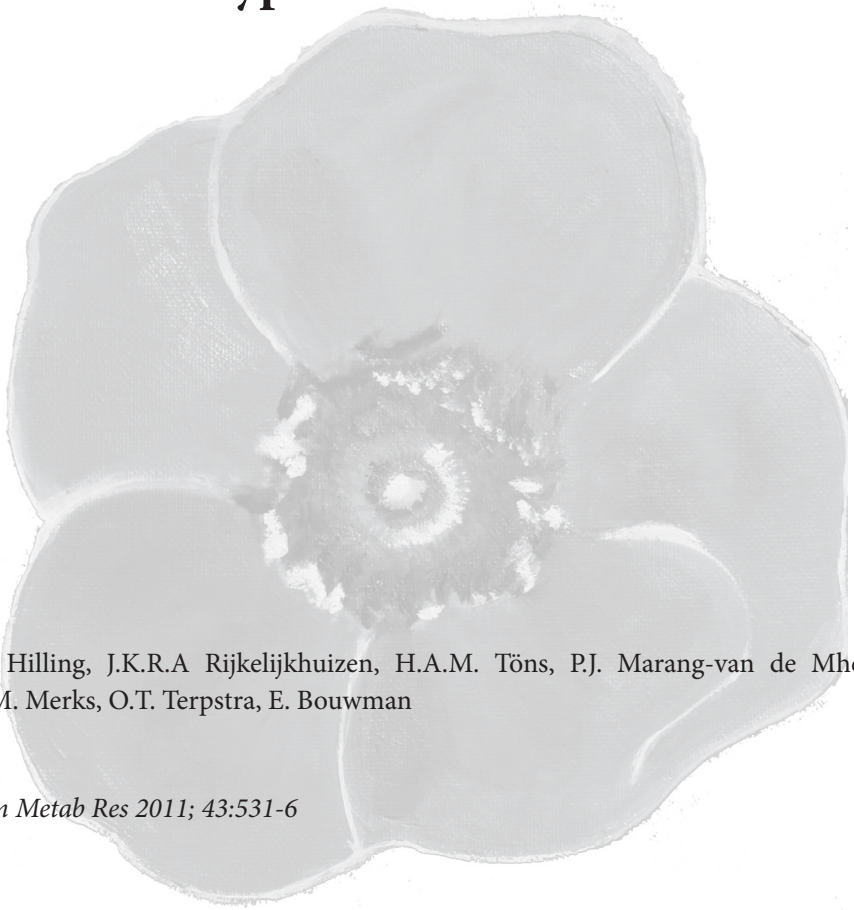
Part III

Porcine islet transplantation

Chapter 6

Reduced porcine islet isolation yield in the presence of hyperemic islets



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Horm Metab Res 2011; 43:531-6

ABSTRACT

Background

When studying histological characteristics of porcine pancreata in relation to islet isolation, a remarkably high number of hyperemic islets (HIs) was encountered. The abnormalities observed in these HIs ranged from a single dilated vessel to hemorrhages extending into the surrounding exocrine tissue. The aim of the present study was to compare pancreata with and without HIs on islet isolation outcomes.

Methods and Materials

This study involved a histological examination of 143 purebred (74 juvenile and 69 adult) and 47 crossbred (only juvenile) porcine pancreata. Islet isolation was performed in 48 purebred adult pigs and in 25 crossbred pigs. Tissue samples were stained with Aldehyde Fuchsine. The presence of HIs was scored semi-quantitatively (HI-, HI+).

Results

We observed HIs in 48% of the purebred and in 68% of the crossbred pigs. However, only 3.3 ± 3.1% and 3.1 ± 4.7% of all assessed islets was hyperemic in HI+ pancreata in purebred and crossbred pigs, respectively. In both groups, significantly higher endocrine cell mass was found in the HI+ pancreata ($p < 0.01$). When the higher endocrine cell mass was taken into account, we found significantly lower yields in the HI+ pancreata in both purebred and crossbred pigs ($p = 0.03$ in both groups).

Conclusions

The presence of HIs occurs frequently in porcine donor-pancreata and is associated with reduced isolation outcomes.

INTRODUCTION

Much research has been conducted to optimize the outcome of porcine islet isolation. The amount of endocrine tissue present in a specific pancreas is undoubtedly an important factor in determining the islet isolation outcome. However, a high endocrine cell mass does not ensure a high isolation yield. Other factors, such as collagen and other matrix elements are thought to play a role (1-6). It is presently unclear to what extent such factors are dependent on breed, sex and age.

When studying histological characteristics of the porcine pancreas in relation to islet isolation, a remarkably high number of hyperemic islets (HIs) was encountered, a phenomenon that has been described only anecdotally, but not been studied in detail before (7-11). The abnormalities observed in these HIs ranged from a single dilated vessel through multiple widely dilated vessels to hemorrhages extending into the surrounding exocrine tissue. We have previously reported our study of human donor-pancreata where we found that the presence of HIs is associated with reduced isolation outcomes (12).

The aim of the present study was to assess whether this is a general phenomenon also present in porcine pancreata, and to determine the frequency of HIs in porcine pancreata compared to human pancreata. Furthermore, we studied the occurrence of HIs in relation to the amount of endocrine tissue in situ and in relation to the outcome of islet isolation to assess whether a similar relation was found as in human pancreata. Because external factors (e.g. breed, nutrition, age and transportation) could potentially have a confounding effect on islet isolation outcome, we assessed 2 distinctly different groups: purebred and crossbred pigs.

MATERIALS AND METHODS

Organ Procurement and Pancreatic Sampling

A total of 143 purebred and 47 crossbred porcine pancreata were harvested in different commercial slaughterhouses. The purebred population consisted of a juvenile group (6 – 12 months) of 74 animals and involved 8 different breeds (Great Yorkshire n = 18, Dutch Landrace n = 25, Norwegian Landrace n = 3, Large White n = 11, Hampshire n = 2, Finnish Landrace n = 7, Duroc n = 8) The adult purebred group (12 – 78 months) consisted of 69 animals and involved 8 different breeds (Great Yorkshire n = 14, Dutch Landrace n = 17, Norwegian Landrace n = 6, Large White n = 7, Hampshire n = 3, Finnish Landrace n = 10, Duroc n = 10). The crossbred group consisted of only juvenile animals (6 – 12 months) and were a cross of 2 or 3 different breeds.

All animals were killed by electric stunning and exsanguination. Warm ischemia times were between 20 – 30 min. After dissection, the pancreata were stored in cold

(4°C) Hank's Balanced Salt Solution (HBSS) and transported on ice to the laboratory for further processing. Cold ischemia times were between 2 – 5 h. Biopsies were taken from the splenic, duodenal and connecting lobes, immersed in Bouin's fixative, cleared and stored in ethanol 70%.

Histology

From all pancreata, paraffin-embedded sections of 4 μm were stained using the Aldehyde Fuchsine-Halmi (AF) technique: Rehydration, 90 seconds 2.5% potassium permanganate, 2' distilled water, 2' 1% oxalic acid, 10' running water, 1' distilled water, 15' AF, 3' 90% ethanol (2 times), 1' 70% ethanol, 1' 50% ethanol, 5' aquadest, 10-60 s Halmi, 10 s 0.2% acetic acid, 2' 100% ethanol (2 times), 5' xylene (2 times). Sections were embedded in malinol (Chroma-Gesellschaft, Köngen, Norway).

Microscopic evaluation

The same method was used as reported previously for human pancreata [12]. We have assessed the relation between the presence of HIs and endocrine cell mass, because pancreata with higher endocrine cell mass could produce higher yields simply because there is more endocrine cell mass to begin with. This could potentially confound our results when assessing the relation between the presence of HIs and isolation outcome.

Islet sizes were determined in each pancreas using a calibrated grid and grouped into 8 categories: >50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400 and >400 μm diameter. As is generally practiced in the endocrine quantification in islet isolation procedures, islets smaller than 50 μm were not included because of their neglectable contribution to the total endocrine cell mass. For determination of the endocrine cell mass and the islet numbers, all islets in a tissue area of 1 cm^2 per slide were assessed. The endocrine area density was determined by summation of all calculated (πr^2) islet areas and expressed as percentage of the total area. According to the principle of Delesse (area density = volume density) and the assumption that islets are spherical, the determined endocrine area represents the endocrine cell mass. Assuming that the islet density does not significantly differ throughout the pancreas, the endocrine cell mass that is found in 1 cm^2 tissue represents the percentage of endocrine tissue in the pancreas.

Islets described as hyperemic in this study ranged from islets showing only a single slightly dilated vessel to hemorrhages extending into the surrounding exocrine tissue (Fig.1). The presence of HIs was scored semi-quantitatively (HI-, HI+) as done in our study of human pancreata (12). HI- pancreata contained none and HI+ pancreata at least one hyperemic islet.

To assess the influence of the severity of the hyperemia, the HI+ pancreata were subdivided into HI+ and HI++ pancreata, also on a semi-quantitative basis. HI++ pancreata were more severely affected than the HI+ pancreata. Because of the

enormous diversity in the occurrence of hyperemic islets both the amount of affected islets and the severity of hyperemia in the tissue sample were considered. For example, a tissue sample with 2 severely affected islets (with hemorrhages extending into the surrounding exocrine tissue, as shown in Fig. 1F) was scored as ++, while another tissue sample with 3 only slightly affected islets (with 1 dilated vessel, as shown in Fig. 1B) was scored as +.

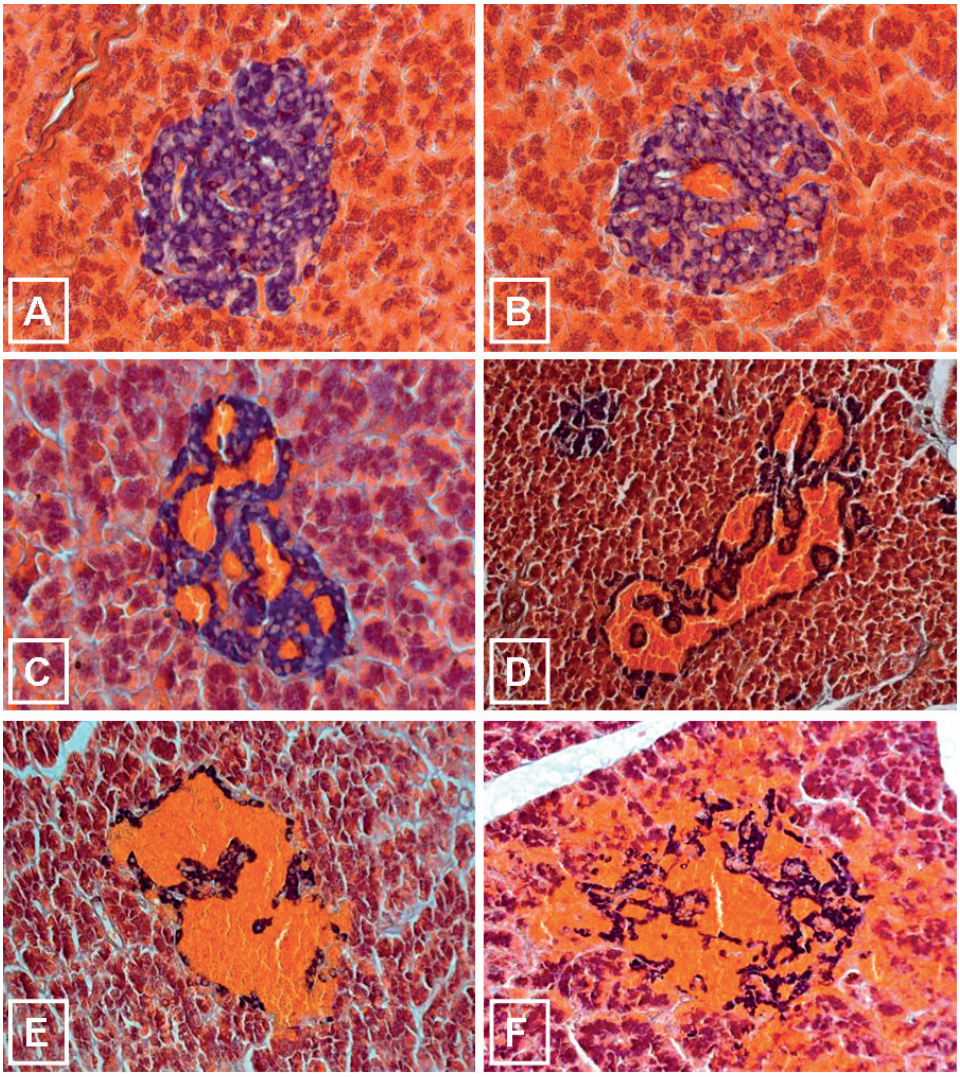

Figure 1: Histology of porcine pancreata (AF staining) showing different stages of hyperemic islets.
(A) Normal islet. (B) Islet with a single dilated vessel. (C) Islet with multiple dilated vessels. (D) Swollen islet with vastly dilated vessels and only a rim of islet cells left. (E) As in picture D, with several small hemorrhages breaking through the rim. (F) Grossly enlarged and distorted islet with multiple hemorrhages penetrating deeply into the surrounding exocrine tissue.

The pancreata were independently allocated to the different categories by 2 observers. The observers were blinded for the isolation outcome when allocating the pancreata to the different categories. Any differences between observers in the allocation of the pancreata, were resolved by discussion.

Average beta cell/islet area ratio per islet and total number of islets

To assess possible swelling of the islets, the average beta cell/islet area ratio per islet was compared in HI+ and HI- pancreata, consistent with the method used previously (12). This was done in a smaller sample of 44 randomly selected pancreata (22 HI+ and 22 HI-). Furthermore, the total number of islets was assessed by counting all islets, including islets < 50 μm, in an area of 1 cm^2 tissue in these pancreata.

In paraffin-embedded sections of 4 μm, beta cells were stained with a polyclonal peroxidase-labeled guinea pig anti-porcine insulin antibody (Zymed, Invitrogen, Carlsbad, CA, USA) at a dilution of 1:200 overnight and visualized with 3,3'-diaminobencidine (DAB) dissolved in Tris/HCl (0.05 M) with 15 μl H_2O_2.

An average of 75 islets per tissue sample was assessed. To quantify the beta cell area, the brown stained area was quantified using the Zeiss KS400 image analysis system (Zeiss-Vision, Germany). Only islets larger than 50 μm in diameter were included. To quantify the islet area, every islet used in the assessment of the beta cell area, was surrounded by a drawn line, manually delineated with a pen tablet using ImageJ (freeware image processing tool). In human islets, beta cells represent 50-60% of the endocrine cell mass. Because alpha, beta and delta cells have been shown to appear scattered throughout the islets and porcine islets have been shown to have similar cytoarchitecture as human islets (13,14), the area inside the surrounding line was considered to represent the islet area. This area was also quantified using the Zeiss KS400 image analysis system. To quantify a possible swelling of the islets, the ratio between the beta cell and islet area was calculated. The greater the swelling of the islets due to vasodilatation or edema, the lower the beta cell/islet area ratio would be.

Islet isolation

In the purebred group, islet isolation was performed in 48 adult animals. In the crossbred group, islet isolation was performed in 25 animals. The pancreata were cut clean in the laboratory. The arm of the pancreas was intraductally injected with a solution of Liberase PI (0.5 mg/ml, Roche Applied Science, Germany) in University of Wisconsin solution (UW). The distended tissue was cut into pieces and incubated in HBSS at 37°C for 20 – 30 min. The digested tissue was placed in a beaker on ice, which was manually shaken to gently dissociate and dilute the tissue. Subsequently, the tissue was poured over a filter with 1000 μm mesh and washed in cold HBSS for 3 times. Islet samples of 25 μl were stained with an equal volume of Dithizone solution (DTZ, diphenylthiocarbazone, Sigma, Steinheim, Germany), freshly prepared by the addition

of 5% DTZ stock-solution (13 mM DTZ dissolved in 91.2% ethanol supplemented with 1.2% NH_4OH) to HBSS. Islet numbers, expressed as islet equivalents (IE, representing islets of 150 μm diameter), were determined in duplicate.

Data analysis

Statistical analysis was performed using SPSS 16.0 statistical software. HI- and HI+ pancreata were compared on endocrine cell mass, number of islets, isolation outcome and the average beta cell/islet area ratio per islet using student *t*-tests. HI-, HI+ and HI++ pancreata were compared to assess the influence of the severity of hyperemia on the yield/islet area ratio using one-way analysis of variance. Post hoc Bonferroni's multiple comparisons tests were performed to determine which groups differed from each other. To assess the influence of the percentage HIs in a pancreas on isolation outcome, linear regression analysis was performed with yield/islet area ratio as dependent and percentage HIs as independent variable. P values ≤ 0.05 were considered to be statistically significant.

RESULTS

Presence of HIs

Histological analysis showed that 48% of the pancreata in the purebred group contained at least one HI and were scored as HI+. HIs were found more frequently in juvenile than in adult purebred pigs (64% vs. 32%, $p < 0.001$). In the crossbred group, HIs were found in 68% of the pancreata. In purebred HI+ pancreata, 3.3 ± 3.1% of all assessed islets was hyperemic. In crossbred pancreata 3.1 ± 4.7% of all assessed islets in HI+ pancreata was histological abnormal.

We assessed the presence of HIs in different parts of both purebred and crossbred pancreata. There were no differences in the occurrence of HIs in either the splenic, duodenal or connecting lobes in both groups. We also found no significant differences in the occurrence of HIs between the different breeds (results not shown).

An experienced pathologist assessed a random selection of 10 HI+ and HI- pancreata. The exocrine tissue and the non-hyperemic islets appeared to be visually normal in both groups of pancreata. In the HI+ pancreata no macrophages or iron deposits were seen and no evidence for an ongoing chronic process was found. Beside the presence of HIs, no differences were seen between the HI+ and HI- pancreata.

Endocrine cell mass in relation to HIs

In both the purebred and crossbred group, significantly higher endocrine cell mass was found in the HI+ pancreata ($p = 0.006$ and $p < 0.001$, respectively) (Fig. 2A,C). HI+ pancreata were also found to contain significantly higher number of islets in 1

cm^2 tissue ($p = 0.009$ and $p < 0.001$, respectively) (Fig. 2B,D). To assess the influence of the severity of the hyperemia on the endocrine cell mass and number of islets, the HI+ pancreata were subdivided into HI+ and HI++ pancreata. One-way analysis of variance showed that the mean endocrine cell mass differed between the groups in both purebred and crossbred pigs ($p = 0.001$ and $p < 0.001$, respectively). In both purebred and crossbred pigs, Bonferroni's test revealed that the endocrine cell mass was significantly higher in the HI++ group compared to the HI- ($p < 0.001$ and $p < 0.001$, respectively) and HI+ ($p = 0.02$ and $p = 0.001$, respectively) groups. One-way analysis of variance showed that the mean number of islets differed between the groups in both purebred and crossbred pigs ($p < 0.001$ and $p < 0.001$, respectively). In both purebred and crossbred pigs, Bonferroni's test revealed that the number of islets was significantly higher in the HI++ group compared to the HI- ($p < 0.001$ and $p < 0.001$, respectively) and HI+ ($p = 0.003$ and $p = 0.007$, respectively) groups.

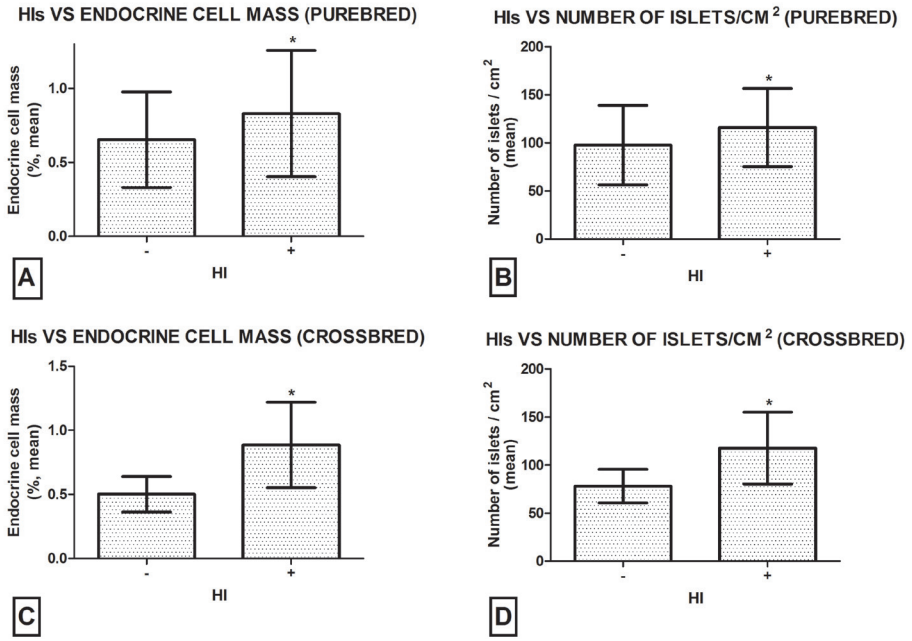

Figure 2: The endocrine cell mass and number of islets in relation to hyperemic islets.
The bars represent the mean endocrine cell mass (A,C) and the mean number of islets per cm2 (B,D). The error bars represent the standard deviation.
(A,B) Pancreata from purebred pigs. (A) Mean endocrine cell mass in HI- pancreata was 0.65 ± 0.32%. In HI+ pancreata this was 0.83 ± 0.43% *p = 0.006 (B) Mean number of islets per cm2 was 97.8 ± 41.2 in HI- and 116.0 ± 40.7 in HI+ pancreata *p = 0.009
(C,D) Pancreata from crossbred pigs. (C) Mean endocrine cell mass in HI- pancreata was 0.50 ± 0.14%. In HI+ pancreata this was 0.89 ± 0.33% *p < 0.001 (D) Mean number of islets per cm2 was 78.2 ± 17.5 in HI- and 117.7 ± 37.3 in HI+ pancreata *p < 0.001.

When we subdivided the purebred group into juvenile and adult pigs and assessed them separately, similar results were observed (data not shown).

These results suggest that the higher endocrine cell mass in the affected pancreata is associated with a higher number of the islets. The endocrine cell mass in HI+ pancreata was found to be 1.7 and 1.3 times higher compared to HI- pancreata in both purebred and crossbred pigs. It is not immediately obvious how this difference can be explained. Although the increased endocrine volume is paralleled by an increase in islet numbers, a straightforward comparison is confounded by the fact that only islets of greater than 50 μm were counted. When the underlying cause of the increased endocrine cell mass would be expansion of the individual islets, then a substantial number of islets smaller than 50 μm might cross the 50 μm limit and enlarge the total pool of countable islets. Alternatively, the most affected pancreata might simply have 1.7 and 1.3 time greater islet numbers. To elucidate this, we have assessed the average beta cell/islet area ratio per islet and furthermore counted all islets, including islets < 50 μm, in a limited series of 22 HI+ and 22 HI- pancreata. Furthermore, to assess and quantify possible swelling of the islets, we determined the average beta cell/islet area ratio per islet. The mean beta cell/islet area ratio in the HI- group was 0.57%, which was significantly higher ($p = 0.004$) than in the HI+ pancreata where this was 0.52%. No significant difference was found between the numbers of islets/cm^2 in HI- and HI+ pancreata (785 and 916, respectively, $p = 0.169$). This suggests that the higher endocrine cell mass in HI+ pancreata is the result of islet expansion, (probably through vasodilatation, edema or both) rather than to an increase in the number of islets.

Islet isolation yield in relation to HIs

In a series of isolations in 48 purebred adult and 25 crossbred porcine donors we assessed whether the presence of HIs might influence isolation outcome. Lower yields were found in HI+ pancreata compared to HI- pancreata in both purebred and crossbred pigs, but these differences were not significant ($p = 0.125$ and $p = 0.190$, respectively) (Fig. 3A,C). However, these outcomes may be confounded by the higher endocrine cell mass in HI+ pancreata given that pancreata with higher endocrine cell mass produce higher yields simply because there is more endocrine cell mass to begin with. Adjusted for the differences in endocrine cell mass, significantly lower yields were found in HI+ pancreata compared to HI- pancreata in both purebred and crossbred pigs ($p = 0.03$ in both groups) (Fig. 3B,D).

To assess the influence of the severity of the hyperemia on the yield/endocrine cell mass ratio, the HI+ pancreata were subdivided into HI+ and HI++ pancreata. One-way analysis of variance showed that the mean yield/endocrine cell mass ratio differed between the groups in both purebred and crossbred pigs ($p = 0.007$ and $p = 0.02$, respectively). Bonferroni's test revealed that the yield/endocrine cell mass ratio was significantly higher in the HI++ group compared to the HI- group ($p =$

0.02) in purebred pigs. In crossbred pigs the yield/endocrine cell mass ratio was also significantly higher in the HI++ group compared to the HI- group (p = 0.02). No significant differences were found between HI- vs HI+ and HI+ vs HI++ pancreata in both groups.

Linear regression analysis showed that the yield/endocrine cell mass ratio after was significantly lower in pancreata for every increase in the percentage of HIs in both purebred and crossbred pigs (B = -115.90, p = 0.005 and B = -106.25, p = 0.05, respectively).

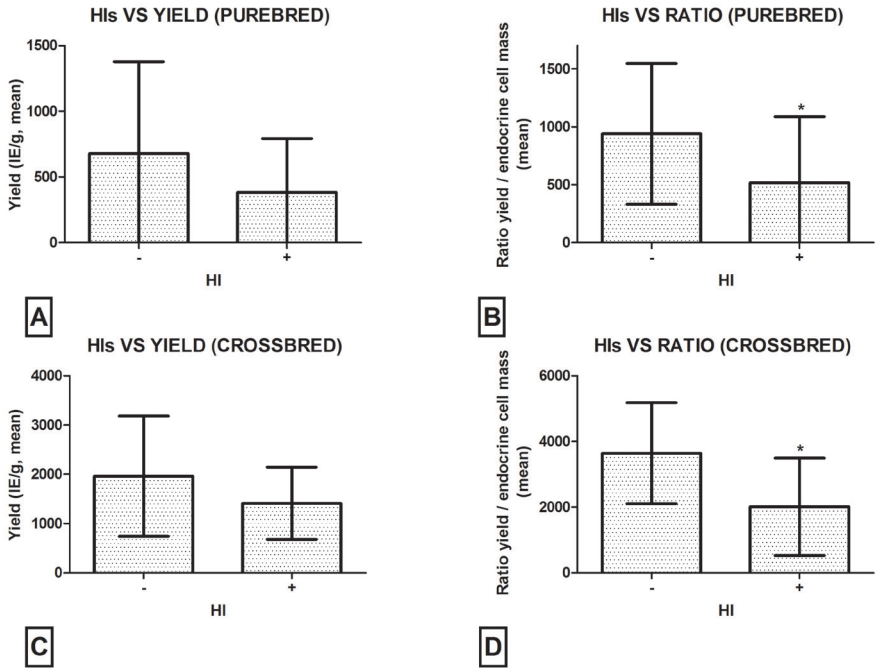

Figure 3: Islet isolation yield and yield/endocrine cell mass ratio in relation to hyperemic islets.
The bars represent the mean islet isolation yield (A,C) and the mean yield/endocrine cell mass ratio (B,D). The error bars represent the standard deviation.
(A,B) Pancreata from purebred pigs (A) Mean yield in HI- pancreata was 679.2 ± 697.5 IE/g. In HI+ pancreata this was 383.4 ± 408.9 IE/g. (B) Mean yield/endocrine cell mass ratio was significantly lower in HI+ pancreata *p = 0.03
(C,D) Pancreata from crossbred pigs. (C) Mean yield in HI- pancreata was 1960.5 ± 1220.6 IE/g. In HI+ pancreata this was 1411.0 ± 733.6 IE/g. (D) Mean yield/endocrine cell mass ratio was significantly lower in HI+ pancreata *p = 0.03

DISCUSSION

Similar to our findings in human donor-pancreata (12), a remarkably high number of hyperemic islets was encountered when studying histological characteristics of the porcine pancreas in relation to islet isolation. Similar islets have been reported

anecdotally in the literature but no mechanisms were described regarding their origination (7-11). Also, no relevance has been determined from the perspective of the isolation of islets for transplantation. Although a slight hyperemia in islets is seen in normal metabolic situations when higher insulin release is demanded (11,15), the phenomena encountered in the present study are far more dramatic and are most likely not related to the normal glucose metabolism. In the literature only one study mentioned the finding of dilated blood vessels in islets, caused by congestion (7). The authors excluded the affected porcine pancreata from their study and gave no data on incidence and severity of the phenomenon, or on possible consequences for isolation outcome.

In the present study, HIs were found in 48% of the pancreata in purebred and in 68% of crossbred pigs. However, the HI+ category consisted of pancreata with at least 1 HI. This could potentially lead to an overestimation of the HI- group, when a hyperemic islet was missed by an observer or when an islet appeared just outside the sectioning plane. However, because 2 observers independently assessed the tissue samples with good agreement and since we have found no differences in the presence of HIs between different parts of the pancreata, it is unlikely that our results were influenced by this. Even if such a misclassification would have occurred, it would have lead to an underestimation of the relationship between HI and isolation outcome, given that HI+ pancreata have lower yields than true HI- pancreata. As a result, the data presented here are a conservative estimate of the strength of the relationship between HI and isolation outcome. Furthermore, we have found similar results in our study of 102 human donor-pancreata (12). In this previous study, HI's were found in 54.6% of all human donor-pancreata, with 4.5% of all islets in the affected pancreata being hyperemic. The affected human pancreata contained slightly more endocrine tissue, and produced significantly lower yields similar to the findings in porcine pancreata. It thus seems to be a general phenomenon occurring in about half of the pancreata.

We have investigated the possible relevance of the presence of HIs from the perspective of islet isolation. A higher endocrine cell mass was found in the affected pancreata. Although the increased endocrine volume was paralleled by an increase in islet numbers, a straightforward comparison is confounded by the fact that only islets of greater than 50 μm were counted. To elucidate this, we have assessed the average beta cell/islet area ratio per islet and furthermore counted all islets, including islets < 50 μm, in a limited series of 22 HI+ and 22 HI- pancreata. We found a significantly lower beta cell/ islet area ratio in HI+ pancreata, without a difference in the total number of islets compared with HI- pancreata. This suggests that islets in these pancreata are more swollen, probably through vasodilatation, edema or both.

When we corrected for the fact that the HI+ pancreata contained significantly more endocrine tissue, significantly lower yields in the HI+ pancreata were seen compared to the HI- pancreata in both purebred and crossbred pigs. This is remarkable

since in HI+ pancreata only a small percentage of all islets was histological abnormal. Most likely, the substantial increase in islet volume, or the reduced isolation outcome is not caused by this small fraction itself. A possible explanation could be that the abnormal islets are just an indicator of the actual damage of the islets. No macrophages or iron deposits were seen and no evidence for an ongoing chronic process was found, a possible explanation is that the HIs arose shortly before the exsanguination and death of the animal and that for instance a sudden rise in blood pressure is responsible for the formation of HIs. In veterinarian literature, it is widely recognized that the slaughtering process induces a rise of the blood pressure with hemorrhages of different extent (16-18). However, further research is needed into the mechanisms by which these hyperemic islets arise e.g. by comparing pigs killed by electric stunning to pigs killed by another method. In our human study, prolonged ICU stay and pre-procurement hypertension were associated with the presence of HIs (12). On the other hand, HIs were also found in 43.3% of "normal" pancreatic tissue samples obtained from elective surgical procedures. So apparently the presence of HIs is not limited to donor organs. However, these "elective patients" were most likely not admitted to the ICU before undergoing surgery and were probably hemodynamically more stable than the pancreas donors. Therefore, it seems likely that, besides the pre-procurement hemodynamic status, the handling of the pancreas during surgery could also be a contributing factor in the development of HIs. This could also be a contributing factor in porcine pancreata.

We have found a relation between the severity of the hyperemic islets in the pancreata in relation to the endocrine cell mass and number of islets, with an increase in severity of the hyperemia being paralleled by a higher endocrine cell mass and number of islets. On the other hand, an increase in severity of the hyperemia did not seem to have a relation with isolation outcome. However, the absence of such a statistical difference could have been the result of the numbers in our study being too small, since there is a statistically lower isolation outcome in HI+ pancreata when compared to HI- pancreata. Furthermore, linear regression analysis showed significantly lower isolation outcome in pancreata containing a higher percentage of HIs. We have found similar results in human donor-pancreata (12). To establish the importance of HIs for islet transplantation, further research, involving post-purification yield, purity, viability etc. would be necessary.

Taken together, we hypothesize that hyperemic islets in porcine pancreata are just an indicator of the actual damage of the islets in the pancreas. These damaged islets could be more prone to fragmentation during the isolation procedure, and thus have more chance to become smaller than 50 μm, thereby resulting in lower isolation yields given the common practice of not including islets smaller than 50 μm when determining the isolation yield. This hypothesis should be tested in future research.

In conclusion, these data confirm that the presence of hyperemic islets may negatively influence isolation outcome in porcine pancreata as they were shown to do in human pancreata and possibly affect the islet quality in the procurement procedure. It is tempting to speculate that this eventually may explain part of the generally experienced difficulties in obtaining sufficient amounts of porcine islets.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. I.M. Bajema for the histological assessment of the differences between most and not affected tissue samples and K.G. van der Ham for the images of the hyperemic islets.

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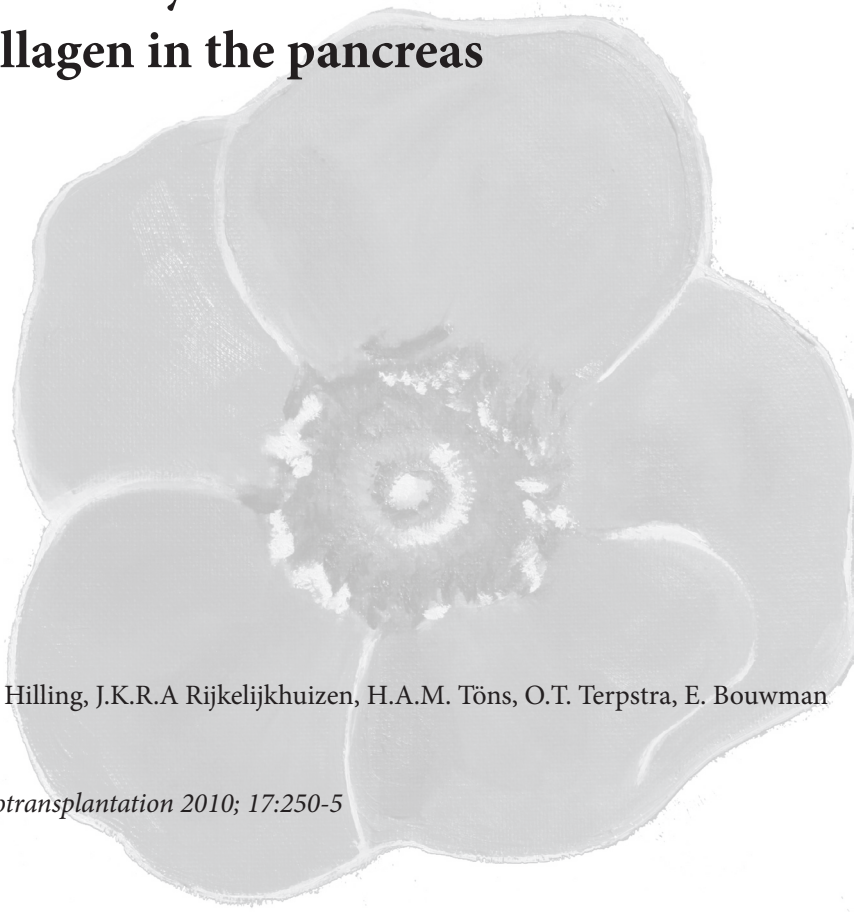
Chapter 7

Porcine islet isolation outcome is not affected by the amount and distribution of collagen in the pancreas



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Xenotransplantation 2010; 17:250-5

ABSTRACT

Background

Variable islet yields in porcine islet isolation may be caused by the collagen substrate within the pancreas. The aim of the present study was to determine the total amount and distribution of collagen within porcine pancreata and their relationship to islet isolation outcome.

Method and Materials

A total of 64 juvenile and 76 adult porcine pancreata of eight purebred breeds were histologically examined. The amount of collagen was quantitatively assessed in tissue samples stained with Sirius Red. Collagen distribution was semi-quantitatively determined by assessing the presence of collagen in the endocrine-exocrine interface and within the islet, in tissue samples stained with Sirius Red and anti-insulin. Islet isolation was performed in 58 pancreata of the adult group.

Results

Total collagen content and islet encapsulation ranged widely in both adult and juvenile pigs. However, the majority of islets in adult and juvenile pigs had no or only a limited collagen capsule. The difference in collagen content between adult and juvenile pigs could not be explained by age. Furthermore, no differences between adult and juvenile pigs were found in islet encapsulation or the amount of intra-islet collagen. In adult pigs, no significant relationships were found between obtained islet yield and total collagen content, islet encapsulation or amount of collagen within the islet.

Conclusions

Considering the limitations in experimental design (staining method) and study material, isolation outcome does not seem to be affected by the total collagen content or collagen distribution. The influence of other matrix elements and collagen subtypes should be investigated.

INTRODUCTION

The pig is considered to be a potentially alternative for human donors of islets of Langerhans (1-5). However, porcine islet isolation procedures have been shown to be notoriously difficult. A possible explanation could be related to donor age and to the relative fragility of the islets of juvenile pigs (4,6,7). Collagenase digestion of the young porcine pancreas usually results in a complete dissociation of both the exocrine and endocrine tissue (7). On the other hand, islet isolation procedures using pancreata from adult pigs have resulted in large islet yields (8-10).

Furthermore, despite improvement of isolation procedures, islet isolation is still associated with a considerable loss of endocrine tissue. This indicates that collagenase digestion of the pancreas is not limited to the exocrine pancreas but affects the islets as well. Because collagen is the major target in the enzymatic dissociation of the pancreas, the collagen substrate within the pancreas is one of the variables that could account for the unpredictable, highly variable islet yields (7,10-12). As successful islet isolation depends upon effective separation of islets from exocrine tissue, a more detailed knowledge of the composition of the connective tissue of the pancreas on which collagenase is acting is necessary.

Previous studies reporting on the collagen content of porcine pancreata have based their conclusions on a small number of animals and furthermore, did not correlate their results to the actual isolation outcome of the pancreata used in their collagen assessment (10-14).

The aim of the present study was to determine the total amount and distribution of collagen within a large study population of adult and juvenile porcine pancreata and assess the relationship of these determinants to the outcome of islet isolation in adult pigs.

MATERIALS AND METHODS

Organ Procurement and Pancreatic Sampling

A total of 140 purebred porcine pancreata were harvested in different slaughterhouses. The population consisted of a juvenile group (6 to 12 months) of 64 animals and involved five different breeds (Great Yorkshire n = 21, Dutch Landrace n = 25, Norwegian Landrace n = 2, Large White n = 9, Finnish Landrace n = 7). The adult group (12 to 78 months) consisted of 76 animals and involved eight different breeds (Piétrain n = 8, Great Yorkshire n = 10, Dutch Landrace n = 15, Norwegian Landrace n = 7, Large White n = 7, Hampshire n = 5, Finnish Landrace n = 14, Duroc n = 10). All animals were killed by electric stunning and exsanguination. Warm ischemia times were between 20 and 30 minutes. After dissection on ice, the pancreata were

stored in cold (4°C) Hank's Balanced Salt Solution (HBSS) and transported on ice to the laboratory for further processing. Cold ischemia times were between 2 and 5 h. Biopsies were taken, immersed in Bouin's fixative, cleared and stored in ethanol 70%.

Amount of collagen

To detect collagen, paraffin-embedded tissue samples of 4μm were stained with 0.1% Sirius Red F3B (Klinipath, Duiven, The Netherlands) in a saturated solution of picric acid for 90 minutes, washed with a saturated solution of picric acid, dehydrated and cleared with xylenes. Sirius Red, when dissolved in a saturated solution of picric acid, stains both structural as well as basement membrane collagen and consequently stains all types of collagen without differentiating between collagen subtypes (15-17).
Quantitative measurements were made using the Zeiss KS-400 image analysis system (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK). The collagen stained area was quantified by the assessment of 30 random fields in each tissue sample (Fig. 1 A, B). The KS-400 image analysis system quantified the red stained area per field, by calculating the percentage of red staining in each field. The average percentage of red staining in these 30 fields was considered to be a valid representation of the amount (percentage) of collagen in the whole pancreas.

Distribution of Collagen

Distribution of collagen was evaluated in 4-μm sections of the same paraffin-embedded tissue samples as used in the determination of the amount of collagen. Collagen was stained with 0.1% Sirius Red F3B (Klinipath, Duiven, The Netherlands) and the identification of islets was facilitated by staining the beta cells with a polyclonal guinea-pig anti-porcine insulin antibody (Zymed, Invitrogen, Carlsbad, USA) at a dilution of 1:200 overnight. To visualize the immunoreactions, sections were incubated with 3,3'-diaminobencidine (DAB) dissolved in Tris/HCl (0.05 M) with 15 μl H_2O_2.

Distribution of collagen was determined by assessing the presence of collagen within the islet and in the endocrine-exocrine interface (the "islet-capsule"), in an average of 100 islets per tissue sample. The presence of collagen in the islet-capsule (prior to isolation) of each assessed islet was scored in four categories: 0% to 25%, 25% to 50%, 50% to 75%, 75% to 100% collagen encapsulation (Fig. 1 C,D). The average score of all islets in a tissue sample was taken to represent the islet encapsulation with collagen in the whole pancreas. The amount of collagen within the islets was scored semi-quantitatively: 0 (absent), 1 (intra-islet collagen present in $\leq$ 25% of islet area), 2 (intra-islet collagen present in >25% of islet area) (Fig. 1 E,F). The average score of all islets in a tissue sample was considered to be a valid representation of the amount of collagen within the islets in the whole pancreas.

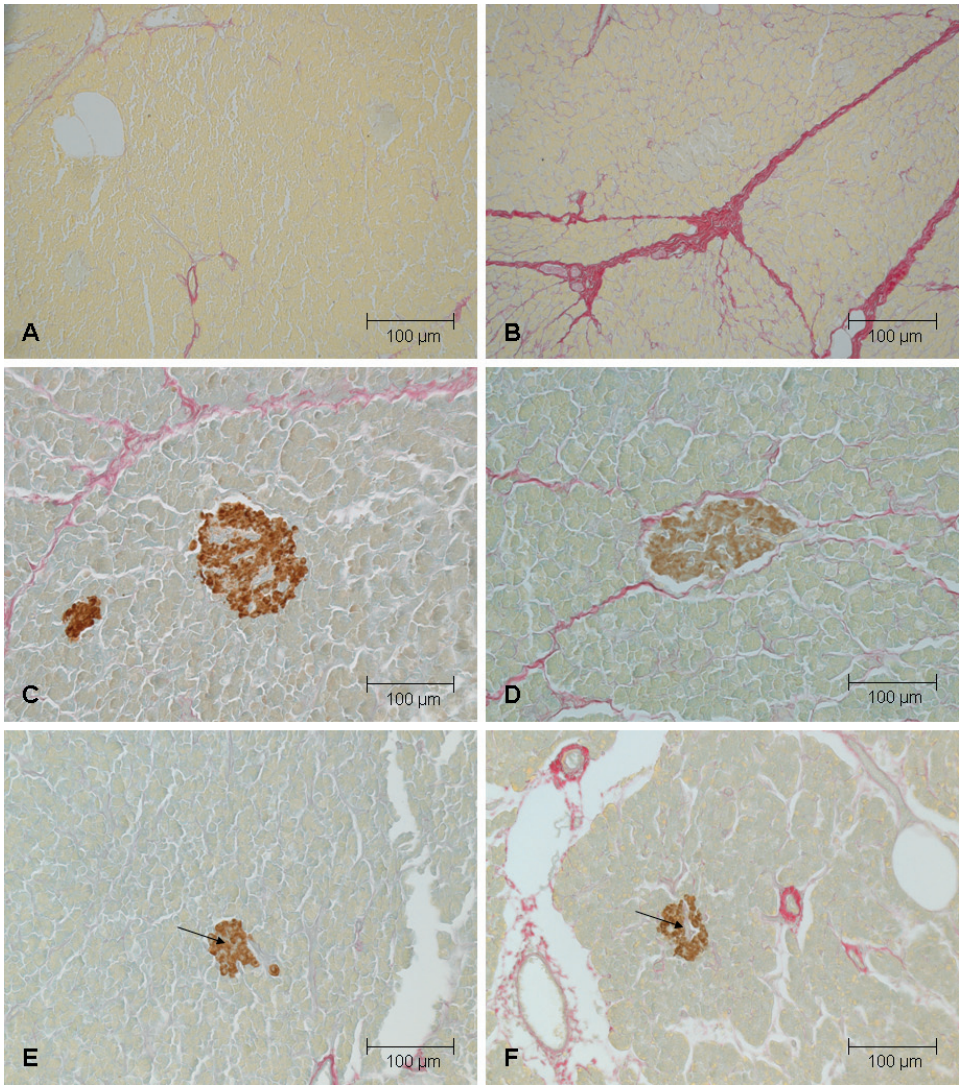

Figure 1: Total amount of collagen and collagen distribution
A,B) The total amount of collagen (red) was determined in tissue samples stained with Sirius Red in a saturated solution of picric acid. The collagen stained area was quantified by the assessment of 30 random fields in each tissue sample
A) 1 of the 30 fields of a pancreas with an average amount of collagen of 3.0%. This field displays part of the exocrine tissue of the pancreas neck B) 1 of the 30 fields of a pancreas with an average amount of collagen of 10.2%. This field displays part of the exocrine tissue of the pancreas neck.
C,D) Collagen encapsulation (red) was assessed in tissue samples double stained with Sirius Red and anti-insulin (brown)
C) Islet with 0-25% collagen encapsulation D) Islet with 75-100% collagen encapsulation
E,F) Intra-islet collagen (arrows) was assessed in tissue samples double stained with Sirius Red and anti-insulin (brown)
E) Islet with no visible intra-islet collagen, score: 0 F) Islet with intra-islet collagen present in <25% of the islet area, score: 1

Islet isolation

Islet isolation was performed in 58 porcine pancreata of the adult group. The isolations in this study were performed after obtaining sufficient experience with porcine islet isolations to achieve consistent results. The pancreata were cut clean in the laboratory. The arm of the pancreas (20 to 40 g in weight) was intraductally injected with a solution of Liberase PI (0.5 mg/ml, Roche, Basel, Switzerland) in University of Wisconsin solution. The distended tissue was cut into pieces and incubated in HBSS at 37°C for 20 to 30 min. The digested tissue was placed in a beaker on ice, which was manually shaken to dissociate gently and dilute the tissue. Subsequently, the tissue was poured over a filter with 1000-μm mesh and washed in cold HBSS for three times. Islet samples of 25 μl were stained with an equal volume of Dithizone solution (DTZ, diphenylthiocarbazone, Sigma, Steinheim, Germany), freshly prepared by the addition of 5% DTZ stock-solution (13 mM DTZ dissolved in 91.2% ethanol supplemented with 1.2% NH_4OH) to HBSS. Islet numbers, expressed as islet equivalents (IE, representing islets of 150 μm diameter), were determined in duplicate.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). After visual inspection of the data, these were found to be normally distributed so that parametric tests were used. Juvenile and adult pigs were compared by the parametric student *t*-test. To determine the effect of age on collagen content and islet encapsulation, multiple linear regression was performed with either collagen content or islet encapsulation as dependent factors and groups of pigs (juvenile vs. adult) and age in months as independent variables. One way analysis of variance was used to compare isolation outcomes between different breeds. Multiple linear regression was performed with yield as dependent factor. Independent factors were either total amount of collagen, islet encapsulation or intra-islet yield, breed and age in months. P values ≤ 0.05 were considered to be statistically significant.

RESULTS

Amount of Collagen

The mean collagen content (± SD) in the adult group was 5.5 ± 2.0%, this was significantly higher ($p < 0.001$) than in the juvenile group where this was 3.7 ± 1.3% (Fig. 2A). The collagen content ranged in adult pigs from 1.7 to 11.0% and in juvenile pigs from 1.1 to 7.1%, showing a widespread range in collagen content between pancreata.

When we corrected for age in months, a difference in collagen content in juvenile vs. adult groups still remained ($B = -2.08$, $p < 0.001$). However, this difference

could not be explained by the age in months (B = -0.01, p = 0.40), and must therefore be explained by another (unknown) difference between these groups.

In a limited series of 15 (randomly selected) pancreata, the collagen content was assessed in tissue samples from two different parts of the pancreas. No clear differences in collagen content were observed between the different parts of the pancreas (results not shown).

Collagen distribution

In adult pancreata, the mean degree of encapsulation of the islet with collagen was 38.9 ± 12.8%. In juvenile pancreata, the mean percentage was 35.2 ± 8.7% (p = 0.04) (Fig. 2B). This percentage ranged in adult pigs from 14 to 78% and in juvenile pigs from 19 to 64%, showing a widespread range in islet encapsulation between pancreata.

However, when adjusted for age in months, no difference in islet encapsulation between adult and juvenile groups was found (B = -4.76, p = 0.09).

The majority of the assessed islets was 0% to 25% encapsulated with collagen. Moreover, 82% of the assessed islets in adult and 88% in juvenile pigs was encapsulated < 50% (Table 1).

We found no significant difference between juvenile and adult pigs with regard to the presence of collagen within the islet (p = 0.69) (Fig. 2C). In all instances, collagen was exclusively located around the capillaries.

In a limited series of 15 (randomly selected) pancreata, the islet encapsulation and intra-islet collagen were assessed in tissue samples from two different parts of the pancreas. No clear differences in islet encapsulation or intra-islet collagen were observed between the different parts of the pancreas (results not shown).

Amount of collagen and collagen distribution vs. islet yield

After isolation, the average obtained islet yield in 58 adult pigs was 545.4 ± 601.2 IE/g pancreas (range 18 to 3849). One way analysis of variance showed that the mean obtained yield did not differ between the different breeds (p = 0.28)

When corrected for breed and age in months, no significant relations were found between isolation outcome and collagen content, islet encapsulation and intra-islet collagen (B = -33.28 p = 0.52, B = -1.51 p = 0.81 and B = 103.02 p = 0.85, respectively).

Table 1. Percentage collagen encapsulation in adult and juvenile pancreata

	Percentage collagen encapsulation, mean ± SD			
	0-25%	**25-50%**	**50-75%**	**75-100%**
Adult pigs (n = 76)	59.6 ± 21.3	22.6 ± 8.8	10.6 ± 8.9	7.3 ± 7.7
Juvenile pigs (n = 64)	68.7 ± 16.8	19.7 ± 8.8	7.1 ± 6.2	4.6 ± 4.2

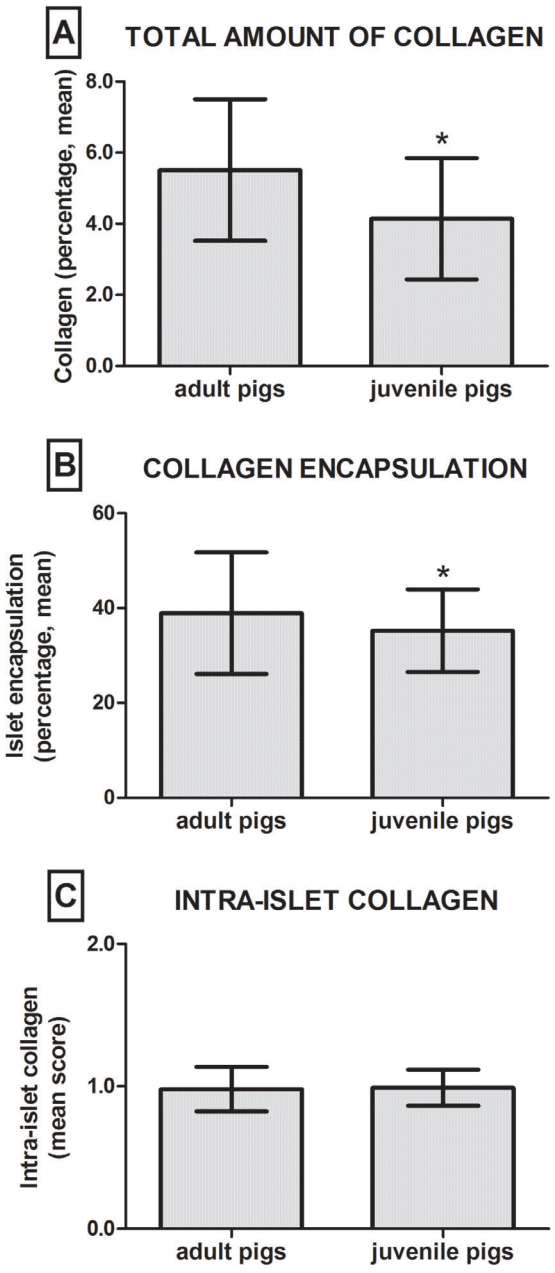

Figure 2: Total amount of collagen and collagen distribution

The bars represent A) the mean percentage of collagen B) the mean percentage of the circumference of the islets that is encapsulated with collagen C) the mean score for intra-islet collagen. The error bars represent the standard deviation.

A) The mean percentage of collagen in adult pigs was significantly higher than in juvenile pigs, * $p<0.001$

B) The mean islet encapsulation with collagen was significantly higher in adult than in juvenile pigs * $p=0.04$

C) No significant differences ($p=0.94$) in intra-islet collagen were found between adult and juvenile pigs

DISCUSSION

Variable yields in porcine islet isolation may be caused by the collagen substrate within the pancreas. If this were true, we would be able to select eligible pigs in advance, to achieve higher isolation yields. However, considering the limitations in experimental design (staining method) and study material, in our study of 58 adult pigs we failed to observe a relationship between isolation outcome and total amount of collagen, islet encapsulation and intra-islet collagen. Juvenile pigs are generally shown to produce lower islet yields than adult pigs and collagenase digestion usually results in complete dissociation of the pancreas (7-10). These contrasting results may also be caused by differences in the collagen substrate between adult and juvenile pigs. However, because we found no relationship in adult pigs and the variance of total amount of collagen, islet encapsulation and intra-islet collagen was even smaller in juvenile pigs, it is unlikely that these would have an effect on isolation outcome in juvenile pigs.

The total amount of collagen in porcine pancreata has been considered to be an important factor in determining the isolation result (11). Yet our results do not support this., the difference in collagen between adult and juvenile pigs in our study could not be explained by the age-difference, but may be explained by another (unknown) difference between those groups. A collagen capsule surrounding the islet could potentially provide protection against enzymatic disintegration of islets and consequently their fragmentation, it has been suggested that a factor in the differing results is a more extensive capsule surrounding the islets of the adult pig pancreas as compared to the young pig pancreas (7,10). However, in our study, we found no difference in islet encapsulation between adult and juvenile pigs. Both adult and juvenile pancreata had no or only a very limited collagen capsule. This is in accordance with van Suylichem et al. (11) and van Deijnen et al. (13) who found that in pigs, the adhesion between islets and exocrine tissue almost exclusively depends on cell-to-cell adhesion.

Our isolation results could have been influenced by (i) the isolation method and (ii) the procurement method and pancreas sampling. The isolation method we used is an accepted method of islet isolation, which is performed in many centers in every day practice and provides consistent results. Even though other methods may result in higher yields, it is unlikely that the observed relationship between isolation outcome and collagen was influenced by the isolation method. Ideally, pancreas samples should be taken before any manipulation takes place and warm ischemia times should be as short as possible. However, as the same procedure was used for all the pancreata in our study, it is unlikely that it will explain any relationship between collagen and isolation outcome.

In the present study, Sirius Red was used to stain collagen. Hence, our observations concern the amount and distribution of collagen in general, without differentiating between subtypes. Previous studies have shown that collagen types I,

III, IV and VI are present in the peri-islet capsule of the porcine pancreas (12,18). Compared with rat, human, and canine tissues, expression of most collagen types and laminin in the porcine pancreas seems to be rather weak (11). It can be speculated that the composition of the islet capsule, and the relative concentration of the components, could influence islet isolation outcome and possibly confound the observed relation between islet isolation outcome and the complete islet capsule. However, as we found no or only a very limited collagen capsule in our study population when we stained tissue samples for all types of collagen, we expect that collagen subtypes play no or only a minor role.

In conclusion, although collagen is the major target in the enzymatic dissociation of porcine pancreata and although total collagen content and collagen distribution show high variability, we found no relationship between islet isolation outcome and these determinants in adult pigs. However, other matrix elements and collagen subtypes could play a role and should be further investigated.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. P.J. Marang-van de Mheen for the statistical advice and K.G. van der Ham for the images of the total amount of collagen, islet encapsulation and intra-islet collagen.

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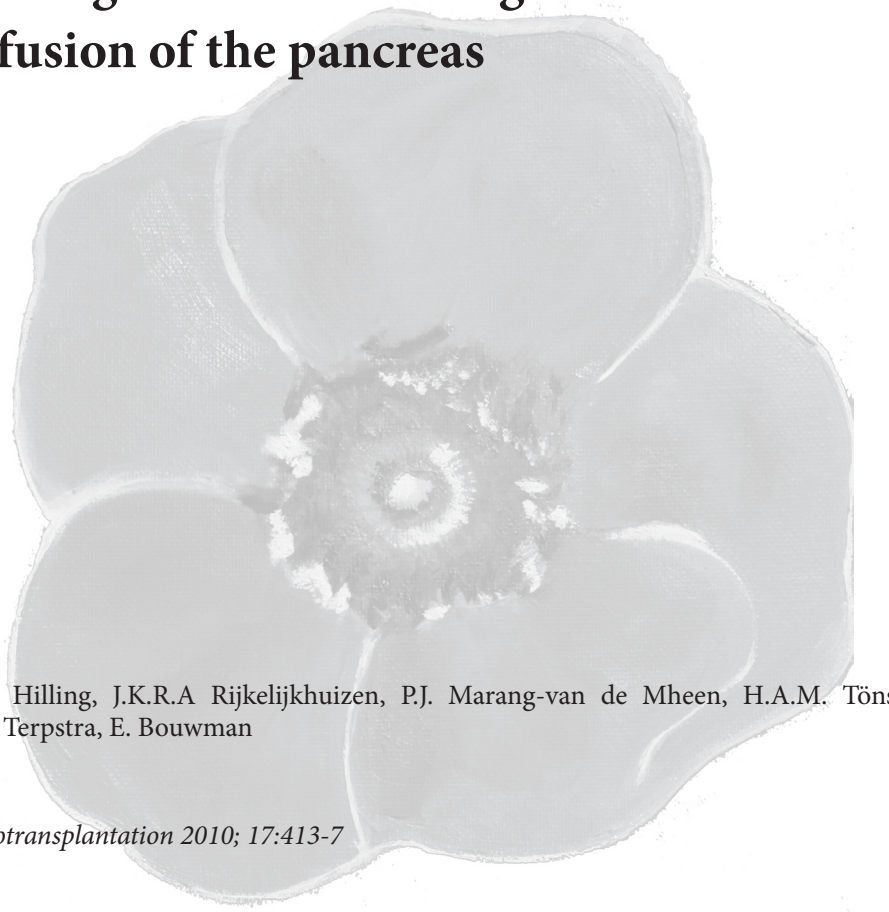
Chapter 8

Morphological changes of porcine islets of Langerhans after collagenase and HBSS infusion of the pancreas



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Xenotransplantation 2010; 17:413-7

ABSTRACT

Background

A remarkable change in porcine islet morphology was observed after infusion of the pancreas with collagenase. The aim of the present study was to quantify these morphological changes and to assess whether these changes were due to the volume expansion caused by the collagenase entering the islet or the result of its digestive effects.

Methods and Materials

This study was performed in pancreata of 28 crossbred pigs. First, eight pancreata were intraductally injected with collagenase by a continuous controlled pressure of 180 mmHg. Pancreas samples before collagenase infusion were used as controls. All tissue samples, both before and after infusion, were stained with anti-insulin. To quantify the morphological change of the islets, the mean beta cell/endocrine content ratio of the infused and not-infused tissue samples was compared. In a second experiment, 20 pancreata were similarly assessed after intraductal injection with Hank's Balanced Salt Solution (HBSS).

Results

In both the collagenase- and HBSS-infused groups, mean beta cell/endocrine content ratio was lower than in the control samples. The observed decline in the beta cell/endocrine content ratio was not significantly different between collagenase- and HBSS-infused pancreata. This suggests that the lower beta cell/endocrine content ratio and thus the morphological change in the infused tissue samples is caused by volume expansion of the fluid entering the islet and that the digestive effect of collagenase plays no or only a minor role.

Conclusions

Morphological changes of islets are observed after infusion of pancreata with collagenase and HBSS, most likely caused by volume expansion due to fluid entering the islets.

INTRODUCTION

The pig is considered to be a potential alternative for human donors of islets of Langerhans. However, porcine islet isolation procedures have been shown to be notoriously difficult (1-4). Although procedures have been improved, porcine islet isolation is still associated with considerable loss of endocrine tissue. This indicates that not only the exocrine pancreas is affected by collagenase digestion, but that collagenase affects the islets as well. If we can find explanations for the loss of endocrine tissue, this may be a first step into finding a solution for this problem so that isolation procedures can be improved.

When studying histological characteristics of porcine pancreata in relation to islet isolation, a remarkable change in morphology of the islets was observed after infusion of the pancreas with collagenase. Previous studies have shown collagenase located within the islets after standard intraductal infusion (at a perfusion pressure of 180 mmHg) of collagenase in human and also at lower perfusion pressures (as low as 50 mmHg) in porcine pancreata (5,6). The observed morphological changes could therefore be a result of either volume expansion of collagenase entering in the islet, leading to disruption of cell-cell contacts or be the result of the digestive effect of collagenase, subsequently leading to islet fragmentation.

Other studies have reported on islet morphology after isolation (7,8). However, both studies have assessed the shape and size of islets after isolation, but did not perform a quantitative study of morphologic changes in islets after collagenase distension. Furthermore, it is not known whether the morphological changes are specific for collagenase or might also be observed for other fluids. If these are also shown for other fluids, this would suggest that the morphological changes are the result of volume expansion caused by fluid entering the islets.

The aim of the present study was to quantify the observed morphological changes after collagenase infusion. Furthermore, we aimed to discriminate whether these morphological changes are the result of volume expansion of collagenase entering in the islet or the result of the digestive effect of collagenase. To distinguish between these two hypotheses, a second series of experiments was performed in which pancreata distended with HBSS.

METHODS AND MATERIALS

Organ procurement and pancreatic sampling

A total sample of 28 pancreata of crossbred pigs (6-12 months) was harvested in a commercial slaughterhouse. All animals were killed by electric stunning and exsanguination. Warm ischemia times were between 20 to 30 min. After dissection

on ice, the pancreata were stored in cold (4°C) HBSS and transported on ice to the laboratory for further processing. Cold ischemia times were between 2 to 5 h, with most pancreata having a cold ischemia time around 3 h. Per pancreas, 1-cm biopsies of the duodenal part, splenic part and arm of the pancreas were taken directly before and some time after collagenase and HBSS infusion, immersed in Bouin's fixative, cleared and stored in ethanol 70%.

Collagenase and HBSS distension

Eight pancreata were distended with Liberase PI (Roche Applied Science, Manheim, Germany). Liberase PI was stored at a temperature of -20 °C and defrosted directly before injection. The pancreata were dissected free of surrounding fat and vessels in the laboratory. The main pancreatic duct was cannulated at the arm of the pancreas and intraductally injected with a solution of Liberase PI (0.5 mg/ml, solution temperature 4 °C at the start of infusion) in University of Wisconsin Solution (UW) by a continuously controlled pressure of 180 mmHg for 10 min. We have chosen 180 mmHg as perfusion pressure because it is used in standard infusion protocols. The syringe was removed before taking the biopsies. In a second experiment, 20 pancreata were intraductally injected with HBSS in the same way.

Histology

In paraffin-embedded sections of 4 μm, beta cells were stained with a polyclonal peroxidase-labeled guinea pig anti-porcine insulin antibody (Zymed, Invitrogen, Carlsbad, CA, USA) at a dilution of 1:200 overnight and visualized (brown colored staining) with 3,3'-diaminobencidine (DAB) dissolved in Tris/HCl (0.05 M) with 15 μl H2O2. The specificity of the antibody was confirmed by a negative control. Infused and not-infused tissue samples were stained in the same way.

Microscopic evaluation

An average of 75, randomly selected, islets per tissue sample were assessed. As is generally practiced, islets smaller than 50 mm were not included. To quantify the beta cell content (Fig. 1A,D), the brown stained area was quantified using the Zeiss KS400 image analysis system (Zeiss-Vision, Germany). The Zeiss KS-400 image analysis system quantified the brown-stained area per field, by calculating the percentage of brown staining in each field. Image J (freeware image processing tool) was used to manually delineate every islet used in the assessment of the beta cell content (Fig. 1B,E). The islets were independently delineated by two observers. The observers were blinded for the category of the tissue samples (collagenase/HBSS-infused or not-infused tissue samples) when delineating the islets.

In human islets (consisting of alpha, beta, delta and PP cells), beta cells represent the majority of the endocrine content. Because porcine islets have comparable

cytoarchitecture as human islets, where the outermost cells are commonly beta cells (9-11), the area inside the surrounding line was considered to represent the endocrine content and colored black using Image J (Fig 1C,F). This black colored area was also quantified using the Zeiss KS400 image analysis system by calculating the percentage of black staining in each field. The ratio between the beta cell and endocrine content was calculated by dividing both percentages (of brown and black staining) in both the infused and not-infused tissue samples. Per tissue sample an average beta cell/endocrine content ratio was calculated.

To quantify alterations in islet morphology, the average beta cell/endocrine content ratio of not-infused and infused tissue samples of the same pancreas were compared. An in- or decline in beta cell/endocrine content ratio after infusion would implicate a change in islet morphology. For example, the greater the decline in beta cell/endocrine content ratio, the greater the morphologic change after collagenase/HBSS infusion would be.

Data analysis

Statistical analysis was performed using SPSS 16.0 (IBM Corporation, Somers, NY, USA) statistical software. After visual inspection of the data, these were found to be normally distributed so that parametric tests were used. Because the beta cell/endocrine content ratio after infusion is related to the beta cell/endocrine content ratio before infusion, these ratios were compared for every individual pancreas using paired student t tests in both HBSS- and collagenase-infused pancreata. To assess the influence of the infused fluid, we calculated the difference between the beta cell/endocrine content before and after infusion for every individual pancreas and consequently compared the average difference between collagenase- and HBSS-infused pancreata using independent student t tests Results are presented as mean ± SD.

RESULTS

In a pilot experiment, three observers (DEH, EB and a medical student) independently assessed infused and not-infused tissue samples of eight collagenase-distended pancreata. The tissue samples were blinded in such a way that the observers were unable to distinguish from the outside whether the samples were infused or not-infused tissue samples. All observers could discriminate the infused and not-infused tissue samples with 100% accuracy, based on the substantial morphological differences observed in the histological structure of the islets and exocrine tissue.

No significant difference was found between the two observers in the quantification of the endocrine content ($p = 0.90$). The average of the two observers was therefore used in further assessments.

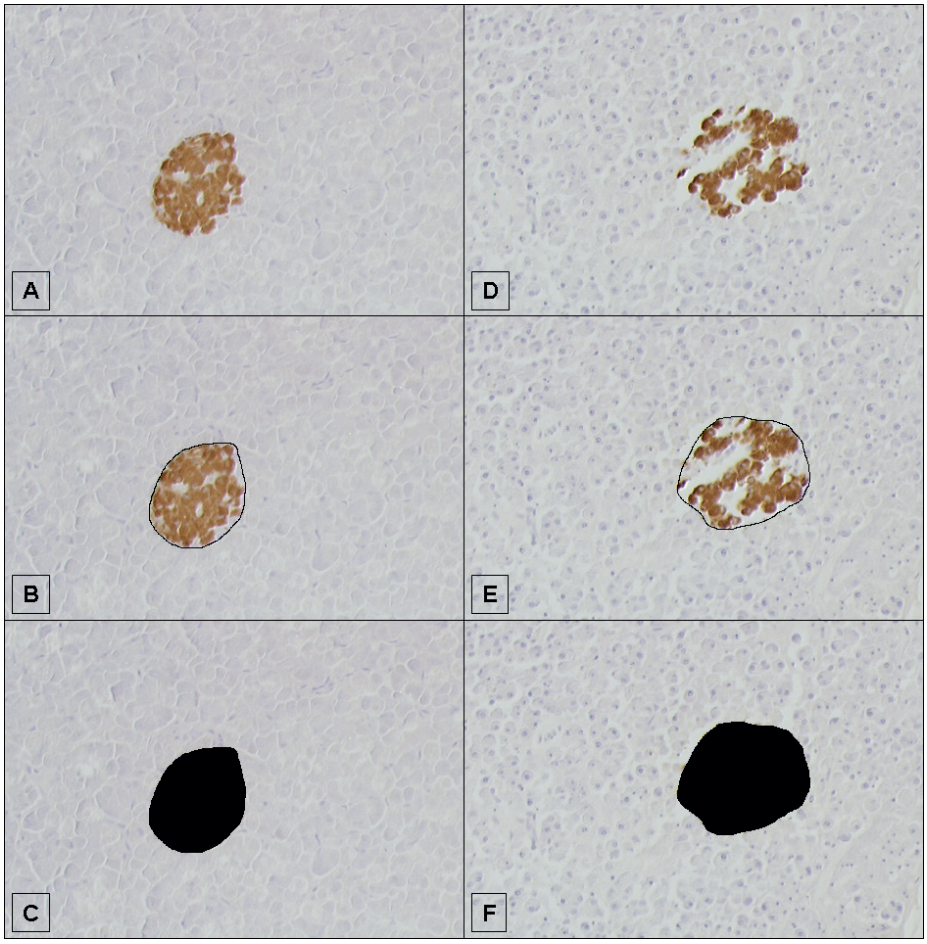

Figure 1: Calculation of the beta cell/endocrine content ratio
(A) Islet of a not-infused tissue sample. The brown stained (beta cell) area was quantified usinag an image analysis system. To quantify the endocrine content, every islet used in the assessment of the beta cell content, was surrounded by a manually drawn line (B). The area inside the surrounding line was considered to represent the endocrine content (C). The ratio between the beta cell and endocrine content was calculated: area A/area C = ratio not-infused.
(D) Islet of the same pancreas, infused with HBSS. The brown-stained (beta cell) area and endocrine content were determined in similar fashion as in the not-infused tissue sample (E, F). The ratio between the beta cell and endocrine content was calculated: area D/area F = ratio infused.
To quantify the morphological change after infusion, the ratios of the not-infused and infused tissue samples were compared.

In eight collagenase-infused pancreata, mean beta cell/endocrine content ratio (± SD) in the not-infused tissue samples was 0.44 ± 0.08. After infusion this ratio was significantly lower: 0.34 ± 0.07 ($t = 2.545$, $p = 0.04$) (Fig. 2). In 20 HBSS-infused pancreata, mean beta cell/endocrine content ratio in the not-infused tissue samples was 0.49 ± 0.09. After infusion this ratio was significantly lower: 0.38 ± 0.09 (t =

3.795, p = 0.001) (Fig. 2). No significant differences in beta cell/endocrine content ratios were found between different parts of the pancreas in both groups (data not shown). Furthermore, we found no significant differences between the beta cell mass or endocrine content before and after infusion of the pancreas with either collagenase or HBSS (data not shown).

To assess the influence of the infused fluid, the difference between the beta cell/endocrine content ratio before and after infusion was compared between collagenase- and HBSS-infused pancreata. In collagenase-infused pancreata the mean decline of the beta cell/endocrine content ratio after infusion was 0.10 ± 0.11. In HBSS-infused pancreata, the mean decline of the beta cell/endocrine content ratio after infusion was 0.11 ± 0.13 which is similar to the decline in collagenase-infused pancreata (t = 0.316, p = 0.76). This suggests that the beta cell/endocrine content ratio in the infused tissue samples is lower regardless of the infused fluid, so that it seems likely that this lower ratio is caused by volume expansion of the fluid entering in the islet. Because the lower

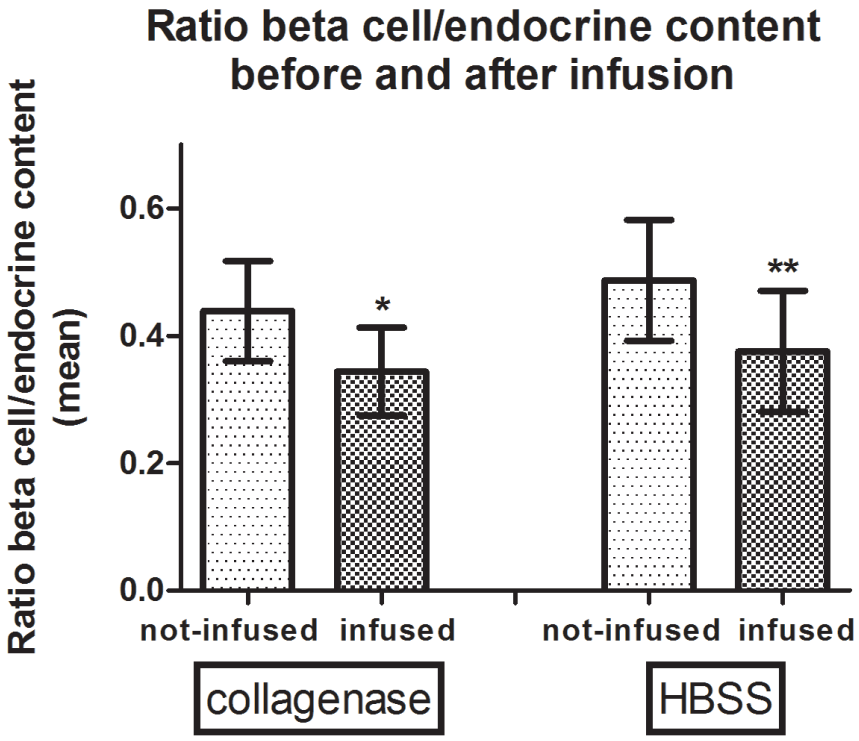

Figure 2: Ratio beta cell/endocrine content before and after collagenase and HBSS infusion
The bars represent the mean and the error-bars the standard deviation.
In the collagenase infused pancreata, mean beta cell/endocrine content ratio in the not-infused tissue samples was 0.44 ± 0.08. After infusion this ratio was significantly lower 0.34 ± 0.07, * p = 0.04
In the HBSS infused pancreata, mean beta cell/endocrine content ratio in the not-infused tissue samples was 0.49 ± 0.09. After infusion this ratio was significantly lower: 0.38 ± 0.09, ** p = 0.001.

ratio is not specific for collagenase, it seems that the digestive effect of collagenase plays no or only a minor role. After infusion, the weight of the pancreas was found to be 2.8 times higher than the weight before infusion, in both groups. This supports the hypothesis that there is an overall swelling of the organ, because the fraction endocrine content is too small to account for this increase.

DISCUSSION

Ductal injection of collagenase has been shown the technique to produce the highest isolation yields. However, even when collagenase is delivered to the pancreas in this way, there is still a considerable loss of endocrine tissue. As a first step in finding explanations, this study showed that islets undergo a morphological change during an islet isolation procedure currently used in most porcine and human islet isolations. The observed decline in the beta cell/endocrine content ratio was not specific for collagenase, but also shown for HBSS, so the morphological changes seem most likely to be due to volume expansion. These results are supported by previous studies by Johnson et al. (6) and Cross et al. (5) which showed the presence of collagenase within the islets of porcine and human pancreata after collagenase distension. The present study adds that other fluids may have the same effect.

In the present study, the endocrine content was determined by manual delineation of the islets used in the assessment of the beta cell content. Because the assessment of the endocrine content could potentially have been influenced by the observer performing the delineation, this could have influenced our results. However, because the delineation was performed by two observers and no significant difference between these observers was found, our study results do not seem greatly affected by this.

Peak activity of collagenase can be found at 37°C. In our study, we did not heat the enzyme to this temperature, as we would have performed when isolating the islets. This may have underestimated the potential effect of collagenase. However, because the biopsies after infusion were taken some time after infusion of the pancreas and the pancreas was not chilled during infusion, the temperature of the enzyme at that point would most likely lie close to room temperature. At this temperature collagenase already has considerable activity and could have influenced islet morphology. However, we cannot exclude that, when heated to 37°C, the digesting effect of collagenase does influence islet morphology.

We have compared HBSS-infused pancreata with pancreata infused with collagenase dissolved in UW. This could potentially have influenced our results since the enzyme-free solution is different than the solution in which the enzyme is dissolved. However, since UW is commonly used in porcine islet isolation procedures and is not

known to have a negative influence on porcine isolation outcome, our results do not seem greatly affected by this.

Moreover, we only have assessed the change in morphology of the islets some time after infusion of the pancreas with collagenase and HBSS and did not assess the effect of the rest of the isolation procedure on islet morphology, further research is necessary to assess whether these effects remain over time or are resolved during further processing of the pancreas.

The results of this study may provide part of the explanation for the loss of endocrine tissue during isolation procedures. The volume expansion caused by collagenase entering into the islet could lead to disruption of cell-cell contacts, leading to islet fragmentation and eventually reduced islet yields. Also the pressure of the infusion of the fluid could lead to disruption of cell-cell contacts. Another explanation could be a change in islet density. After digestion of the pancreas, separation of exocrine and endocrine tissue by a gradient is based on a difference in density, with the exocrine having a higher density than the endocrine tissue. When collagenase enters the islet, this could change the density of the endocrine tissue and lead to a smaller difference in density between the endocrine and exocrine tissue. It would consequently be more difficult to separate these two fractions by a gradient leading to a reduction in isolation outcome.

Besides lower yields, the volume expansion caused by collagenase could also play a role in impaired function after isolation, simply as a result of mechanical pressure from ductal infusion on the membranes of acinar cells leading to fluid entering vasculature and islets. This is supported by Dufrane et al. (11) who showed that large, well-structured porcine islets with central capillaries possessed a better potential for cellular engraftement than disrupted islets. In addition, other studies have shown that disruption of the microanatomy of the islets results in alteration of insulin secretory responses (12) and that glucose hemostasis not only depends on the number and integrity of beta cells, but also on their interaction with neighboring beta and non-beta cells (13).

In conclusion, morphological changes of islets after collagenase infusion seem to depend on volume expansion caused by fluid entering the islets. This could potentially lead to islet fragmentation, resulting in reduced islet isolation outcome and impaired function. If future research finds a way to inject collagenase without the fluid entering the islets, this may result in a considerable improvement of isolation yields.

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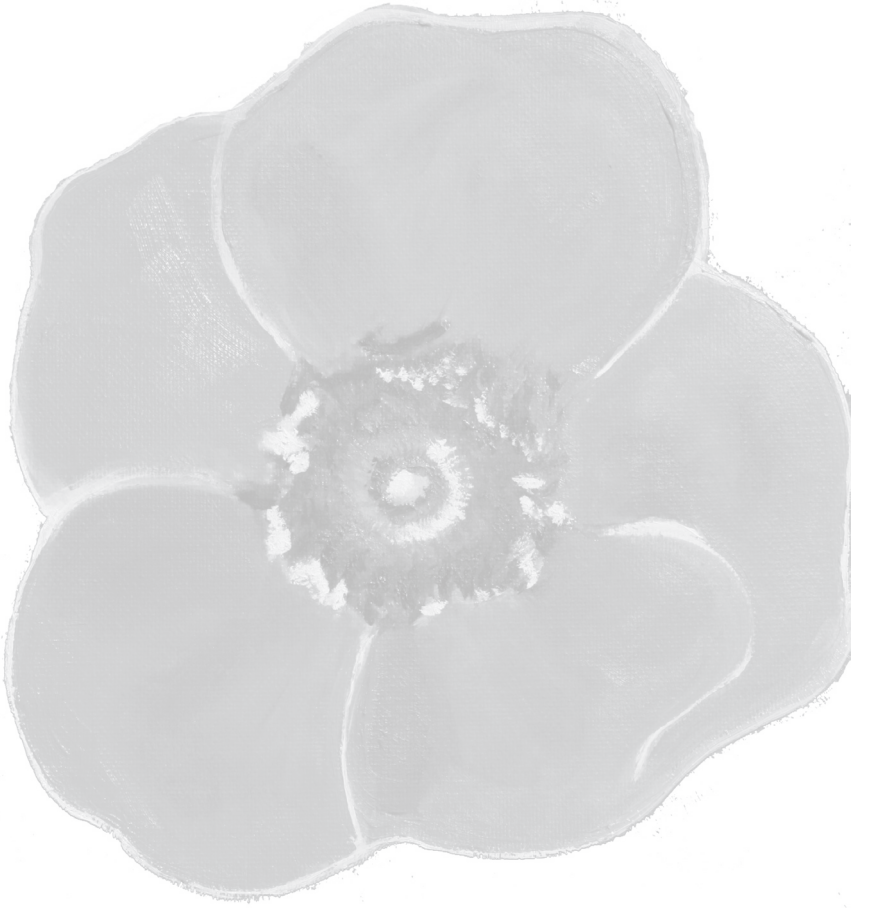
Chapter 9

Summary and discussion



SUMMARY AND DISCUSSION

Pancreas transplantation and islet of Langerhans transplantation are potential solutions to treat patients with type 1 diabetes. Both procedures have shown to abolish the need for exogenous insulin and to restore normoglycemia. However, pancreas grafts are scarce and there is a shortage of donor pancreata relative to the number of patients needing a transplant (1). Therefore, optimal use of the available donor organs is essential.

The aim of this thesis was to contribute to further optimization of pancreas graft survival in pancreas transplantation and optimization of islet isolation outcomes in islet of Langerhans transplantation, leading to better use of available organs. Over the last years, human islet transplantation has become routine clinical practice in approximately 30 centers worldwide. In the Leiden University Medical Center it is performed on a regular basis and currently making the transition to becoming routine clinical practice. Porcine islet transplantation is also, slowly but surely, making a transition from the laboratory to clinical practice worldwide. To achieve this aim, we first assessed the importance of several factors that may optimize the outcome of either pancreas or islet transplantation, using both registry-based comparative effectiveness research and systematic review techniques. This was combined with biomedical research to study the mechanisms through which some factors may influence the outcomes of transplantation or isolation outcome in more detail.

In the Netherlands, the Leiden University Medical Center is the largest center performing pancreas transplantations. In 2011, 86% of all pancreas transplantations in the Netherlands were performed in our center. In **chapter 2** we have shown that both donor and recipient characteristics as well as donor-recipient matching influence graft survival. Pancreas graft survival was reduced in female patients who received a graft from a donor with a similar BMI with enteric drainage of the graft. Recipient factors remain most important and explain the largest proportion of the variance in both 1-year and overall survival whereas donor factors were less important in both short-term and long-term pancreas graft survival.
Our method of quantifying the impact of donor versus recipient factors has not been shown before. The advantage of our method is that besides the assessment of which factors significantly influence pancreas graft survival, their importance in terms of their contribution to graft survival can also be established. Optimizing recipient factors thus seems more important for long-term survival than optimizing donor factors. This seems logical when considering that pancreas donors are highly selected prior to procurement and transplantation. Because of this selection, the variation in donor factors (e.g. variation in age) is much smaller than in recipient factors. This smaller variation in donor factors is likely to result in a smaller variation in survival,

thus explaining a smaller part of the differences in pancreas graft survival. Recipients on the other hand are selected to a smaller extent, in particular in more recent years in which pancreas transplantation is also offered to more high-risk patients (e.g. older patients with comorbidity) so that they differ far more in various characteristics that may influence survival. Further research may lead to an improvement of this model by including other factors, which may result in an even higher ability to explain differences in survival.

Apart from donor or recipient factors, the procurement technique of a pancreas graft has also been shown to influence pancreas graft and patient survival. Surgical injuries that occur during pancreas procurement may lead to complications after transplantation, impaired function of the allograft, graft loss or even death of the patient. In **chapter 3** we determined how often pancreata were refused for transplantation after procurement during back-table inspection, which type of problems were responsible for the decision not to transplant the pancreas and whether different problems were encountered in transplanted versus refused pancreata. Reasons to refuse pancreata for transplantation were for example: severe atherosclerosis, severe injuries of the pancreas parenchyma, superior mesenteric or splenic vein and of the splenic or dorsal pancreatic artery such that reconstruction and transplantation became impossible. We evaluated all procured pancreata transported to our center for transplantation in the period February 2002 until May 2008. Of these, 82.8% were transplanted while 17.2% were refused for transplantation during back-table inspection, regardless of procurement region. Thirteen percent of the pancreata were refused solely due to surgical injuries. As one would expect, in refused pancreata a higher number of critical and non-critical problems per pancreas were found than in transplanted pancreata. Chances of refusal increased in pancreata from older donors procured by centers not performing pancreas transplantations. When pancreata were procured by these centers, chances of refusal were eight times higher compared with centers that did perform pancreas transplantations. These results have important implications for current practice in pancreas procurement. More extensive and recurrent training of pancreas procurement surgeons might lead to a better quality of the organs and thus to a reduction in refusal rates. Surgeons with experience in pancreas transplantation may be excellent teachers in such a training program. Another possibility to reduce pancreas refusal may be to leave pancreas procurement to those centers also performing pancreas transplantations, but this seems difficult (if not impossible) to implement in practice. It seems better to complement training with annual feedback to each center on the proportion of procured organs that could be transplanted compared to other centers, which may lead to further improvement if rates are lower than expected.

In selected patients, the alternative to pancreas transplantation is transplantation of isolated islets as a free graft. Sufficient islet numbers can be obtained from a

single-donor, but even in the most successful studies multiple transplantations were necessary to obtain (temporary) normalization of hyperglycemia in the recipients (2-7). Furthermore, islet isolation outcome is highly variable. Given the relative shortage of donor pancreata, this emphasizes the need to optimize isolation yields so that sufficient islet numbers are routinely obtained from a single-donor.

In **chapter 4** we present a systematic review of donor, pancreas and isolation procedure related factors shown to influence islet isolation outcome. Higher pre-and post-purification islets yields and a higher proportion of successful islet isolations were obtained when pancreata were preserved with the "two-layer method", rather than with the University of Wisconsin solution in donors with shorter cold ischemia times (one hour longer cold ischemia time resulted in an average decline of pre-purification post-purification yields, and proportion of successful isolations of 59 IEQ/g, 54 IEQ/g and 21%, respectively). Higher pre-purification yields and a higher percentage of successful islet isolations were found in younger donors with higher BMI. Lower yields were found in donation after brain death (DBD donors) compared to donation after cardiac death (DCD donors). Higher post-purification yields were found in isolations with Serva collagenase.
However, these results were obtained by including only a selection of studies, as not all studies reported the same factors. To obtain more reliable evidence, standardized reporting of these factors would be necessary. In univariate analysis 66.3-87.5% of the available pancreata were analyzed on the effect on pre- and post-purfication yield or percentage of successful isolation. In multivariate analysis this percentage was only 10.3-20.6%, due to missing data on at least 1 of the variables included. This indicates that the studies differ to such a great extent in the variables that are reported, even when only those variables reported in most studies were selected. Standardized reporting of a minimal set of variables in all future studies would also lead to a better fit of the model used in any meta-analysis. In the current analyses on post-purification yield 19% of the variance in islet isolation outcome could be explained by the variables included. In pre-purification islet yield and proportion of successful isolations, this percentage was better, but still only 50% of the variance could be explained. This suggests that besides the variables included in our systematic review, other factors also influence isolation outcome.

Among these other factors influencing isolation outcome may be the occurrence of hyperemic islets (HIs), for which the mechanism describing its origin as well as their relevance for islet isolation outcome is unknown. In **chapter 5** we studied histological characteristics of the human pancreas in relation to islet isolation. HIs were found in approximately half of the assessed pancreata. It is most likely that the HIs arose shortly before or during pancreas procurement and that a rise in blood pressure in combination with hemodynamic instability (associated with prolonged ICU stay),

are responsible for the formation of HIs. In addition, besides the pre-procurement hemodynamic status, the handling of the pancreas during surgery could also be a contributing factor in the development of HIs.
With respect to the consequences of HI occurrence, we found substantially lower yields in pancreata with HI (HI+) than in pancreata without HI (HI-). It can be speculated that besides the reduced isolation outcome this phenomenon could also provide a possible explanation for the variable, unexplained loss of islets during culture and after transplantation. When islets are obtained from donor pancreata containing HIs, these islets would appear to be "normal" (since only a small proportion of the assessed islets was hyperemic) when in fact, the entire islet population of these pancreata may be affected to some extent. Therefore, these islets are more likely to fail in culture or have impaired function when transplanted. To establish the importance of HIs for islet transplantation, further research is needed focused on the impact on post-purification yield, purity and viability. Systematic reporting of the presence of hyperemic islets in future studies (by taking a biopsy prior to the isolation procedure for example) would make it possible to include this as a factor in a meta-analysis to determine their relevance on isolation outcome compared to other known factors.

A potential solution for the shortage of human donor pancreata is xenotransplantation of porcine islets. However, porcine islet isolation procedures have been shown to be notoriously difficult. Morphological characteristics of the porcine pancreata could also be responsible for the highly variable islet yields. Similar to our findings in human donor pancreata, a remarkably high number of HIs was encountered when studying histological characteristics of the porcine pancreas in relation to islet isolation, as described in **chapter 6**. HIs were found in 48% of the pancreata in purebred pigs and in 68% of the pancreata in crossbred pigs. Similar to our results in human pancreata, significantly lower yields in the HI+ pancreata were found compared to the HI- pancreata in both purebred and crossbred pigs. No evidence for an ongoing chronic process was found, so it can be speculated that the HIs arose shortly before the exsanguination and death of the animal and that for instance a sudden rise in blood pressure could be responsible for the formation of HIs.
Since HIs were found in both human and porcine pancreata and have a similar effect on islet isolation outcome in both species, HIs are potentially an important factor in islet isolation outcome.

Given that collagen is the major target in the enzymatic dissociation of the pancreas, the collagen substrate within the pancreas is another variable that could account for the unpredictable, highly variable islet yields. In **chapter 7** we have described our findings in pancreata of 64 juvenile and 76 adult pigs. Islet isolation procedures in adult porcine pancreata are known to result in large islet yields (8, 9), whereas these procedures have been shown to be more difficult in juvenile porcine pancreata,

possibly as a result of the relative fragility of the islets of juvenile pigs (10, 11). Even though we found a difference in total amount of collagen between adult and juvenile pigs, this difference in collagen could not be explained by the age-difference, and should thus be explained by another (unknown) difference between adult and juvenile pigs. A collagen capsule surrounding the islet could potentially provide protection against enzymatic disintegration of islets and consequently their fragmentation. It has been suggested that a factor in the differing islet isolation outcomes is a more extensive capsule surrounding the islets of the adult pig pancreas as compared to the young pig pancreas (12, 13). However, in our study, we did not find a difference in islet encapsulation between adult and juvenile pigs. Both adult and juvenile pancreata had no or only a very limited collagen capsule. Furthermore, previous studies have shown that the amount of collagen in porcine pancreata was related to the isolation outcome (12). However, we did not observe a relation between islet isolation outcome and total amount of collagen, islet encapsulation or intra-islet collagen in 58 adult pigs.
It can be speculated that the composition of the islet capsule, and the relative concentration of the components could influence islet isolation outcome and possibly confound the observed relation between islet isolation outcome and the complete islet capsule. However, since we found no or only a very limited collagen capsule in our study population when we stained tissue samples for all types of collagen, we expect that collagen subtypes play no or only a minor role. Other matrix elements on the other hand could play a role and should be further investigated.

Ductal injection of collagenase has been shown to be the technique to produce the highest isolation yields. However, even when collagenase is delivered to the pancreas in this way, there is still a considerable loss of endocrine tissue. We showed in **chapter 8** that islets undergo a morphological change during most porcine and human islet isolation procedures. To quantify the morphological change of the islets, the mean beta cell/endocrine content ratios of the infused and not-infused tissue samples were compared. In a second experiment, 20 pancreata were similarly assessed after intraductal injection with Hank's Balanced Salt Solution (HBSS). The observed decline in the beta cell/endocrine content ratio was shown to be not specific for collagenase, but was also shown for HBSS, so that the morphological changes most likely seem to be due to volume expansion. This could potentially lead to islet fragmentation, resulting in reduced islet isolation outcome and impaired function.

In conclusion, this thesis has added evidence that the focus in pancreas transplantation should be on optimizing recipients to improve graft survival and on improving quality of pancreata procured by centers not performing pancreas transplantation (for example, by training the procurement surgeons to optimize pancreas procurement thus resulting in more transplantable organs.

In islet transplantations, it is recommended that the reporting of donor, pancreas and isolation factors should become more standardized, which would enable us to determine more accurately which factors are important predictors for islet isolation outcome. Furthermore, if more biomedical factors (e.g. the presence of hyperemic islets) would be reported in addition to the other factors, we would be able to assess the independent effect of these biomedical factors on islet isolation outcome and eventually the effect on islet transplantation in the clinical setting.

FUTURE PERSPECTIVES

Pancreas transplantation

In December 2010, more than 36,000 pancreas transplantations have been reported to the International Pancreas Transplant Registry (IPTR): more than 24,000 transplantations were performed in the US and more than 12,000 outside the US (14). Recipient age at transplantation increased over the course of 24 years of pancreas transplantation as well as transplantations in type 2 diabetes mellitus patients. Donor criteria have become more strict over time, with a concentration on younger donors, preferably trauma victims, with short preservation time. Surgical techniques for drainage of the pancreatic duct also changed over time. In the US, enteric drainage is the predominantly used technique in combination with systemic drainage of the venous effluent of the pancreas graft. In the Leiden University Medical Center, a two-step approach is routinely performed in most patients. Pancreas transplants are bladder drained initially, with patients undergoing elective pancreas conversion to enteric drainage 6 – 12 months after transplantation (15). Immunosuppressive protocols developed towards antibody induction therapy with Tacrolimus and Mycophenolate Mofetil (MMF) as maintenance therapy. The rate of transplantations with steroid avoidance increased over time. All of these changes together have resulted in improved patient and graft survival. Patient survival is now 95% at one year and over 83% 5 years after transplantation. Because donors are already highly selected prior to pancreas transplantation, future improvements in patient and pancreas graft survival may be realized primarily by optimizing recipient factors (e.g. BMI). Alternatively, living donor segmental pancreatectomy has been reported as a therapy in selected patients (16). An initial technical failure rate of more than 33% has been reported; nearly twice the rate in deceased donors, but this has declined to less than 1% since the start of this therapy. Living donor and deceased donor graft survival rates are more or less equivalent. If the use of living donors would increase, it is likely that eventually not only highly selected donors will be considered to be suitable, but criteria will become less strict over time, leading to more variation in donor characteristics that may influence survival. In this way, the use of living donors could potentially increase the

importance of donor factors in explaining survival differences. The potential downside of this therapy is that the donor must face a major surgical intervention, and even if a minimally invasive technique is used, the donor faces risks of surgical diabetes and potential risks for complications such as pancreatic fistula or infection.

Human islet transplantation

Clinical islet transplantation is currently being offered to a subset of approximately 15% of patients with type 1 diabetes with refractory hypoglycemia or marked hypoglycemic episodes. With over 750 islets transplants performed in over 30 international centers yearly, this therapy has been transferred from research to becoming a standard recognized clinical therapy (5). In the Leiden University Medical Center, 19 transplantations have been performed in 13 patients since the start in 2007. Currently, islet transplantation offers a means of endogenous, regulated insulin secretion, thereby stabilizing glycemic control, preventing hypoglycemia, and correcting glycosylated hemoglobin (HbA1C) to a level predicted to prevent and reverse secondary complications of diabetes. However, patients require immunosuppressive therapy for the rest of their lives. Islet transplantation is also being offered after kidney transplantation, where the choice is simpler as these patients already require lifelong immunosuppressive therapy, and the intraportal islets implantation procedure is a simple nonsurgical intervention with a relatively low risk.

Islet transplantation is thus likely to become a standard therapy once islet transplantation becomes more readily available. This means that the islet supply should be expanded (e.g. through expansion of existing islets, stem cell approaches or when xenotransplantation sources become available). The remaining challenges of inducing immunological tolerance, preventing islet destruction due to autoimmunity or alloimmune rejection and avoiding all potential side-effects due to immunosuppressive therapies, will all need to be addressed to facilitate this transition towards becoming a standard therapy. Furthermore, routine attainment of single-donor islet transplants success remains an important goal in islet transplantation. This would allow for many more subjects to be treated with islets, and would reduce the potential risk of donor HLA-sensitization by avoiding exposure to multiple donors. Islet allograft transplantation has also been performed with islets from three living donors, the last one successfully, showing the potential for further application (17-19).

Moving from multiple-donor to single-donor success will require a multimodal approach, including optimization of the pre-procurement condition of donor pancreas organs, protection of islets from cold and warm ischemic injury and the process of islet isolation, access to effective, stable and consistent human compatible collagenase enzyme blends for digestion, and several multimodal strategies for treatment of the recipient to suppress immunological, inflammatory and thrombosis pathways, while at the same time stimulating neovascularization and metabolic function of the islet graft.

Such a multimodal approach will transform short- and long-term islet transplantation success and will continue to facilitate the rapid transition from research to routine clinical care. This emphasizes the importance of optimizing islet isolation outcome.
In contribution to optimization of islet transplantation outcome, research in the Leiden University Medical Center is focused on the development of devices (in collaboration with the Technical University Twente) to create an optimal microenvironment for transplanted islets and alternative cell sources (e.g. precursor cells).

Porcine islet transplantation

Porcine islets could be an alternative to human islet transplantation, particularly if delivered in a way that evades the host immune system rejection (20). This can be achieved by protecting xenogeneic islets from immune rejection by selective semi-permeable barriers. Designated pathogen-free herds (21, 22) could provide a supply of wild-type porcine islets that are well tolerated when administered in a suitable protective delivery vehicle. Such barrier systems have enabled amelioration of diabetes in a variety of animal models and preliminary evidence suggests that similar results could be obtained in humans. Ongoing trials using encapsulated islets, without immunosuppressive therapy, are sponsored by Living Cell Technologies (LCT), either in Russia or New Zealand, as well as trials that are still in the planning phase in the US. The trial in New Zealand started in 1995 with six type 1 diabetes patients receiving either encapsulated or non-encapsulated neonatal porcine islets. One individual, receiving encapsulated islets, showed a reduction in average monthly insulin dose, a reduction in glycosylated hemoglobin and the detection of porcine C-peptide in urine. A biopsy from this patient 9,5 years after transplantation showed encapsulated islets expressing insulin (23). These improvements in diabetic state, although temporary, were encouraging and prompted further expansion. However, trials were temporarily put on hold because of concerns raised by the documentation of in vitro pig-to-human transmission of porcine endogenous retroviruses. This resulted in a long process of communication with the New Zealand regulatory authorities to fulfill requirements associated with the health status of the source pigs, pancreas processing and islet encapsulation, nationwide public consultation and implementation of a safety strategy. In the Russian trial, commenced in 2007, eight patients were transplanted with 5000-10000 IEQ/kg. Presenting the three- and six-month post-transplant data, evidence for efficacy was demonstrated in some, but not in all, patients. Five patients manifested a reduced insulin need and two patients (one at three months and one at six months after transplantation) temporarily did not need any insulin. Six patients showed a reduction in circulating glycosylated hemoglobin, average levels being 8.9% before transplantation, 6.9% at three months after transplantation and 7.3% at six months after transplantation. The procedure was found to be safe and could be repeated safely.

The trial in New Zealand continued in October 2009. It is an open-label dose-range study for one implant with 10000-15000 IEQ/kg in eight patients with unstable diabetes and severe hypoglycemic episodes. All patients, with a follow-up of 20-36 weeks, showed a clear reduction in hypoglycemia score. At the XXIII international congress of the Transplantation Society (24) one patient was presented in more detail, showing a 20% insulin dose reduction at 4 weeks after transplantation
These data show that clinical porcine islet transplantation is a safe procedure that might benefit patients with hypoglycemia unawareness and gives a modest improvement in diabetes control. In upcoming studies, the dose and timing of possible repeat doses will be addressed. Optimization of porcine islet isolation outcome would become of even greater importance once porcine islet transplantation becomes a more standard recognized clinical therapy.

This thesis is a first step in the direction of these future developments. By combining several research methods (registry-based comparative effectiveness research, systematic review techniques and biomedical research) we have accomplished a profitable interaction. On one hand we have assessed the general overview (by determining the relative importance of factors on outcomes of transplantation) and on the other hand we have unraveled some of the mechanisms through which potential factors may influence the outcomes of transplantation. The advantage of such a combination is that we do not only investigate how certain factors may influence transplantation outcomes, but at the same time try to quantify which part of the variation in outcomes may be explained by this factor, and thereby determine the room for improvement in those outcomes.

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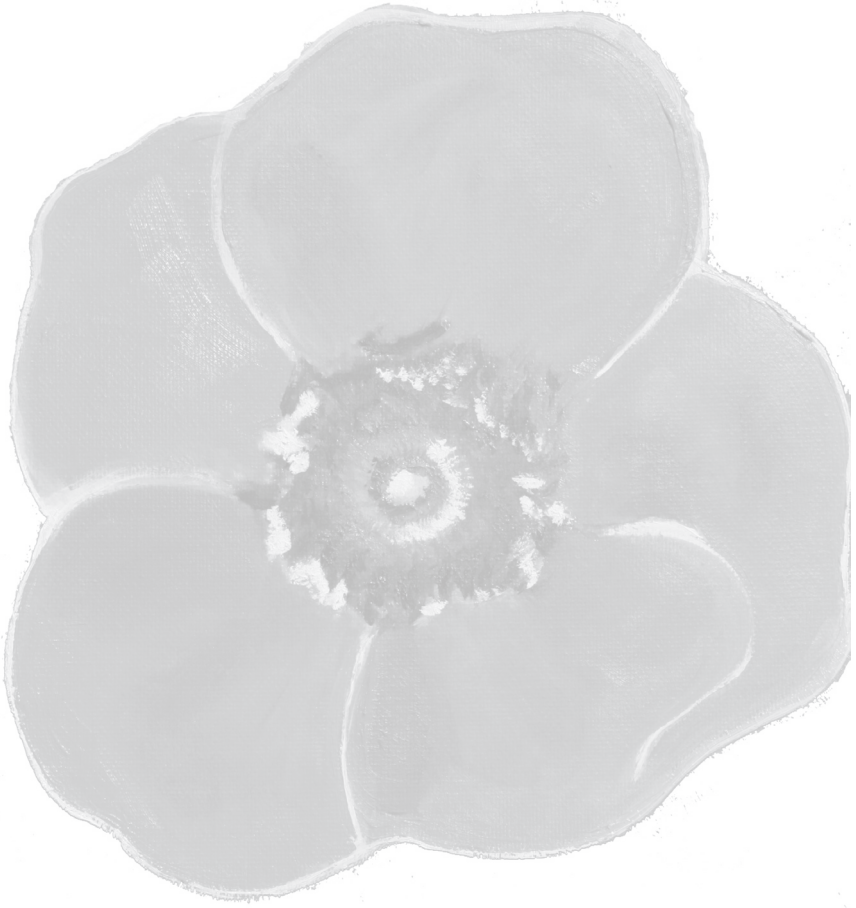
Chapter 10

Nederlandse samenvatting



NEDERLANDSE SAMENVATTING

Pancreastransplantatie en eilandjes van Langerhans transplantatie zijn potentiële oplossingen voor de behandeling van patiënten met Diabetes Mellitus type 1. Beide procedures hebben bewezen dat zij het gebruik van exogeen insuline overbodig maken en kunnen leiden tot normoglycemie. Pancreasdonororganen zijn echter schaars en er is een relatief tekort aan donorpancreata in vergelijking met het aantal patiënten dat op de wachtlijst staat voor een transplantatie (1). Het is daarom essentieel dat beschikbare organen optimaal benut worden.

Het doel van dit proefschrift is om een bijdrage te leveren aan de voortschrijdende verbetering van pancreastransplantaatoverleving en de eilandjesisolatieopbrengst, zodat optimaal gebruik kan worden gemaakt van beschikbare organen. Hiervoor richten wij ons in deel I op pancreastransplantatie en in deel II en III op eilandjestransplantatie. Humane eilandjestransplantatie heeft zich in de afgelopen jaren ontwikkeld tot een routinebehandeling in ongeveer 30 centra in de wereld. In het Leids Universitair Medisch Centrum vindt momenteel de transitie van experimentele naar routinematige behandeling plaats. De transplantatie van varkenseilandjes maakt ook langzaam maar zeker de overgang van het laboratorium naar klinische toepassing.

Om dit doel te bereiken hebben wij, middels vergelijkend onderzoek naar de effectiviteit in registraties en via systematische reviewtechnieken, eerst verschillende factoren bekeken die in potentie de uitkomsten van pancreas- of eilandjestransplantatie kunnen verbeteren. Dit werd gecombineerd met biomedisch onderzoek om het mechanisme, waardoor verschillende factoren de uitkomst van transplantatie of isolatieopbrengst kunnen beïnvloeden, in meer detail te bestuderen.

Het Leids Universitair Medisch Centrum is het grootste centrum in Nederland dat pancreastransplantaties verricht. In 2011 werd 86% van alle pancreastransplantaties hier verricht. **Hoofdstuk 2** laat zien dat zowel donor- als ontvangerfactoren als ook matching van donor met ontvanger de transplantaatoverleving beïnvloeden. Pancreastransplantaatoverleving is korter bij vrouwen die een orgaan ontvingen van een donor met eenzelfde BMI met enterale drainage van het transplantaat. Ontvangerfactoren zijn het meest belangrijk en verklaren het grootste deel van de variatie in overleving zowel na 1 jaar als op de lange termijn. Donorfactoren zijn minder belangrijk dan ontvangerfactoren. Dit geldt in ongeveer gelijke mate voor de korte en de lange termijn.

Onze methode, waarbij het effect van donorfactoren wordt gekwantificeerd ten opzichte van ontvangerfactoren, is niet eerder beschreven. Het voordeel van deze methode is dat niet alleen onderzocht wordt welke factoren een significante invloed hebben op pancreastransplantaatoverleving, maar dat ook de relatieve bijdrage aan de transplantaatoverleving bepaald kan worden. Voor transplantaatoverleving op

lange termijn lijkt het optimaliseren van ontvangerfactoren dus belangrijker dan het optimaliseren van donorfactoren. Dit is waarschijnlijk een logisch gevolg van het feit dat pancreasdonoren voor de uitname en transplantatie al uitgebreid geselecteerd worden. Door deze selectie is er een kleinere variatie in donorfactoren (bijv. variatie in leeftijd) dan het geval is bij ontvangerfactoren. Deze kleinere variatie resulteert zeer waarschijnlijk in een kleinere variatie in overleving en zal daarom een geringer deel van de verschillen in pancreastransplantaatoverleving verklaren. Ontvangers worden in veel mindere mate geselecteerd, zeker in de afgelopen jaren waarbij pancreastransplantatie ook wordt aangeboden aan meer hoog-risico patiënten (bijv. oudere patiënten met comorbiditeit). Hierdoor verschillen de karakteristieken die van invloed kunnen zijn op de overleving bij ontvanger veel meer. Verder onderzoek zal leiden tot een verbetering van dit model als er bijvoorbeeld ook andere factoren meegenomen worden, zodat we nog beter in staat zijn om verschillen in overleving te verklaren.

Naast donor- en ontvangerfactoren beïnvloedt ook de uitnametechniek van een pancreas de overleving van het transplantaat en van de patiënt. Chirurgische schade die ontstaat bij de uitnameprocedure kan leiden tot complicaties nadat het orgaan getransplanteerd is, een verminderd functioneren van het transplantaat, transplantaatverlies en zelfs overlijden van de patiënt. In **hoofdstuk 3** hebben wij bekeken hoe vaak pancreata werden afgewezen voor transplantatie nadat zij zijn uitgenomen, tijdens de zogenaamde "back-table" inspectie. Daarnaast hebben wij bekeken welk type problemen hier verantwoordelijk voor waren en of in getransplanteerde pancreata andere problemen werden gezien dan in afgewezen pancreata. Redenen om pancreata af te wijzen voor transplantatie waren: ernstige atherosclerose, ernstige schade aan het pancreasparenchym en dusdanige beschadiging van de normale anatomie van de v. mesenterica superior, de v. lienalis en de a. lienalis of van de a. pancreatica dorsalis dat reconstructie en daardoor transplantatie niet mogelijk waren.

Alle uitgenomen pancreata die getransporteerd werden naar ons centrum in de periode februari 2002 – mei 2008 zijn onderzocht (n = 134). Van deze pancreata werd 82,8% getransplanteerd en 17,2% werd afgewezen voor transplantatie tijdens de "back-table" inspectie. Dertien procent van de pancreata werd afgewezen op basis van chirurgische schade. Zoals te verwachten viel, werden in afgewezen pancreata meer kritieke en niet-kritieke problemen gevonden dan in getransplanteerde pancreata. De kans dat een pancreas werd afgewezen voor transplantatie was hoger in pancreata van oudere donoren, uitgenomen door centra waar geen pancreastransplantaties worden verricht. Pancreata hadden een 8 keer hogere kans om afgewezen te worden wanneer zij werden uitgenomen door deze centra in vergelijking met centra die wel pancreastransplanties verrichten. Deze bevindingen hebben belangrijke implicaties voor het huidige pancreasuitnamebeleid. Uitgebreidere en frequentere training van pancreasuitnamechirurgen kan leiden tot betere kwaliteit van de uitgenomen organen

en dus voor een afname van het percentage afgewezen organen. Chirurgen met ervaring met pancreastransplantaties zouden goede instructeurs zijn in een dergelijk trainingsprogramma. Een andere manier om het percentage afgewezen organen te verlagen zou bereikt kunnen worden door pancreasuitnames alleen te laten verrichten door centra die ook pancreastransplanties uitvoeren. Dit is in de praktijk echter moeilijk (zo niet onmogelijk) te bewerkstelligen. Een betere oplossing lijkt te zijn om naast het geven van training ook jaarlijks het percentage niet-transplanteerbare pancreata terug te koppelen aan uitnamecentra.

Bij een geselecteerde groep patiënten is de transplantatie van eilandjes van Langerhans een goed alternatief voor pancreastransplantatie. Voldoende eilandjesopbrengst kan worden verkregen van een enkele donor, maar zelfs in de meest succesvolle studies zijn vaak multipele transplantaties nodig om (tijdelijke) normalisatie van hyperglycemie in de ontvangers te bewerkstelligen (2-7). Daarnaast is de opbrengst van eilandjes uit een pancreas erg variabel. Omdat er een relatief tekort aan donorpancreata is, is het noodzakelijk om dit proces te optimaliseren zodat er routinematig voldoende eilandjes van een enkele donor worden verkregen.

H**oofdstuk 4** is een systematische review van donor-, pancreas- en isolatieprocedure gerelateerde factoren die van invloed zijn op de eilandjesisolatieopbrengst. Hogere pre- en post-purificatie opbrengst en een hoger percentage succesvolle isolaties werden bereikt wanneer pancreata werden gepreserveerd met de "two-layer method" in plaats van University of Wisconsin oplossing en wanneer ze afkomstig waren van donoren met een korte koude ischemietijd. Een uur langere koude ischemietijd resulteerde in een gemiddelde daling van pre-purificatie, post-purificatie opbrengst en percentage succesvolle isolaties van respectievelijk 59 IEQ/g, 54 IEQ/g en 21%. Hogere pre-purificatie opbrengst en een hoger percentage succesvolle isolaties werden bereikt in jongere donoren met een hoger BMI. Lagere opbrengst werd gevonden in heartbeating donoren in vergelijking met non-heartbeating donoren. Hogere post-purificatie opbrengst werd gevonden wanneer de isolatieprocedure werd verricht met Serva collagenase. Omdat niet alle studies dezelfde factoren rapporteerden, werden bovenstaande resultaten gevonden op basis van slechts een deel van de studies. Om deze bevindingen met grotere betrouwbaarheid vast te stellen is het noodzakelijk dat er een gestandaardiseerde rapportage van deze factoren komt. Bij de univariate analyse werd 66,3-87,5% van alle beschikbare pancreata geanalyseerd terwijl bij de multivariate analyse slechts 10,3-20,6% van alle beschikbare pancreata werd geanalyseerd als gevolg van missende data. Dit laat zien dat er grote variatie is tussen de gerapporteerde variabelen in de verschillende studies, zelfs als alleen die variabelen geselecteerd worden die in de meeste studies worden gerapporteerd. Gestandaardiseerde rapportage van een minimale set van variabelen zal ook leiden tot een betere "fit" van het model dat gebruikt werd in de multivariate analyse. In

onze huidige analyse van post-purificatie opbrengst werd 19% van de variatie in isolatieopbrengst verklaard door de geïncludeerde variabelen. In de analyses van pre-purificatie opbrengst en percentage succesvolle transplantaties was dit percentage hoger, maar werd ook slechts 50% van de variatie verklaard. Dit suggereert dat naast de factoren die in onze systematische review werden geïncludeerd, ook andere (nog onbekende) factoren de isolatieopbrengst beïnvloeden.

Het voorkomen van hyperemische eilandjes (HE's) zou een van de andere factoren kunnen zijn die de isolatieopbrengst beïnvloeden. Het ontstaansmechanisme van deze eilandjes en hun relevantie voor isolatieopbrengst is onbekend. In **hoofdstuk 5** hebben wij histologische karakteristieken van het humane pancreas in relatie tot eilandjesisolatie bestudeerd. HE's werden in ongeveer de helft van de onderzochte pancreata gevonden. De meest waarschijnlijke verklaring is dat de HE's kort voor of tijdens de uitname van het pancreas ontstaan als gevolg van een stijging in de bloeddruk gecombineerd met hemodynamische instabiliteit (geassocieerd met een langere IC opname). Daarnaast kan ook de manipulatie van het pancreas tijdens de uitnameprocedure bijdragen aan het ontstaan van HE's.

Wij vonden een substantieel lagere isolatieopbrengst in pancreata met HE's (HE+) dan in pancreata zonder HE's (HE-). Speculatief kan gesteld worden dat naast de lagere isolatieopbrengst de aanwezigheid van HE's ook een verklaring is voor het variabele, onverklaarde verlies van eilandjes tijdens het kweken en na transplantatie. Wanneer eilandjes worden geïsoleerd uit pancreata die HE's bevatten, lijken deze eilandjes "normaal" (omdat slechts een klein percentage van de eilandjes in een pancreas hyperemisch was), terwijl eigenlijk de hele populatie aan eilandjes in dergelijke pancreata in meer of mindere mate is aangedaan. Deze eilandjes overleven waarschijnlijk eerder het kweken niet of hebben een verminderde functie wanneer zij getransplanteerd worden. Om het belang van HE's te bepalen in het licht van eilandjestransplantatie zal verder onderzoek moeten worden gedaan waarbij de focus moet liggen op de invloed op post-purificatie opbrengst, zuiverheid en levensvatbaarheid van de eilandjes. Systematische rapportage van het voorkomen van hyperemische eilandjes in toekomstige studies (door bijvoorbeeld het nemen van een biopt voor de isolatieprocedure) zal het mogelijk maken deze factor te includeren in een meta-analyse, zodat de relevatie hiervan voor eilandjesisolatieopbrengst bepaald kan worden bepaald in vergelijking met andere bekende factoren.

Een mogelijke oplossing voor het tekort aan humane donorpancreata is xenotransplantatie van varkenseilandjes. De isolatie van varkenseilandjes is echter moeizaam en sterk variabel gebleken. Morfologische karakteristieken van varkenspancreata zijn waarschijnlijk verantwoordelijk voor de variabele opbrengst. Wanneer histologische kenmerken van varkenspancreata werden bestudeerd in relatie tot isolatieopbrengst, werd evenals bij onze studie in humane donorpancreata,

een verrassend hoog aantal hyperemische eilandjes gevonden, zoals beschreven in **hoofdstuk 6**. HE's werden gevonden in 48% van de pancreata van raszuivere en in 65% van de pancreata van hybride varkens. Net als in onze humane studie werden significant lagere isolatieopbrengsten gevonden in HE+ pancreata in vergelijking met HE- pancreata in zowel raszuivere als hybride varkens. Er werden geen aanwijzingen gevonden voor een chronisch ontstaansproces, zodat kan worden gespeculeerd dat HE's kort voor de dood van het varken ontstaan door bijvoorbeeld een plotselinge stijging van de bloeddruk.

Omdat HE's in zowel humane als varkenspancreata gevonden worden en in beiden eenzelfde effect lijken te hebben op de isolatieopbrengst, zou het voorkomen van HE's mogelijk een belangrijke factor kunnen zijn in eilandjes isolatieopbrengst.

Bij de enzymatische vertering van de pancreas (om eilandjes te isoleren) is collageen het belangrijkste doelwit. De hoeveelheid collageen in het pancreas is daarom een andere factor die de onvoorspelbare, variabele isolatieopbrengst kan verklaren. In **hoofdstuk 7** beschrijven wij de resultaten van onze studie in 64 jonge en 76 volwassen varkens. Hierbij hebben wij eerst gekeken naar het verschil tussen jonge en volwassen varkens. Van isolatieprocedures in volwassen varkens is beschreven dat zij over het algemeen resulteren in hoge eilandjesopbrengst (8, 9). In jonge varkens blijkt deze procedure echter moeilijker te zijn, mogelijk als gevolg van de relatief fragiele eilandjes (10, 11). Hoewel wij een verschil in hoeveelheid collagen vonden tussen volwassen en jonge varkens, bleef dit verschil bestaan ook als wij rekening hielden met het verschil in gemiddelde leeftijd tussen de groepen. Dit verschil moet daarom worden veroorzaakt door een ander verschil tussen jonge en volwassen varkens.

Een collageen kapsel rondom het eilandje zou in potentie het eilandje kunnen beschermen tegen de enzymatische desintegratie en zodoende tegen de fragmentatie van het eilandje. Er is beschreven dat een uitgebreider collageen kapsel rond eilandjes van volwassen varkens in vergelijking met jonge varkens het verschil in isolatieopbrengst tussen jonge en volwassen varkens zou kunnen verklaren (12, 13). In onze studie vonden wij echter geen verschil in de collageenomkapseling van eilandjes tussen volwassen en jonge varkens. Zowel in volwassen als in jonge varkens waren de eilandjes niet of slechts zeer beperkt omkapseld met collageen.

Naast het verschil tussen volwassen en jonge varkens hebben wij ook gekeken naar de invloed van collageen op de isolatieopbrengst in een studie van 58 volwassen varkens. Eerdere studies lieten zien dat de totale hoeveelheid collageen invloed had op de isolatieopbrengst (13). Wij vonden echter geen relatie tussen totale hoeveelheid collageen, collageenomkapseling van eilandjes en collageen binnenin de eilandjes en de uiteindelijke isolatieopbrengst.

De samenstelling van het kapsel van het eilandje en de relatieve concentratie van de afzonderlijke componenten zouden van invloed kunnen zijn op de isolatieopbrengst en een mogelijke bias kunnen veroorzaken in de geobserveerde relatie tussen

isolatieopbrengst en het gehele kapsel rondom het eilandje. Omdat wij geen of slechts een zeer beperkte collageenomkapseling van de eilandjes vonden in onze studie (wanneer wij weefselbiopten kleurden voor alle typen collageen), verwachten wij dat collageen subtypes slechts een beperkte rol spelen. Andere matrixelementen zouden echter wel een rol kunnen spelen en dienen nader onderzocht te worden.

Ductale injectie van collagenase is de techniek gebleken waarbij de hoogste isolatieopbrengst wordt verkregen. Er is echter nog steeds een aanmerkelijk verlies van endocrien weefsel bij gebruik van deze techniek. In **hoofdstuk 8** laten wij zien dat eilandjes een morfologische verandering ondergaan tijdens isolatieprocedures bij varkens en bij humane procedures. Om deze morfologische verandering te kwantificeren hebben wij de gemiddelde beta cel/endocrien weefsel ratio bepaald van opgespoten en niet-opgespoten weefselbiopten. In een 2^{e} experiment werden 20 pancreata op eenzelfde manier beoordeeld na intra-ductale injectie van Hank's Balanced Salt Solution (HBSS). De geobserveerde daling in beta cel/endocrien weefsel ratio bleek niet specifiek te zijn voor collagenase, maar werd ook gezien in het experiment met HBSS. De morfologische verandering van eilandjes lijkt daarom meest waarschijnlijk het gevolg te zijn van volume-expansie door de ingespoten vloeistof. Dit zou kunnen leiden tot fragmentatie van de eilandjes met verminderde isolatieopbrengst en een verminderde functie tot gevolg.

Concluderend stellen we dat dit proefschrift bijdraagt aan de bewijslast dat de focus zou moeten liggen op de optimalisatie van ontvangers om pancreastransplantaatoverleving te verbeteren en op de verbetering van de kwaliteit van de uitgenomen pancreata door centra die geen pancreastransplantaties verrichten (bijv. door uitnamechirurgen te trainen waardoor pancreasuitname wordt verbeterd, hetgeen resulteert in meer transplanteerbare pancreata).

Bij eilandjestransplantatie zou de rapportage van donor-, pancreas- en isolatiefactoren meer gestandaardiseerd moeten worden waardoor er nauwkeuriger bepaald kan worden welke factoren belangrijke voorspellers zijn voor isolatieopbrengst. Als daarnaast meer biomedische karakteristieken (bijv. de aanwezigheid van hyperemische eilandjes) worden gerapporteerd, dan zouden we in staat zijn om het onafhankelijke effect van de biomedische factoren te bepalen en uiteindelijk de invloed van deze factoren op eilandjestransplantatie in de klinische setting.

TOEKOMSTPERSPECTIEF

Pancreastransplantatie

In december 2010 zijn meer dan 36.000 pancreastransplantaties geregistreerd bij de International Pancreas Transplant Registry (IPTR). Meer dan 24.000 transplantaties zijn uitgevoerd binnen de VS en 12.000 daarbuiten (14). Gedurende de afgelopen 24 jaar is de leeftijd van de ontvangers toegenomen evenals het aantal patiënten met Diabetes Mellitus type 2. De criteria voor donoren zijn in deze periode strikter geworden waarbij de nadruk ligt op jongere donoren, bij voorkeur traumaslachtoffers met korte preservatietijd. Ook chirurgische technieken voor drainage van de ductus pancreaticus zijn veranderd in de loop van de tijd. In de VS is enterale drainage in combinatie met systemische veneuze afvloed van het pancreastransplantaat de meest gebruikte techniek. In het Leids Universitair Medisch Centrum wordt een 2-staps benadering routinematig toegepast bij de meeste patiënten. Hierbij wordt het pancreastransplantaat in eerste instantie gedraineerd via de blaas en vindt na 6-12 maanden een electieve conversie naar enterale drainage plaats (15). Protocollen voor immunosuppresieve therapie hebben zich ontwikkeld in de richting van inductietherapie met antilichaam, gevolgd door Tacrolimus en Mycophenolaat Mofetil (MMF) als onderhoudstherapie. Het aantal transplantaties zonder behandeling met steroïden is ook toegenomen in de afgelopen jaren. Deze ontwikkelingen hebben geresulteerd in een toegenomen patiënt- en transplantaatoverleving. Patiëntoverleving is nu 95% na 1 jaar en 83% 5 jaar na transplantatie. Omdat donoren al uitgebreid geselecteerd worden voor de transplantatie, zal verdere verbetering van patiënt- en transplantaatoverleving gerealiseerd moeten worden door de optimalisatie van ontvangers (bijv. optimalisatie van het BMI). Als alternatief wordt ook segmentele pancreastransplantatie van levende donoren beschreven (16). Initieel was er een technisch falenpercentage van 33% (bijna 2 keer zo hoog als in overleden donoren), maar dit is inmiddels gedaald tot minder dan 1%. Percentages transplantaatoverleving zijn in beide groepen ongeveer even hoog. Bij toename van het gebruik van levende donoren zullen in de toekomst waarschijnlijk niet alleen streng geselecteerde donoren worden gebruikt, maar zullen de selectie criteria voor donoren in de loop van de tijd minder stringent worden. Uiteindelijk zal dit leiden tot meer variatie in donorkarakteristieken die de overleving beïnvloeden. Op deze manier zal het gebruik van levende donoren de invloed van donorfactoren - bij het verklaren van verschillen in overleving - vergroten. Nadelen van deze therapie zijn de grote operatie die de donor moet ondergaan (zelfs als minimaal-invasieve technieken worden toegepast), en het risico van de donor om diabetes, pancreasfistels of infecties te ontwikkelen.

Humane eilandjestransplantatie

Klinische eilandjestransplantatie wordt momenteel uitgevoerd bij ongeveer 15% van de patiënten met Diabetes Mellitus type 1 met refractaire hypoglycemie of hypoglycemische episodes en is inmiddels een gestandaardiseerde klinische behandeling. Jaarlijks worden 750 transplantaties verricht wereldwijd in ongeveer 30 centra (5). In het Leids Universitair Medisch Centrum zijn, sinds de start in 2007, 19 transplantaties uitgevoerd bij 13 patiënten. Eilandjestransplantatie zorgt voor een endogene regulatie van insulinesecretie en daardoor voor een stabilisatie van de glucoseregulatie waardoor hypoglycemieën worden voorkomen en het geglycosyleerde Hb (HbA1c) wordt gecorrigeerd tot een dusdanig niveau dat secundaire complicaties van Diabetes Mellitus worden voorkomen en gecorrigeerd. Patiënten moeten echter de rest van hun leven behandeld worden met immunosuppresiva. Transplantatie van eilandjes wordt ook uitgevoerd na een eerdere niertransplantatie. Hier is de overweging gemakkelijker, omdat deze patiënten vanwege de niertransplantatie al behandeld moeten worden met immunosuppresiva. Verder is de procedure voor eilandjestransplantatie een simpele, niet chirurgische interventie met een relatief laag risico op complicaties.

Transplantatie van eilandjes zal zich, om bovenstaande redenen, waarschijnlijk ontwikkelen tot een standaardtherapie, wanneer het meer voorhanden zou zijn. Dit betekent dat de voorraad eilandjes moet toenemen (bijv. door toename van de voorraad bestaande eilandjes, door stamcellen of wanneer xenotransplantatie bronnen meer beschikbaar zouden komen). Daarnaast moeten problemen van immunologische tolerantie (waarbij destructie van eilandjes door auto- of alloimmuun reacties wordt voorkomen) en potentiële bijwerkingen van immunosuppressieve therapie worden aangepakt om de transitie naar een standaardtherapie te bewerkstelligen. Verder blijft het routinematig verkrijgen van voldoende eilandjes van een enkele donor een belangrijk doel. Dit zorgt ervoor dat meer patiënten kunnen worden behandeld met eilandjes en zorgt eveneens voor een verlaging van het potentiële risico op HLA-sensitisatie doordat de blootstelling aan multipele donoren wordt voorkomen. Allotransplantatie van eilandjes is ook uitgevoerd met eilandjes van drie levende donoren. De laatste hiervan was succesvol en biedt dus perspectief voor toekomstige behandelingen (17-19).

De overstap van multipele-donoren naar enkelvoudige-donor succes vraagt om een multimodale aanpak. Dit vereist optimalisatie van de conditie van het donorpancreas voor de uitname, bescherming van eilandjes tegen schade als gevolg van koude en warme ischemie, optimalisatie van het eilandjesisolatieproces, toegang tot een effectief, stabiel, humaan collagenase-enzymmengsel en verschillende strategieën voor de behandeling van de ontvanger om immunologische, ontstekings- en trombosereacties te onderdrukken, waarbij tegelijkertijd de neovascularisatie en de metabole functie van het transplantaat moeten worden gestimuleerd. Een dergelijke

multimodale aanpak zal van invloed zijn op het succes van eilandjestransplantatie op korte en lange termijn en zal de snelle overschakeling van onderzoek naar routinematige klinische behandeling ondersteunen. Dit benadrukt de noodzaak om de isolatieopbrengst van eilandjes te optimaliseren.

Om deze optimalisatie van de eilandjesisolatieopbrengst te bewerkstelligen ligt de focus van het onderzoek in het Leids Universitair Medisch Centrum op de ontwikkeling van 'devices' (in samenwerking met de Technische Universiteit Twente) die een optimale micro-omgeving van getransplanteerde eilandjes creëren en op alternatieve celbronnen (bijv. precursorcellen).

Xenotransplantatie

Varkenseilandjes kunnen een alternatief zijn voor humane eilandjestransplantatie, met name als zij op een dusdanige manier worden getransplanteerd dat het immuunsysteem van de ontvanger omzeild wordt (20). Dit kan worden bereikt door de xenogene eilandjes te beschermen tegen immuunreacties van de ontvanger door ze te omkapselen met selectieve semipermeabele barrières. Speciaal gefokte pathogeen-vrije kuddes (21, 22) kunnen voor een voorraad varkenseilandjes zorgen die goed getolereerd worden wanneer zij in zo'n beschermend transportvehikel toegepast worden. Deze barrièresystemen zijn succesvol gebleken in de behandeling van diabetes in verschillende diermodellen en eerste studies laten zien dat dergelijke resultaten ook kunnen worden bereikt in mensen. Lopende studies waarin omkapselde varkenseilandjes worden gebruikt zonder immunosuppressieve therapie, worden gesponsord door Living Cell Technologies (LCT) in Rusland en Nieuw-Zeeland, evenals studies die in de planningsfase zijn in de VS. De studie in Nieuw-Zeeland startte in 1995 met zes patiënten met DM type 1 die een transplantatie met omkapselde, dan wel niet-omkapselde eilandjes ontvingen. Eén individu, dat een transplantatie met omkapselde eilandjes kreeg, liet een daling van de gemiddelde maandelijkse insulinedosis zien, een afname van het HbA1C en er werd in het bloed varkens C-peptide gedetecteerd. Een biopt bij deze patiënt, 9,5 jaar na de transplantatie, liet omkapselde eilandjes zien met expressie van insuline (23). Deze, alhoewel tijdelijke, vooruitgang in diabetische status was veelbelovend en gaf aanleiding tot verder onderzoek. Helaas werd de studie tijdelijk stopgezet vanwege bezorgdheid over in vitro varken-naar-humane transmissie van endogene retrovirussen. Dit resulteerde in een lang proces van overleg met de Nieuw-Zeelandse autoriteiten om te voldoen aan kwaliteitseisen met betrekking tot de gezondheidsstatus van de varkens, pancreasverwerking en eilandomkapseling, voorlichting aan het publiek en implementatie van een veiligheidsstrategie.

In de Russische studie, gestart in 2007, werden bij acht patiënten 5000-10000 IEQ/kg eilandjes getransplanteerd. Na 3 en 6 maanden was er effect merkbaar in een aantal, maar niet in alle, patiënten.

Vijf patiënten lieten een gereduceerde insulinebehoefte zien en twee patiënten (één na 3 en één na 6 maanden), waren tijdelijk insulineonafhankelijk. Zes patiënten hadden een lager HbA1C (gemiddeld 8,9% voor transplantatie, 6,9% 3 maanden na transplantatie en 7,3% na 6 maanden). De procedure werd veilig bevonden en ook het herhaald geven van eilandjestransplantaties werd veilig bevonden.

De studie in Nieuw-Zeeland werd hervat in oktober 2009. Het is een open-label dose-range studie met een transplantatie van 10000-15000 IEQ/kg in acht patiënten met instabiele DM en ernstige hypoglycemische periodes. Op het XXIIIe internationale congres van de Transplantation Society (24) werd één patiënt meer gedetailleerd gepresenteerd. Hierbij werd een insuline reductie van 20% bereikt, 4 weken na transplantatie.

Deze data laat zien dat klinische varkenseilandjestransplantatie een veilige procedure is die voordelen kan hebben voor patiënten die zich niet bewust zijn van hypoglycemische episodes en die een bescheiden verbetering van de diabetescontrole geeft. In komende studies zal de dosis en de timing van de toegediende eilandjes onderzocht worden. Optimalisatie van isolatieopbrengst van varkenseilandjes zal nog belangrijker worden als de transplantatie van varkenseilandjes een standaardtherapie wordt.

Dit proefschrift is een eerste stap in de richting van deze toekomstige ontwikkelingen. Door het combineren van diverse onderzoeksmethodes, namelijk vergelijkend onderzoek naar de effectiviteit in registraties, systematische review technieken en biomedisch onderzoek hebben wij een vruchtbare interactie bewerkstelligd. Aan de ene kant hebben wij het effect van factoren op uitkomsten voor de patiënt onderzocht (door het bepalen van de relatieve invloed van factoren op transplantatie uitkomsten) en aan de andere kant hebben wij enkele mechanismen proberen te ontrafelen waardoor deze potentiële factoren de transplantatieuitkomst beïnvloeden. Het voordeel van een dergelijke combinatie is dat wij niet alleen onderzocht hebben hoe sommige factoren de transplantatieuitkomst beïnvloeden, maar tegelijkertijd geprobeerd hebben te kwantificeren welk deel van de variatie in uitkomst wordt verklaard door deze factor. Hierdoor kan bepaald worden waar de ruimte voor verbetering ligt in deze uitkomsten.

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CURRICULUM VITAE

Denise Eline Hilling was born on 29 May 1981 in Rotterdam. After graduating high school (VWO, C.S.G. Angelus Merula, Spijkenisse) in 1998, she studied Technical Chemistry at Technical University Delft for one year. In 1999 she started the study Medicine at Erasmus University Rotterdam. In 2004 she obtained her masters degree and obtained her medical degree cum laude in 2006. Her graduate research project was on long-term aesthetic results of fronto-orbital correction of craniosynostosis conducted at the department of Plastic and Reconstructive Surgery, Erasmus University Medical Center Rotterdam (Dr. I.M. Mathijssen), which resulted in her first two publications. During her medical training, she took an elective clerkship in General Surgery at the department of Surgery, Albert Schweitzer Hospital Dordrecht (Dr. P.W. Plaisier) and she participated in an elective clerkship Emergency Medicine at the department of Accident and Emergency, Prince of Wales Hospital, Sydney, Australia.

After obtaining her medical degree, she worked for one year as a Surgical Resident (not in training) at Reinier de Graaf Hospital (Prof. dr. L.P.S. Stassen). During this period, she conducted a pilot study of a new transabdominal preperitoneal approach for endoscopic correction of umbilical hernias (Drs. I.H. Oei). From October 2007 onward she conducted the work described in this thesis at the department of Surgery, Leiden University Medical Center under supervision of Prof. dr. O.T. Terpstra.

In January 2010 she started her training as a Surgical Resident at MC Haaglanden (Dr. J.C.A. de Mol van Otterloo and Dr. S.A.G. Meylaerts) and she will continue her training from January 2014 onward at LUMC (Prof. dr. J.F. Hamming).

DANKWOORD

Een boekje met alleen mijn naam, maar zeker niet het resultaat van alleen mijn werk.

Iedereen die heeft bijgedragen aan de totstandkoming van dit proefschrift, veel dank hiervoor!