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Review

Advances in Human Islet Processing: Manufacturing Steps to Achieve Predictable Islet Outcomes from Research Pancreases

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Abstract:

***Background*:** This presentation of a six-year study processing human islets for research and transplantation includes a review of multi-center transplant studies identifying key variables critical for successful islet processing and defines standardized processing procedures required to provide highly purified, functional Human Islets.

***Methods*:** Human islet processing methods are defined in detail with pancreas retrieval, shipping, trimming for processing, collagenase distension, controlled digestion by digestion/filtration method, islet purification and islet culture. Islet processing results are summarized from 27 published reports (2003-2017) from 21 international clinical islet transplant centers with 13 single islet centers and 8 from multi-center clinical trials that averaged islet yields of 5,680 IEQ/Gm (Pre-Purification), 4,101 IEQ/Gm (Post-Purification), and 3,599 IEQ/Gm (Post-Culture) with 59.2% purity at time of islet transplant into the liver.

***Results*:** Their results were compared to this study of 226 Non-DM (Non-Diabetes Mellitus) donor processing islet yields that averaged 6,942.7±201 IEQ/Gm (Pre-Purification), 5,484.5±199 IEQ/Gm (Post-Purification), and 4,351.0±167 IEQ/Gm (Post-Culture) per pancreas with 90.0% purity at time of islet distribution. Islet processing from 29 Type 2 Diabetes donors resulted in reduced islet yields of 5,797±734 IEQ/Gm (Pre-Purification), 4,371±866 IEQ/Gm (Post-Purification), and 3,323±423 IEQ/Gm (Post-Culture) with similar purity but reduced insulin content. Glucose Stimulated Insulin Release testing in T2D islets showed significantly reduced insulin release at 20mM and 20mM+IBMX versus Non-DM islets and showed significantly reduced Total Stimulated Insulin Release of 0.722±0.142 (T2D) versus 1.069±0.067 ng insulin/ng DNA (Non-DM).

***Conclusions*:** Of the significant process variables, short Switch Times were predominant in greatly increasing islet yields, GSIR results, and insulin content with any time <10 minutes with Donor Age and Body Mass Index also important. Increased Cold Ischemia times decreased islet yields and insulin content. In terms of pancreas preservation solutions, University of Wisconsin solution (UW) use was most effective for optimal islet quantity, quality, and function post preservation.

Keywords

Clinical; collagenase; diabetes; human; islet culture; islet equivalents; islet processing; islet purification; islets; islet transplantation; manufacture; pancreas; pancreatic islets; preservation; research; transplantation; transportation

1. Introduction

Prodo Laboratories (Prodo) in Aliso Viejo, CA, has been processing adult cadaver donated pancreases for distribution of *Human Islets for Research (HIR)* in conjunction with the Scharp-Lacy Research Institute (SLRI) since 2007. The first few years were initially focused on establishing the ability to process human islets to the level required for a weekly distribution of consistently high quality *HIR*. The first requirement was to utilize improved islet tissue culture media that Prodo had developed in 2006: PIM(R)® for islet recovery and culture post-isolation, PIM(S)® for standard islet culture, and PIM(T)® for islet distribution. The required two supplements for these media are PIM(G)® (glutamine and glutathione) and pre-tested, high quality human AB serum, PIM(ABS)®. A tissue culture triple antibiotic product, PIM(3X)®, has recently been released that contains appropriate levels of Ciprofloxacin, Gentamycin, and Amphotericin B for human islet culture. Due to limited pancreas procurement opportunities, many human islets were processed in our early years at longer cold ischemia times than ideal, but permitted a higher quality of *HIR,* as published in 2010 [1]. The last seven years have been focused on the standardization of the multitude of variables involved in human pancreas retrieval, shipping, and pancreas processing into purified, functional islets that are optimally cultured and shipped weekly on a global basis to diabetes investigators. Due to these improvements and standardization, the analyses reported here describe the critical variables involved for each aspect of this process that have resulted in a more predictive outcome of high quality, adult human islet yields with highly functional results on a

more consistent basis. This study not only compares the outcomes of islet processing and function from Non-Diabetes Mellitus (Non-DM) adult cadaver organ donors, but also those from donors with Type 2 Diabetes (T2D). In addition, an evaluation of organ donors following Donation after Cardiac Death (DCD) was completed to assess their utility in providing *HIR* and *HIT* on a routine basis.

The USA Federal Drug Administration (FDA) issued a "Guideline for Industry: Considerations for Allogeneic Pancreatic Islet Cell Products" in 2009 defining all of the aspects that must be considered to perform clinical islet transplantation under FDA requirements and approval [2]. It is planning to release a Biological License Approval (BLA) for effective clinical islet transplantation to a limited number of university-based programs. To optimize islet yields and islet quality in order to comply with this BLA, the university based islet transplant centers will likely be held to the highest quality and uniformity of processing human islets. For each center involved, this means that standardization and introduction of manufacturing technology for process-to-process uniformity of human islet preparations would be ideally developed, demonstrated, and followed. In order to achieve this goal, "the first license-enabling trial of a cellular product for treatment of Type 1 Diabetes (T1D) demonstrating multi-site compliance with common manufacturing processes and release criteria" was published in Diabetes Care in October, 2016 [3] and the "National Institutes of Health – Sponsored Clinical Islet Transplantation Consortium Phase 3 Trial: Manufacture of a Complex Cellular Product at 8 Processing Centers" was published in Diabetes in 2016 [4]. This trial was focused on islet transplantation to treat impaired awareness of hypoglycemia and severe hypoglycemic events in 48 T1D recipients that had failed standard medical therapies. It was designed as a multi-center, single arm, consortium Phase 3 study conducted at 8 North American clinical islet transplant centers, as the first type of study that can anticipate ongoing, multi-center clinical trials to develop islet transplantation as a potential therapy. The primary endpoints of restoring hypoglycemic awareness and prevention of severe hypoglycemic events was achieved in 87.5% of recipients in year one and in 71% in year two with safety events primarily limited to the infusion procedure and the required immunosuppression. The secondary one-year endpoint of achieving insulin independence following clinical islet transplantation was achieved in 52.1% of the recipients with an insufficiency of data response in 14.5% of the recipients. While 33% of the recipients failed to achieve insulin independence with an average of 1.6 islet transplant doses, these recipients were still able to demonstrate their ability to eliminate their critical problems of hypoglycemic unawareness as a major accomplishment that reduces the risks they had experienced prior to their implants. While Prodo has chosen to not be involved in human islet processing for research under these new BLA clinical trials, it has already developed much of the manufacturing improvements that will be required by the clinical centers to routinely produce islets under these BLA requirements. The primary reason to publish Prodo's processing technology information and results at this time is to assist these university BLA programs to adapt their methods towards more standardized, manufacturing practices as required. Prodo has had to focus its development of these manufacturing processing steps in order to routinely provide the delivery of their consistently high quality *HIR* on a weekly basis to their global corporate and university based investigators.

Another recent development of note in the human islet processing for clinical transplantation efforts was published in 2016 as a retrospective, multi-center study by eleven academic islet transplant centers to establish the North American Islet Donor Score (NAIDS) for optimal selection

of human pancreases for processing into islets for clinical islet transplantation [5]. In this reference, the multiple centered authors develop methods to score human pancreases for processing into islets for clinical applications. They identify variables in pancreas weight and quality that they will use to scale which human pancreases have the potential of delivering >400,000 Islet Equivalents (IEQ) per process post-purification. If followed, this approach could reduce the required number of human pancreases to process per donor successfully down to a single donor to eliminate insulin requirements when implanted into each diabetic recipient. Of the 1,056 pancreas donors from the eleven centers reported, 286 processes were successful in achieving >400,000 IEQ per process post-purification, that was 27% of the processes performed. If they ranked the results of islet production in these 1,056 pancreas processes using the NAIDS method of predicting human islet processing success, then they predict that 53.7% of the pancreas processes would have been successful. Thus, this pancreas selection ranking could reduce the numbers of pancreases to process with lower scores and end with a much higher percentage of successful quality islets processed for clinical transplantation.

While the use of the NAIDS ranking could certainly reduce the number of processes from less promising pancreas donors, their basis of utilizing this pancreas ranking method in their published opinion is that human pancreas processing into islets has reached its maximal success and no longer needs to be improved. Thus, they state "…(human) islet processing techniques appear to have reached a mature and stable stage." [5] The recent outcomes in the publication of the first 8 center clinical Phase 3 trial [3, 4] demonstrate that significant islet processing variability clearly remain in at least 50% of the participating centers involved in that study. The opinion of the authors reporting this Prodo study is that the field remains far from achieving such a widely practiced, stable endpoint in processing human islets for clinical islet transplantation with its required optimal islet yields, quality, quantity, functionality, and sterility for each human pancreas processed. It is our hope that openly publishing these Prodo islet processing details may permit a higher percentage of clinical islet transplant centers to optimize their ability to achieve the highest levels of clinical islet transplant success required for their ongoing participation in these critical clinical trials.

It is commonly believed in the human islet research community that *Human Pancreases for Research (HPR)* are of sufficiently less quality than *Human Pancreases for Transplantation (HPT)* and clinical islet transplantation *(HIT).* Thus, many investigators believe they have to accept the hypothesis that *Human Islets for Research* (*HIR)* are also of poorer quality since they assume that these poorer quality human islets are all that are available for their research efforts. This report provides reproducible results that demonstrate this hypothesis is incorrect. Instead, *HIR* can be of very high quantity, purity, and functional quality if one properly controls all of the variables known that affect the processing steps required in islet production, culture, and transportation. If one successfully stabilizes the donor variables, process variables, and tissue culture variables while producing at the level of a manufacturing process, then one can predictably provide high quality, functional human islets for global distribution for research on a weekly basis from *HPR*. The results presented here demonstrate that the quality of these *HIR* is similar to those islets being clinically transplanted. Since the current islet purity for clinical trials is reported to only be at the 55%-60%, this study reports islet purity can routinely be increased to >90% purity for shipping *HIR* for research. Yet, a legitimate question is whether islets processed for future clinical trials can be implanted with these kinds of higher purities without giving up significant amounts of total islet

mass per donor. Those performing clinical islet transplantation are focused on attaining maximal islet yields per donor that currently means decreased islet purity, while those performing islet research are focused on attaining maximal islet purity, but not maximal islet yields. A resultant primary goal of processing human pancreas into islets is to bring these two current, divergent objectives into one approach that can achieve both high islet purity and large islet yields of optimally functioning human islets for both research and clinical transplantation.

1.1 Early Efforts in Large Animal and Human Islet Isolation

There have been many improvements since early efforts to isolate human islets for clinical transplantation were initiated. The original islet isolation techniques were begun with rodent islet isolations in 1967 with Lacy and Kostianovsky's demonstration of rat islet isolations [6] that in 1972 brought the first suggestion that islet transplantation could be feasible by partial reduction of induced diabetes following rat islet implants [7]. By improving the islet recoveries and, for the first time, implanting into the portal vein of diabetic rodents [8], it became apparent that these consistently improved functional results permitted consideration of successful human islet transplants. Meanwhile, moving to the larger diabetic animal model of rhesus monkeys brought the new challenge of how to isolate increased quantities of viable islets from a much larger, dense pancreas [9]. The original rodent method placed chopped pancreas pieces in a tube into which powdered collagenase was added followed by hand shaking in a 37^{o}C water bath until the digestate "looked finished" as an endpoint at which time the enzyme was diluted and the freed islets were recovered by handpicking. At that point, rodent islet processing was more of an art than a science. By placing a screen basket within the tube that held the pancreas pieces, it became possible to add the enzyme, shake the tube, and then empty the fluid around the screen holding the digested islets that had passed through the screen basket. Then, one simply added more enzyme solution and shook again repeating this step until all of the pancreatic pieces had been sufficiently digested to pass through the screen having collected the freed islets at each step. The final change in this rodent method was to continuously flow the digesting enzyme through the screen while shaking at 37^{o}C and collecting the freed islets continuously that significantly increased rodent islet yields. This newly developed method was called Digestion/Filtration and was developed by Scharp, as a surgical research fellow in Lacy's lab in 1975 [9], and was quickly confirmed by others [10]. The rat islet isolation yields prior to and after the use of the Digestion/Filtration Chamber (D/FC) for rat islet isolations went from 151 IEQ per rat process (575 isolations) using the original collagenase tube method to 447 IEQ per rat process (50 isolations) using the D/FC technique [9]. Figure 1Aa presents the first D/FC design developed that permitted recovering such an increase in viable islets processed from rodent pancreases and documents the increased islet yields (Figure 1Ab).

The challenge to process the larger and denser primate pancreas into islets by Digestion/Filtration was met by making a much larger screen basket contained within larger, protein separation column tubing that permitted sufficient numbers of higher quality islets to be recovered (Figure 1Ba). These islets were shown to be viable by in vitro glucose stimulation (Figure 1Bb) and were implanted successfully with partial function in the portal vein in 5 diabetic Non-Human Primates (NHP) that reduced their insulin requirements for many weeks [9].

Over the next several years, a number of improvements and modifications of the Digestion/Filtration method were made including chamber design, screen types and sizes, marbles, digestion volumes, pumps, tubings, flow rates, and heating/cooling steps [11-13]. These improvements resulted in an automated method of large scale islet isolation in both large animals and human islet isolation in 1984 by the Scharp team [14]. This Digestion/Filtration technology was applied to dog islet recoveries permitting long-term dog islet auto-transplant success in 1987 [15, 16] and also improved human islet processing as summarized by Scharp in 1988 [17]. This modified canine Digestion/Filtration device (Figure 1Ca) was shaken by an orbital shaker and contained 5 screen baskets each with mixing marbles with freed islets collected within the one outer compartment that connected to 250 ml conicals for tissue recovery in the cold (Figure 1Cb). This D/FC device permitted extensive digestion of the dog pancreas into islets by its flow into collection tubes providing viable canine islets for these implant studies in diabetic auto-transplants and allografts [15-17].

When major efforts contemplated human islet production, a minor modification to the canine and NHP apparatus was made by Scharp and Lacy producing the chamber in stainless steel and converting the 5 separate screens into one exit screen, keeping the five mixing marbles for human pancreas processing and islet recoveries as the human D/FC that was published in 1988 [18] as shown in Figure 1Da with its flow diagram shown in Figure 1Db.

Camillo Ricordi [19] entered Dr. Paul Lacy's laboratory as a post-doctoral fellow at this time from Milan, Italy. While he published many important research activities with Dr. Lacy, became a pioneer in islet research and clinical islet transplantation by his own efforts, and continues making important research and administrative contributions to this field today, his initial laboratory efforts in human islet processing, while a young research fellow, were to place the two halves of the collagenase distended human pancreas for processing directly into this human stainless steel D/FC without cutting open the pancreatic surfaces. While his initial islet yield data were promising, ongoing islet yield results with his approach were replaced by lightly chopping the distended, partially digested pancreas surfaces prior to their placement in the D/FC. The observation of the digesting pancreas that justifies this change is that the outer natural, but this restrictive pancreas coating layer functions as a bag preventing the freed islets from escaping into the open fluid and out of the device. Making this change results in not only consistently higher human islet yields, but also a very important, much quicker release of the digested, free human islets from the chamber. This mechanical cutting and opening of the natural but restrictive, pancreatic surface mantle reduced human islet damage and permitted earlier release of the islets from the ongoing digestion. This chopping the surface of the distended pancreases prior to placing it into the D/FC, stabilized the human islet yields in our experience and has remained one of the standard processing steps in our protocols as outlined in the Methods section. It also contributed to the first partially successful human islets that were implanted in the portal vein as allograft islets processed with the human D/FC published in 1989 and 1991 [20, 21]. Their success led to the first clinically successful human islet transplant recipient to achieve insulin independence following islet transplantation into the portal vein of the liver in 1990 by Scharp and Lacy by supplying the required critical islet mass of free islets [22].

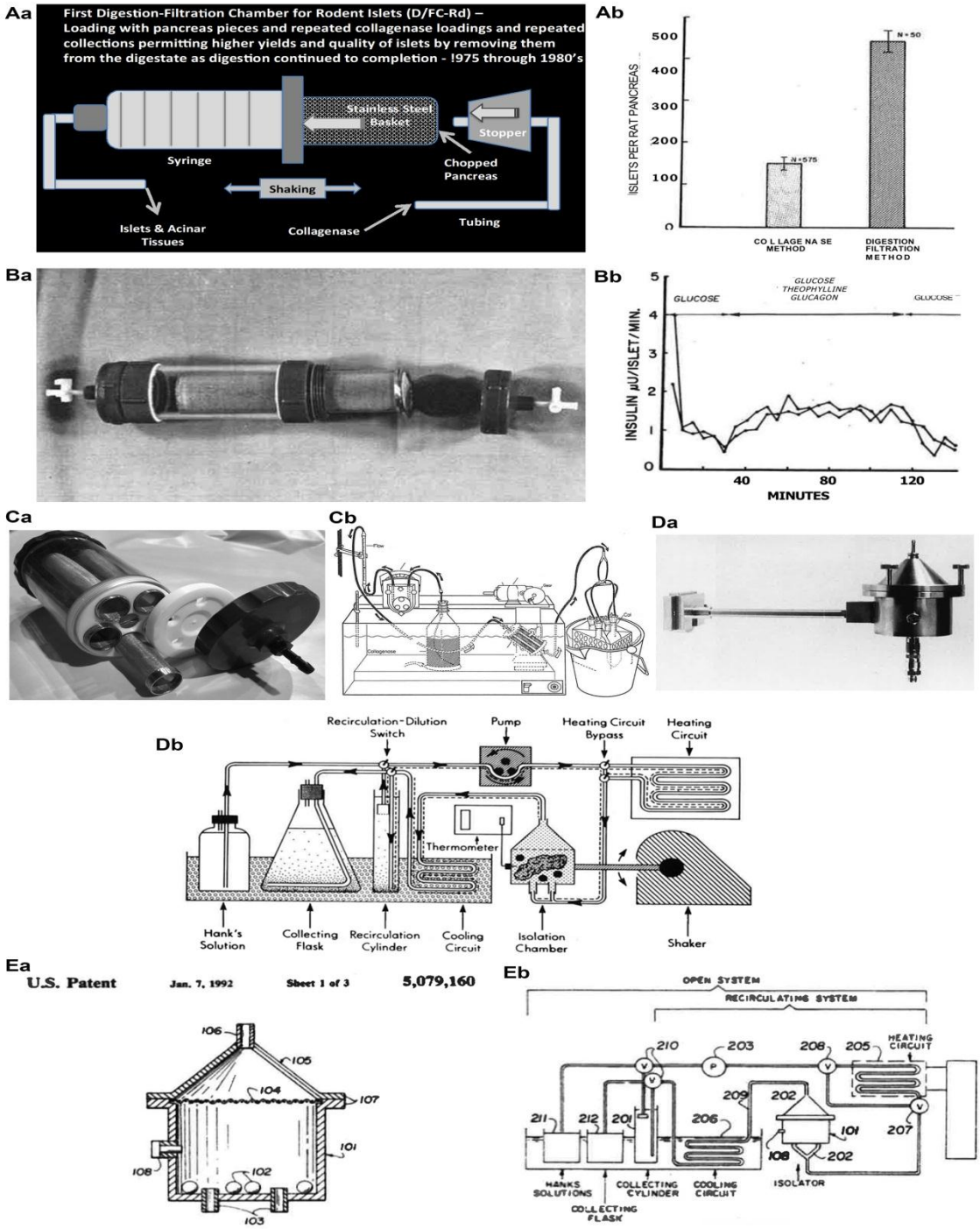


Figure 1 Digestion/Filtration history. A: Initial Digestion/Filtration processing designed for rodent pancreases in 1975 (a: Initial rodent D/FC design; b: Comparative Islet Yields) [9, 10]; B: Original D/FC for processing NHP pancreases in 1975 (a: NHP D/FC; b: Viable NHP Islets) [9]; C: D/FC and circuit design utilized for canine pancreas processing in 1980 - 1988 (a: Canine D/FC; b: Processing Circuit for Canine D/FC) [15-17]; D: D/FC and circuit design used for clinical human pancreas processing and initial clinical trials in 1987-1988 (a: Human D/FC Stainless Steel; b: Human D/FC Circuit Design) [18, 20-22]; E: USPTO #5,079,160 D/FC for Human Islet Production (a: Human D/FC Patent Design; b: Human D/FC Patent Circuit Design) [23] (Filed 1987, Approved 1992).

A United States patent was filed in 1987 with the United States Patent and Trademark Office (USPTO) entitled "Method to Isolate Clusters of Cell Subtypes from Organs" that was issued in 1992 (#5,079,160) that was not assigned, but was held by Washington University with inventors Paul E. Lacy (St. Louis), and David W. Scharp (St. Louis), along with Camillo Ricordi (Milano, IT) [23]. The primary purpose in filing this patent was to protect and permit the development of this technology so that it could remain at the universities in order that this critical technology could freely be developed without corporate restrictions (Figure 1Ea and Figure 1Eb). As expected, this technology was developed at multiple global universities, so that Dr. Lacy and Dr. Scharp purposely never filed any patent infringement actions throughout this patent's protective life of 17 years.

A more recent USPTO application regarding islet processing was submitted in 2001 and approved as a patent in 2004 by Ramon Poo and Camillo Ricordi (USPTO #6,833,270) that was assigned to both Biorep Technologies and the University of Miami [24]. This patent presents essentially the same design of the digestion chamber and flow through system that was shown above in Figure 1D and in Figure 1E, except the chamber construction material was changed from stainless steel to a resin. It appears any claims for the chamber design from this more recent patent (#6,833,270) were not allowed by the USPTO due to the prior art from both the previous publication [18] and the previous patent #5,079,160 [23]. Yet, it was at that time, and continues to be named, the "Ricordi Chamber". This resin chamber for islet processing, sold by Biorep (#500-MVL-03), was a major improvement over the stainless steel chambers and has become universally acceptable in the university based islet programs. The second major improvement from this patent was the development of a glass marble replacement as the "agitation member" that is a non-reactive and relatively indestructible sphere made of silicone nitride that also is sold by Biorep (#SN-01).

Fortunately, one of the most troubling variables that had affected islet yield and quality during these development years was the lot to lot variation in the key enzymatic products used in the digestion of the pancreas: collagenase and thermolysin in 2007 [25] and in 2013 [26]. Thus, highly improved, microbial produced and highly uniform collagenase and thermolysin lots have become readily available since mid-2012, greatly reducing this challenge, as captured in Table 1 below.

Table 1 Recent clinical islet transplant studies with islet processing results.

Islet Trans-plant Center	Pub Date & Ref #	Colla-genase Types	IEQ / Process & / or IEQ/Gm Pre-Pure	IEQ / Process & / or IEQ/Gm Post-Pure	IEQ / Process & / or IEQ/Gm Post-Culture	IEQ/Kg BW Infused / % Purity	Switch Time / Digest Time (min)	GSIR Result (Stim Index)	Donor Age (Yrs)	Donor BMI	Cold Ischemia Time (Hrs)
Single Centers	-	-	-	-	-	-	-	-	-	-	-
U Albert Edmon-ton Can	2007 [25]	Roche Lib HI	348,902 IEQ	24% >300,000 IEQ	-	-	-	-	45.5	26.6	8.3
U Albert Edmon-ton Can	2013 [26]	Serva NB1	604,000 IEQ 6,062 IEQ/Gm	429,000 IEQ 4,307 IEQ/Gm	363,000 IEQ 3,644 IEQ/Gm	- / 55.8%	16.8 min switch/- min total	3.6	46.0	27.9	10.0
DRI, U Miami USA	2005 [27]	Roche Lib HI	595,440 IEQ	469,560 IEQ	-	-	-	3.4	40.6	36.3	9.0
DRI, U Miami, USA	2009 [28]	Roche Lib Hi	512,919 IEQ	203,124 IEQ	322,261 IEQ	-	16.6 min switch/ - min total	2.1	27.9	29.4	7.55
DRI, U Miami USA	2012 [29]	Serva NBI. Roche Hi	Lib HI 434,243 IEQ Serva NB1 375,751 IEQ 5,931.5 IEQ/Gm	Lib Hi 324,256 IEQ Serva NB1 263,389 IEQ 5,672.8 IEQ/Gm	-	-	Lib HI 18.6 min switch/- min total Serva NB1 14.5 min switch/ - min total	Lib Hi 1.65 Serva NB1 2.37	Lib Hi 43.8 Serva NB1 20.0	Lib Hi 29.9 Serva N128. 5	Lib Hi 10.3 NB1 10.6
U MN (1donor	2005 [30]	Roche Lib HI	-	-	301,428 IEQ	7,271 / 64.0%	-	3.4	39	34.3	6.6

tx) USA											
UCSF San Francis USA	2010 [31]	Roche Lib HI / Serva NB1	-	-	472,910 IEQ	11,520 / -	-	3.2	33.9	34.4	6.9
U IL ChicagoUSA	2013 [32]	Roche Lib HI / Serva NB1 / Sigma	318,630 IEQ, 3,378 IEQ/Gm	286,369 IEQ, 2,881 IEQ/Gm	2,934 IEQ/Gm	-	16.4 min switch/ - min total	3.6	48.8	29.1	9.2
U of PA Philadel USA	2003 [33]	Roche Lib HI	-	-	414,788 IEQ	8,294 / 66.3%	-	1.6	49.8	33.6	6.1
U WS Madison USA	2010 [34]	-	-	-	627,330 IEQ	9,061 / -	-	-	-	-	-
Baylor Tx USA	2010 [35]	Serva NB1 / Roche MTF	798,000 IEQ, 7,510 IEQ/Gm	628,000 IEQ, 5,900 IEQ/Gm	-	10,589 / 56.1%	15.2 min switch/ 58.2 min total	-	49.2	28.5	3.7
San Raf Sci Inst Milano Italy	2005 [36]	Roche Coll P/ Lib HI	-	-	347,000 IEQ	5,845 / 50.0%	- /min	2.0	42	24	6.0
Nordic Net490 Clin Islet Tx Sweden	2012 [37]	Roche Lib HI/ Serva NB1	-	-	482,324 IEQ	7,670 / 53.0%	24.7 min switch/ - min total	2.8	52.3	26.7	8.0
Centre Hosp U Lille France	2007 2008 [38]	Roche Lib HI	502,200 IEQ, 5,680 IEQ/Gm	299,300 IEQ, 3,305 IEQ/Gm	385,560 IEQ	- / 59.0%	-	3.2	41	26.6	5.6

	[39]										
Univ Hospital Geneva Swiss	2014 [40]	Serva NB1 Roche Lib Hi Vita-cyte	341,421 IEQ 4,389 IEQ/Gm	244,167 IEQ 3,194 IEQ/Gm	-	- / 59.6%	18 min switch/ - min total	3.0	48.5	26.0	6.2
Oxford, UK (NP- neutral protease,cNP – clostropaine)	2016 [41]	C1a vs C1b +/- Collage-nase NP/CP, Serva +cNP	-	-	C1a +NP/CP 3087 IEQ/gm, C1b -NP/CP 1312 IEQ/gm	-	-	2.4	56.3	24.7	10.8
Oxford, UK (Clostropaine , Neutral Protease Thermolysin)	2017 [42]	Serva NB1 +cNP	-	-	Yields Control 65.2% Clostp 62.2% NProtea 53.7% Therm 52.0%	Viable Control 72.4% Clostp 69.6% NProte 66.1% Therm 67.4%	Insulin Control 51.2 Clostp 43.4 NProte 44.1 Therm 43.4		58	24.5	13.1
Multiple Centers	-	-	-	-	-	-	-	-	-	-	-
City of Hope 2004-08 14 cent USA	2010 [43]	Roche Lib Hi Serva NB1, Sigma	-	-	>315,000 IEQ	- / 63.1%	17.7 min switch/ 59.1 min total	-	0.1-47.5	30.8	7.7

Internat Trial Ed Protocol	2006 [44]	Roche Lib HI	-	-	403,500 IEQ	-	-	-	15-70	-	<12.0
CITR 1999-2010 Global	2012 [45]	Roche Lib Hi Serva NB1	-	-	419,200 IEQ	435,200 IEQ / 62.0%	-	3.1	43.1	29.5	7.8
CITR 1999-2010	2014 [46] Am J Tx	Roche Lib Hi Serva NB1 Vita-cyte	-	-	416,000 IEQ	426,500 IEQ/ 61.9%	-	3.3	43.2	29.1	7.6
Nordic Network 7 centers	2015 [47] Transplant	Vita-cyte, Serva, Roche + or – Clostripain	-	Control 254,765 IEQ 2,498/gm IEQ/gm +Clost 391,565 IEQ 3,598/gm	-	-	-	8.0 10.6	53 53	27.2 27.6	9.8 10.2
Multi-Center 11 centers	2016 [5] Cell Tx		-	-	-	-	-	-	45.8	28.9	9.4
Phase 3 Clinical Trial, 8 centers CITR	2016 [3, 4] Diabetes	Serva NB1, Vita-cyte Roche MTF	708,470 IEQ 6,813 IEQ/Gm	582,370 IEQ 5,471 IEQ/Gm	490,174 IEQ 4,730 IEQ/Gm	-	14 min switch / 48.0 min total	2.3	42.8	33.4	7.7
Multi-Center	2017	Serva	-	296,494			21.5 min	3.7	51.4	25.3	7.3

3 centers	[48] Cell Tx	AF-1 N Prot		IEQ 3,274 IEQ/gm			switch/ - min total				
Center Averages	-	-	-	-	-	-	-	-	-	-	-
13 Separate Centers, 8 Groups of Multi-Centers	27 pub-lica-tions	Roche 40.5%, Serva NB1 37.5%, Vita-cyte 10.0%, Sigma 5.0%, No ID 7.0%	503,634 IEQ, 5,680 IEQ/Gm Pre-Pure	356,307 IEQ, 4,101 IEQ/Gm Post-Pure	411,462 IEQ 3,599 IEQ/Gm Post-Culture	9,286 IEQ/KgBW / 59.2% Pure Implant	16.2 min Switch Time/ 47.6 min Total Digest Time	3.5 Stim Index	42.8 years donor age	27.9% donor BMI	8.4 hrs Cold Ischemia Time

Pipeleers data were not included since results are reported as Beta cells, not islets [49, 50].

1.2 Current Publications Processing Human Islets for Clinical Transplantation

Table 1 reviews 27 of the most recently published reports (2005 – 2017) from 13 global Islet Transplant single centers and from 8 global multiple centers of islet transplantation resulting from their processing adult human pancreases into HIT. There were 18 published reports presented in this table originating from these 13 global single centers (9 American and 9 non-American including Canada, France, Italy, Switzerland, Sweden, and United Kingdom). Most all of the published multiple center reports included investigators from both American and Non-American centers, many within the same report. While the islet transplant publications listed in this table were focused on the details of clinical results, most of these articles also presented some details of islet processing results that are provided in Table 1. Nearly all of these islets provided for clinical islet transplantation in these reports were processed by the Digestion/Filtration method with different modifications from center to center with the collagenase brands and types presented. The islet yields are expressed as published as IEQ per Process and/or as IEQ/gm of pancreas processed at three different processing time points: Pre-Pure, Post-Pure, and Post-Culture. For the islets implanted into diabetic recipients, the infused islets are expressed as IEQ/Kg body weight and their islet purity. For pancreas processing both the Switch Time from enzyme recycling to processed islet collection was compared along with the total Digestion time. The final four evaluations were the Glucose Stimulated Insulin Release (GSIR) of the purified islets expressed as the first Stimulation Index, Donor Age in years, Donor BMI, and the Cold Ischemia Time in Hours.

The broad discussion of the details in Table 1 shown here were multiple collagenase types reported for use by these centers as either single or multiple enzyme combinations. Roche collagenase (Liberase HI or MTF) was used in 40.5% of these processes followed by Serva NB1 in 37.5% of the processes, then by Vitacyte in 10.0%, Sigma in 5.0%, and “not reported” in 7.0% of these processes. For this study, Prodo was using Liberase HI in 2011 at the start of the study and switched to Liberase MTF in June, 2012, for the rest of the study duration through 2016. Returning to Table 1, all islets for clinical transplant (HIT) were purified by density gradients of different types with some using a second purification as a Rescue COBE process to increase the purity of their islet preparations [27, 28]. The published average HIT Pre-Purification islet yield was reported as 503,634 IEQ (n=11) or 5,680 IEQ/gm (n=6) with an average HIT Post-Purification islet yield of 356,307 IEQ (n=11) or 4,101 IEQ/Gm (n=6). The published HIT Post-Culture results were an average of 411,462 IEQ (n=13) per process with 3,599 IEQ/Gm (n=2) Post-Culture. For the 8 centers reporting the number of IEQ/Kg Body Weight infused into diabetic recipients at the time of transplant, the average was 9,286 IEQ/Kg BW (n=8) with an average purity of 59.2% (n=11). Examining the details of the HIT processing, the Switch Time when the circuit was switched from recycling to collection mode averaged 16.2 minutes (n=11) with an average total time of digestion of 47.6 minutes (n=4). The average HIT Glucose Stimulation Index (GSI) reported from the Glucose Stimulated Insulin Release (GSIR) testing of islets was 3.5 (n=20) confirming their islet viability. Additional results from all these publications revealed an average donor age of 42.8 years (n=24), an average donor BMI of 27.9 (n=25), and an average Cold Ischemia Time of 8.4 hours (n=26). The quoted reference numbers for each of these studies are included in Table 1. It should be noted that the Pipeleers publications [49, 50] are important in islet transplantation, but were not

specifically included in Table 1 since their published results are expressed as "Beta Cells", that makes it difficult to specifically compare to "Islets" that were noted in these publications.

2. Materials and Methods – Prodo Laboratories

2.1 Pancreas Procurement

2.1.1 Organ Procurement Organizations (OPO's)

Both Prodo and the SLRI have contracts with several United Network for Organ Sharing (UNOS) regulated OPO's to receive *HPR*. Those that participated in these studies include One Legacy in Los Angeles, CA; Donor Network West in Oakland, CA; Sierra Donor Service in Sacramento, CA; Nevada Donor Network in Las Vegas, NV; Texas Organ Sharing Association in San Antonio, TX; LifeGift in Houston and Fort Worth, Tx; and several others through a contract with the International Institute for the Advancement of Medicine (IIAM) with their main office in Edison, NJ. Most recently, Promethera in Durham, NC, began distributing human pancreas for research, but their number of cases with Prodo accumulated by 2016 were insufficient to analyze for this study. The SLRI has a contract with the Integrated Islet Distribution Program (IIDP) of the National Institutes of Health (NIH) to provide human islets along with others to its over 200 investigators funded for islet research by both the NIH and the Juvenile Diabetes Research Foundation (JDRF). The SLRI contracts with Prodo to both process the human pancreases and distribute human islets to their investigators. These Prodo and SLRI contracts with OPO's define the inclusion and exclusion criteria for human pancreas acceptance for islet processing as well as methods of intact *HPR* removal, packaging, and shipping from the OPO to Prodo in Aliso Viejo, CA. While these methods are universal in the USA, being similar to those used for kidneys for transplantation, different OPO's utilize different organ preservation solutions for flushing the donor and shipping the pancreases that may affect the results of human pancreas processing into islets.

2.1.2 Human Organ Donors for Research Pancreases

Adult human pancreases were only procured for research from donors that had been approved for clinical transplant of any organ including whole pancreas and/or for clinical islet transplants, but were not able to be transplanted. In addition, for our acceptance of a pancreas to be processed into *HIR*, each donor providing a human pancreas for islet research must have donated at least one organ for clinical transplantation under appropriate informed consent from one of the OPO's. The details of the organ donors utilized in this study are presented in multiple tables located in the Results section, including the donor demographics. Since Prodo islet distribution efforts preceded the formation of the International Islet Distribution Program (IIDP) of the NIH and has continued as one of the major producers of *HIR* for this program, Prodo follows a number of current IIDP Standard Operating Procedures (SOP) for pancreas retrieval, processing, and shipping. Prodo follows the IIDP SOP for Pancreas Selection [51] specifically for obtaining appropriate pancreas for research selection.

These studies excluded a number of donors based on our previous experience demonstrating reduced numbers and qualities of islets isolated. Our selected exclusions of the donors for this study were based on our previous lower processing results and included age <18 years and >69

years, BMI >40, Cold Ischemia Time <4 hours and >40 hours, and HgbA1c >12. We purposely excluded all donors <18 years of age due to our experience that these younger donors have increasing quantities of embedded islets after processing that are not able to be purified that reduces their distribution. By age 10 years of age, more than 28% of the islets are embedded. Very young donors have 60%-80% of the islets embedded that cannot be purified by standard means. We hope to enable our ability to process younger donors since their islets have the ability to multiply that needs to be studied since adult islets have lost the ability to multiply. There also is a fall off in high quality islets that can be processed from donors >69 years of age, so we usually exclude these older donors in our routine processing for islet distribution. We also exclude all donors from processing with documented acute and chronic infections that could possibly be transmitted by the processed islets such as HIV I/II, Hepatitis B & C, Syphilis, Cytomegalovirus, Epstein Barr virus, Toxoplasmosis, T. Cruzi virus, and West Nile virus. While these research islets are not being transplanted, we still exclude processing pancreases from these donors since potential contaminations could be spread to those processing the pancreases as well as to those receiving these islets for research.

2.1.3 Ethics Statement

Prodo holds an independent Institutional Review Board (IRB) Exemption from Western Institutional Review Board for the “Use of Human Islets for Research” stating no approval from human subjects is required since all pancreases are from cadaver organ donors under 45 CFR 46.102h. In addition, all pancreases accepted by Prodo for islet processing for HIR, if they were not utilized for clinical pancreas or islet transplantation, are approved by the OPO and included in the informed consent documents for pancreas for research.

The authors have declared that no competing interests exist.

2.2 Human Pancreas Processing

2.2.1 Pancreas Preparation

The donor chart was shipped with the pancreas or electronically submitted from the OPO that is redacted for donor identification information. It becomes part of a process numbered batch record that documents the donor history, medical conditions and medications as well as the documentation of brain death in the heart-beating donors as required. For our accepting pancreases following Donation after Cardiac Death (DCD) or non-heart beating donors for pancreas processing into *HIR*, we restrict them to <15 minutes of downtime without Cardio-Pulmonary Resuscitation (CPR) at the time of the initial hospitalization and, at the time of pancreas procurement, they are restricted to <31 minutes of duration from disconnecting artificial respiration until the onset of cardiac arrest. Prior to initiating the pancreas processing, confirmation of proper informed consent and acceptable serological testing results are confirmed and recorded in the Batch Record. The procedure for pancreas processing into islets takes place within a Class 10,000 (Iso Level 7) clean room isolated by an anteroom with HEPA filtered air-flow. Within the Class 10,000 clean room, there are one 12 foot, one six foot, and three four foot Class 100 (Iso Level 5) laminar flow hoods (Biological Safety Cabinet (BSC), Class 100, Type II-A1). CEPA Operations, Inc certifies the clean room at Iso 7 and all of the hoods at Iso 5 on a regular basis.

One four foot, Iso 5, hood is restricted only to the pancreas surgical dissection with appropriate surgical instruments due to the inherent risk of pancreas contamination coming with the transport solution, duodenum, and pancreas. There also are 5 tissue culture incubators set at 37^{o}C for the culture of the purified islets in flasks. An adjacent anti-room under laminar air-flow contains a refrigerator for holding reagents and purified islets. A second laminar air-flow room holds all the recycled sterilized non-disposable items as well as disposable items sterilely stored for the processes.

Prior to removing the pancreas from the internal shipping container, a pancreas transport fluid sample is taken for microbiological testing, strictly from the inner bag containing the pancreas [52]. The pancreas is removed, brought into the surgical hood, and placed into an empty sterile dissecting pan where 60 mls of 2% sterile chlorhexidine digluconate in distilled water (Sigma, C9394) is injected into the stapled off duodenum through a 18 gauge needle (BD Insyte Autogard, Ref 381447) inserted at the proximal end. The needle is removed and the injection hole clamped with a bulldog clamp (John Hopkins Bulldog Clamp, curved 2.25 inches, Medical Supplies Equipment Co, Cat# 325-546FSI). Prior to this study, we had previously demonstrated 2% chlorhexidine digluconate in distilled water to be superior over Betadine (Medline, NDC 53329-939-25) to rapidly sterilize the duodenal content (unpublished results). The pancreas is then removed from this pan, rinsed with sterile saline, and placed into a beaker containing 1% chlorhexidine in distilled water and held there for a couple of minutes to surface decontaminate the tissues. The pancreas is then removed and placed in a new stainless steel dissection pan with 500 mls of sterile saline (Baxter, NDC 0338-0048-04) that is kept cold with ice packs (Uline, S-7361 (3 cold & 3 frozen) beneath the pan. Initially, if at any time during the dissection step, duodenal content is released, the hole is clamped and the contaminated surface areas are rinsed with 2% chlorhexidine digluconate in distilled water and placed into a new sterile pan. The extra pancreatic fat along with the spleen residual is surgically dissected off the pancreas. The portal vein is identified, if still present, and used to continue dissection of the non-pancreatic fat and vessels. The surgical plane between the duodenum and the head of the pancreas is developed to isolate the primary and secondary pancreatic ducts. Two large bulldog clamps are used on the pancreas side and a curved surgical clamp on the duodenal side of each duct to enable dissecting the intact duodenum off the pancreas without spillage of duodenal content. The dissected pancreas is then weighed (Ohaus Model #Ct1200) with the attached bulldogs within a sterile 500 ml Nalgene beaker (Nalgene, 2116-0500) and returned to the dissection hood. The pancreas is removed and placed into another 500ml sterile Nalgene beaker containing 250mls of 1% chlorhexidine in distilled water that is then removed from the Dissection hood and passed sterilely into the Distension Digestion/Filtration hood (Baker, BSC, Class 100 Type II-A1, 12 foot. Iso 5).

2.2.2 Pancreas Enzyme Distension

Within the 12 foot, laminar flow, Iso 5 hood, the pancreas is removed from the chlorhexidine digluconate solution and rinsed through three 500ml Nalgene jars, each containing 333 mls of sterile, Normal Saline (Baxter, NDC 0338-0048-034) that precipitates out the chlorhexidine digluconate. It is then placed in a stainless steel pan containing 500 mls of Normal Saline that is placed on top of refrigerated ULine ice packs in a stainless steel pan. The pancreas is divided in half by a scalpel with #10 blade on the distal side of the previous site of the portal vein crossing. A

0.5 gm pancreas biopsy is taken routinely from the cut surface for microbiological testing. Additional pancreatic biopsies are taken for requested additional research studies from the same cut surface of the pancreas, prior to pancreas distension. Each of the exposed ducts are cannulated with a 16 gauge intra-catheter (BD Insyte Autogard Winged, #381447) that are secured with 3-0 silk suture ligature (Surgic LC, ESILRC18387530). The pancreas distension step is accomplished manually with the pancreas placed into an empty stainless steel pan. For example, Roche collagenase (#MTF053398800001, Collagenase Liberase MTF C/T Blend 500mg GMP Grade) is combined with Roche Thermolysin (#MTF05339880001 Thermolysin MTF GMP 15mg) and is diluted and filtered through a 0.22 micron filter (Corning, Part #431097), then warmed to 37^{o}C and loaded into a 60 ml syringe (Terumo, #SS-60L) connected to tubing with appropriate connectors. The Collagenase/Thermolysin mixture is injected by hand via the pancreatic ducts of each the head and the tail sections with injections at increasing flow rates with the endpoint being full pancreatic distension with obvious intra-lobular distension. Previous evaluation of this technique measuring injection pressures showed a similar set of pressures generated as is described for processing clinical grade islets for implantation into diabetic recipients. While the pan initially received a 37^{o}C collagenase solution, this temperature readily drops to room temperature (25^{o}C) during this distension step. Upon completion, the two distended pancreas pieces are secured and the residual digestion fluid containing collagenase and thermolysin is collected into a 500ml sterile bottle. The catheters are removed and the residual, non-pancreatic tissue is dissected off, weighed, and recorded. The two enzyme distended pancreatic halves are divided into 6 to 8 pieces, followed by surface chopping of these pieces using scissors to penetrate the pancreas outer capsule that would have restricted and delayed the freed islets from being released during the Digestion/Filtration step. These partially digested pancreatic pieces are then loaded into the D/FC including the collagenase/thermolysin digestion mixture used for distension along with DNAse 1 and the 5 mixing silicon nitride marbles (Biorep #SN-01). It should be noted that a recent publication [53] from the Uppsala group has confirmed that pancreas to pancreas processing variation in islet yields without obvious explanation may be due to variations in Ca^{++} concentrations that can fall below the 5mM required concentration for all of the pancreatic digestive enzymes to be optimally functional.

2.2.3 Digestion/Filtration Chamber Digestion

The current D/FC circuit we utilize in these human islet isolations, previously shown as a diagram in Figure 1Eb, is also shown as a photograph in Figure S1. The resin D/FC (BioRep Technologies, Model #500-MVL-03)) has previously been connected into a circuit of tubing (Tygon3350, SILCNTBG 1/8x1/4), stopcocks (D600 Discofix High flow 4-way, Ref 456060), and two thermal couples (QOSINA, Cat #13138), one in the tubing outlet from the chamber and one in the outlet from the cooling coil. One cooling coil (BioRep Technologies, Model #HC-02) is placed in a 4^{o}C water bath in the collection portion of the tubing (sterile ice and water), and one heating coil (BioRep Technologies, Model #HC-02) is placed in a 50^{o}C water bath (ThermoElectron Corp, Model #2833) in the recycling portion of the tubing with the D/FC included. The circuit is set initially to recycle the content from a 250 ml conical (Corning, Ref 430776) through tubing to the heating coil in the hot water bath into the D/FC past a sampling port and return to the 250 ml conical using a pump that simultaneously reports the controlled flow rates (Cole Palmer, Masterflex, model

#77200-60). The initial circulating temperature within the system is held at 40°C with circulating HBSS to pre-heat the apparatus. With the distended pancreas enzyme mixture readied, the D/FC is emptied of the HBSS, opened and loaded with 5 synthetic marbles (BioRep Technologies, Silicone Nitride marbles, model #SN-01), 5 mls of 1 mg/ml of DNAse (Roche, Ref 10104159001), loaded with the distended, partially digested pancreas pieces, filled with the collagenase mixture, covered with a stainless steel screen (450 microns porosity, Prodo custom), and closed tightly. The residual collagenase mixture is loaded into the recycling 250 ml conical. The initial recycling flow rate of 70 mls per minute is used during the shaking of the loaded D/FC in the recycling mode, monitoring the D/FC temperature rise to 37°C. Prior to the Switch Time, the digestate samples are taken every 2 minutes and stained with diphenylthiocarbazone (DTZ) (Sigma, D5130 (80mg DTZ prepared in 40mls HBSS, & 10mls DMSO, that stores for 2 weeks @ 10°C, light protected) [54]. It should be noted that adding 5 drops of 28-30% ammonium hydroxide (Sigma 320145) to this DTZ standard formulation will increase the speed of islet staining. This staining is documented under the microscope (Olympus Model CKX41) that is also electronically connected to a computer and video screen documenting the digestate components of islets that are free or embedded and the quality of the islets and the acinar cell aggregates. Selected photos of each observation time are recorded as part of the batch record (Figure S2 and Figure S3).

The decision to Switch the process from recycling to collection modes, by chamber sample evaluations is based on the percentage of free islets, the quality of acinar cells, the size of both islet and acinar aggregates, and the disintegration of the pancreas pieces and free islets. When the decision to Switch is made from the recycling to the collection mode, the stopcock is changed for collection of the digestate to proceed to the collection tubes through a cooling coil in a 4°C water bath. The digestate collection temperature is kept between 7°C and 10°C by the cooling coil. After the Switch, Medium 199 (Sigma, M0393) supplemented with horse serum (JR Scientific, cat#44635) at 6°C-10°C is entered into the circuit towards the chamber at a rate to match the output. The collection of the digestate is made in 250 ml conicals that contain a collection solution of supplemented Medium 199 that are placed in containers with cold packs and water. As each 250ml conical is filled for a total of 24 tubes, it is removed from the 10°C collection cycle and centrifuged at 180G for 2 minutes at 8°C-10°C. The supernatant is aspirated off and the residual pelleted islets are rinsed with supplemented Medium 199 and collected into 4 combining tubes with pelleted digestate volumes recorded. Prior to purification the content of the combining tubes are combined into one 250 ml conical, mixed properly and a sample taken from the mid-portion of the conical for recording the islet number, viability, purity, and IEQ calculation. The standard conversion from islet number to islet equivalent is calculated and recorded as the Pre-Purification Count. The specific islet staining method for islet identification follows the IIDP Standard Operating Procedure (SOP) for the use of dithizone [54] plus the addition of ammonium hydroxide. The viability stain uses the IIDP SOP for the use of inclusion and exclusion of fluorescent dyes [55].

An example of the Digestion/Filtration Recycling and Collection islet preparation is provided in Figure S2 that is from a representative islet preparation from a 41 year old T2D pancreas donor (HP-15298-01T2D) not on insulin therapy who developed brain death after a stroke and was 5’ 2”, 251 pounds, with a BMI of 43 and a HgbA1c of 6.5%. Samples (1-2 mls) were taken from the sample port placed after the D/FC at 2 and 4 minutes during the recycling portion and every 3 to 4 minutes during the collection portion and stained with 5 drops of dithizone and 1-2 mls of HBSS.

The photos were taken at 4X magnification and clearly show the mix of islet (red), acinar (tan), and duct cells (clear) occurring throughout the process.

2.2.4 Islet Purification

Following the count sample, the digestate is centrifuged (Beckman Coulter, Allegra 6R) to remove the supernatant and replace it with University of Wisconsin (UW) Solution (CoStorSol, PS-004). Islets are incubated with the UW solution for 100 minutes at 8^{o}C to 10^{o}C. The COBE 2991 (COBE Blood Processor) is prepared by the removal of the foam core and plastic disc and replacement with the COBE bag (Termo BCT, Ref 90819) followed by appropriate set up spinning. Three Density Gradients are made from different combinations of Biocoll and Viaspan that range from 1.100gm/ml to 1.050gm/ml that are all made fresh on the day of isolation. All the densities are confirmed using the Mettler Toledo – Densito30PX (cat#LWC75663) out to three decimal places and recorded. The Density Gradient Maker (Prodo custom) is loaded on the outlet side with the 1.100gm/ml cushion that is pumped into the COBE. To avoid introduction of air into the tubing, the fluid level is adjusted backward to the bottom of the gradient maker to prevent air bubbles from entering the COBE bag. The COBE is then turned on slowly advancing the speed to 3,000 rpm. The next heaviest solution is loaded into the outlet side of the gradient maker with a stir bar. The inlet side of the gradient maker is then loaded with the lighter solution. These two loaded gradient densities can be varied in densities to focus on different sized islets and purities. The gradient maker is positioned on the magnetic stirrer (Termo Scientific, Variomag Max, order #500 94714) with the speed set at 400 rpm. The pump is turned to the appropriate speed for loading the gradient with the mixing lever opened between the inlet and outlet sides and the clamp opened on the tubing connecting to the COBE bag. As the inlet chamber side empties into the outlet chamber, the pancreas digestate should be removed from its cold storage. It is added to the outlet chamber when nearly all of the gradient has completed its loading from the outlet chamber. The COBE run time is 10 to 12 minutes in duration. The 30 pre-loaded and numbered 50ml conical tubes in racks are brought into the hood and prepared to receive the gradient off the COBE. The "Superout" button of the COBE is pushed at the end of the run collecting the output serially into these consecutively numbered collection tubes. Individual mixed samples are taken from each collection tube into separate wells of 24 well plates (Millipore Cat#PIMWS2450) for determination of islet purity and pellet volume. The percent islet purity and pellet volume is used to determine assignment of each well to the most purified Fraction 1 or lower purified fractions below as required. Each of the fractions are combined into a 250ml conical, washed, and loaded with PIM(R)® supplemented medium for taking a count sample from the mixed tube. Highly purified acinar tissue is removed from the bottom of the gradients and distributed to interested investigators as requested since the PIM(R)® media also supports the culture of human acinar cells, unlike the CMRL1066 culture medium.

An example of the human islet Purification is provided in Figure S3 that is from the same islet preparation from the same 41 year old T2D pancreas donor (HP-15298-01T2D) samples that were shown in the D/F step (Figure S2). After the purification in the COBE, during the 'Super-Out" step, samples are sequentially placed in 30 consecutive 50ml conical tubes containing the collection medium. The photos were taken at 4x, stained with dithizone with HBSS added for each of the 30 collection tubes. The volume in each tube is recorded on each sub-photograph. For this example,

Tubes 2-12 were collected with pellets purified as Fraction 1 with 90% purity and distributed to investigators after 3-5 days of culture. A lower Fraction 2 was collected from tubes 13-16 with a combined initial purity of 60% that were cultured and distributed to interested investigators. Purified acinar cells were also distributed to investigators from tube 25. The remaining tubes were discarded.

2.2.5 Islet Tissue Culture

Corning T150, non-tissue culture treated flasks (Corning, cat#431465) are labelled and placed into the 12 foot Laminar flow hood following 70% ethanol spraying of the flasks. The islet culture medium (Prodo Labs, 500 mls, PIM(R)® Recovery, cat#PIM-R001GMP) is supplemented with 5 mls PIM(G)® Glutamine/Glutathione (Prodo Labs, cat#PIM-G001GMP), and 5% PIM(ABS)® Human AB Serum (Prodo Labs, cat#PIM-ABS001GMP), along with triple antibiotics, PIM(3X)®, that includes Ciprofloxacin (Ref 61-277-RF, 10mg/1000ml), Gentamycin (Sigma, G1272, 10mg/1000ml), and Amphotericin B (Omega, FG-70, 2500mcgm/1000ml) and then pipetted into the T150 flasks at 30ml per flask with the bottoms wetted. The purification solutions are removed from the islets after centrifugation and replaced with the supplemented islet culture medium. Calculations are completed using the hand counter to enable placing 10,000 IEQ of purified human islets per each T150 flask with a total of 40 mls of the supplemented islet culture medium. All of the islet loaded flasks are wiped with alcohol and placed on the shelves of the 37^{o}C incubators. From 12-18 hours after the initial post-processing islet culture, a 50% media change is made using the same newly supplemented islet culture medium, PIM(R)®. Additional culture time utilizes 50% media changes every 3-4 days with supplemented PIM(S)®. The majority of highly purified human islet preparations are distributed after 3 to 5 days of tissue culture, shipping in PIM(T)® transport medium using controlled temperature shipping boxes. Glucose stimulated insulin release testing is performed on the residual islets held for the testing that is completed on the 5th to the 8th day post-processing.

2.2.6 Islet Distribution

Prodo has contracts with universities, colleges, institutes, individuals, and pharmaceutical and biotechnology corporations for distribution of *HIR* on a global basis. It also has a contract with the SLRI to process human pancreases into islets for distribution to those investigators with funded grants from the NIH and the JDRF for *HIR* through the IIDP. Most islets are distributed from 3 to 7 days after processing that meet release specifications. Islet distribution from Prodo utilizes Nalgene shipping bottles (ThermoScientific, Sterile Square Media Btl – 30ml-2019-0030, 60ml-2019-0060, 125ml-342040-0125, 250ml-342040-0250) containing PIM(T)® Transport supplemented medium (Prodo Labs, Cat#PIM-T001GMP) with the size of the bottle dependent upon the number of IEQ per order. These islet-containing bottles are packed in temperature controlled and specifically packed boxes that maintain the temperature between 6^{o}C and 15^{o}C from overnight to up to 3 days of transport time on a global basis. For circumstances when additional islets remain available but are not ordered, Prodo follows the IIDP SOP for the flash freezing of human islets that can be stored frozen for future shipping [56].

If requested, Prodo also supplies histologic samples of human pancreas, duodenum, and abdominal fat for research prior to processing the pancreas for islets and utilizes the IIDP SOP for

preparing pancreas sections for histology [57]. For both human islet culture and cold shipping, Prodo utilizes IIDP SOP standardized islet culture prior to shipping based on Prodo protocols [58]. It also uses IIDP SOP standardized shipping packaging the islets and maintaining cold temperatures for shipping based on Prodo protocols [59]. Special shipping requirements are needed for shipping islets internationally and Prodo utilizes IIDP SOP for standard shipping needs [60]. Throughout all of these pancreas and islet processing, purifying, culturing, and shipping steps involved, Prodo utilizes IIDP Guidelines taken from the "2007 Guideline for Isolation Precautions: Preventing Transmission of Infectious Agents in Healthcare Settings" from the Centers for Disease Control and Prevention [61].

2.3 Process Confirmation Testing

2.3.1 Islet Yield Determination

Human islet yields are determined at Pre-Purification, Post-Purification, and after tissue culture (Post-Culture) by counting islet size through the use of a microscope ocular piece that provides a grid graded with 50 to 100 micron squares to measure islet size. An "Islet Equivalent (IEQ)" is defined as an islet with a diameter of 150 microns [62]. The number of IEQ's in each size class is calculated by multiplying the number of islets by a conversion factor for each micron size. Performing islet counts has two major opportunities for error: a) properly mixing the final preparation in a 250ml conical and taking the sample in the quickest time in the mid-portion of the conical to avoid missing the largest islets that drop more rapidly and b) actually estimating the accurate count for each islet size observed. Since these two steps can combine to as much as a 25% error, Prodo has developed a specific image analyzer (Figure S4a) built to reduce this error that scans each T150 flask (Corning, cat#431465) and counts islet number by size excluding those below 50 micron diameter with an error margin of ±10%. In addition, it counts all particles up to 600 microns and marks those larger objects for review, such as cotton fibers. It calculates the IEQ number using the same equation and plots a scan of islet size by particle size (Figure S4b). This image analysis count confirms the manual counts to reduce the error in estimating IEQ numbers prior to islet distribution in order to ship the most accurate quantity of islets to each recipient laboratory. Those human islets of the purest fractions (90% pure and above) are labelled Fraction 1 (Fx1) and cultured in separate T150 flasks. Those of intermediate purity (70-89% pure) are labelled as FX2 and cultured in separate flasks. Those of lower islet purity (50-69%) are labelled as FX3 and cultured in separate flasks. Islet containing flasks that contain islets <50% purity are discarded and are not cultured.

2.3.2 Glucose Stimulated Insulin Release (GSIR) Testing

Prior to the scheduled test date that is usually 5-8 days following the islet processing, the test volume of islets is removed from cultured flasks and placed in a sterile 15ml conical for GSIR testing [62]. The GSIR test plates are prepared using 24 well Cell Culture Receiver Plates (TC, Cat#PIMWS2450) and well-inserts (Millicell Cell Culture Insert, 12mm polycarbonate, 12 μm, Cat# PIXP01250). Prior to loading the samples, 3 inserts are placed in the 24 well testing plate. There is 0.4ml medium placed into each of 3 wells with inserts to pre-wet, and islets are washed 2 times with 3mM glucose test medium. Islets (75 IEQ) are loaded in 0.6ml of 3mM test medium into each

of the 3 wells containing inserts that are covered and cultured at 37^{O}C and 5% CO_2 overnight. The next day, the plate cover is removed and each of the inserts containing islets are transferred out of their wells with medium and placed one row below into empty wells for the medium to drain out of the inserts. The residual media is aspirated out in the 3 original wells and discarded. Then, fresh 3mM glucose medium is added to these wells followed by replacing the inserts with the islets back into their original wells with the fresh medium. The drained media is aspirated from the wells where the inserts had been set to drain. These same steps are repeated two more times with one hour culture periods to enable the islets to reach basal insulin release levels after each, before starting the test media. After the third media change, the wells with the islets inside the inserts are incubated at 37^{O}C for one hour. The additional test media for 12mM Glucose, 20mM Glucose, and 20mM glucose+0.1mM IBMX has to be warmed to 37^{O}C with 5% CO_2 prior to use in the test. The 3 inserts with islets undergo the same incubation periods for one hour duration with each of the test media in order. The final incubation returns to 3mM glucose again. Each of the test media collected from each well is separated from the islets, then sealed, labelled and frozen for ELISA insulin assay (Mercodia Insulin Assay cat#10-1113-10), and DNA Assay (Life Technologies – Quant-iT™ Picogram® ds DNA Assay Kit cat#P7589). The residual islets are removed from the trans-wells, processed and stored for quantifying insulin and DNA content. Prodo utilizes the IIDP SOP to determine islet secretory potency of insulin release [63], but reduces the high glucose concentration from 28mM to 20 mM.

2.3.3 Microbiological Testing

Due to potential bacterial, fungal, and yeast contamination from the donor pancreas, duodenum, and the transport solution as previously presented [52], samples are prepared for bacterial and fungal microbiological testing. The initial sample of the transport solution is taken prior to removal of the pancreas from the transport container inner bag enclosing the pancreas. A second sample is taken from the mid-portion of the pancreas by biopsy prior to inserting the ductal catheters. A 0.5 gram pancreas biopsy is taken from the cut surface for microbial testing and divided into three pieces. Microbiologic culturing of these samples is done in agar (Remel – Tryptic Soy Agar cat#R111570) and read every 2-3 days for 14 days with the first 7 days at 30^{O}C-35^{O}C and the second 7 days at 20^{O}C-25^{O}C. A third sample is taken from the fluid in the dissection pan following duct cannulation. Microbial samples are not only taken during isolation, but also taken at media changes and from the final islet preparation post-culture and observed for 7 days for microbial growth. Gram staining is done on the supernatant on the day of shipping and followed to supplement the culture samples. If microbial contamination is documented prior to shipping, these islets are not distributed. If discovered after shipping, microbial contamination is reported to the islet investigators who received them as contaminated islet preparations with the opportunity to be replaced. Prodo utilizes the IIDP SOP for Microbiology Testing [52]. Since these samples are tested for distribution of human islets for research and not for clinical transplantation, USP guidelines are not followed.

2.3.4 Statistical Analyses

Statistical analyses were run on all the results except those not indicated such as those based on percentage data or those where insufficient numbers of samples per group prevented

calculation of meaningful results. The majority of Student t tests were 2 tailed and assumed unequal variances using the Bonferroni correction to reduce p values based on number of variables. The use of linear regression analysis used scatter plots with calculated straight lines with the appropriate p value for the regression reported. P values are presented as their calculated value with $p<0.050$ and $p<0.001$ showing statistical significance depending upon number of variables tested. (see Acknowledgements)

3. Human Islet Processing Results (2011-2016)

3.1 Human Islet Processing Result Details

3.1.1 Human Pancreas Donors

Organ Donors for Current Study. Prodo and the SLRI have been distributing *HIR* as a service business since 2007 (Table 2) delivering a total of 78.45 million Islets (IEQ) for Research through 2016 that ranged from 7.33 to 12.10 million IEQ per year. This report focuses on the last six complete years of distribution: 2011 - 2016, during which 62.8 million *HIR* were distributed to both corporate and academic investigators that averaged 10.47 million IEQ per year and represents 80.1% of the total islets distributed since 2007. The islets distributed from 2011-2016 had very consistent yields of islets delivered compared to the earlier processing years that were completed without the manufacturing methods and controls. The average IEQ per donor shipped per year ranged from 218,506 IEQ per year to 335,961 IEQ per year with a 6 year average of 280,333 IEQ per year. The islet purities six year average was 88.4% pure per process, ranging from 86.3 % to 91.9% pure human islets. There was an average of 6.3 different OPO's delivering HPR to Prodo with a range of 4 to 9 OPO's with 9 being the current number. Prodo has contracts with a number of global pharmaceutical and biotechnology companies, research institutes, universities, and individual investigators to deliver *HIR* on a weekly schedule. Prodo also has a contract with the SLRI to process *HPR* into islets under their contract with the National Institutes of Health (NIH) for its International Islet Distribution Program (IIDP) that provides human islets to its funded academic investigators. All of these islet research recipients are encouraged to report back regarding any discrepancies in their islets received. If there are found to be major differences from what was sent, then islet replacements are sent for those islets that were not able to be utilized without additional charges. The demands of these contracts for weekly distribution of *HIR* have required the development of a standardized, manufacturing approach to human pancreas processing so that consistent, high quality human islets can be distributed on a weekly basis (see Methods). This report documents the procedures developed and the outcome results achieved by the success of this standardized islet production effort as well as maintaining control of the critical variables involved in the success of islet processing and distribution for diabetes research.

Table 3 presents the number of adult human pancreas processed into *HIR* from both Non-Diabetes Mellitus (Non-DM) donors and Type 2 Diabetes (T2D) donors for this six-year study period. Processes were declared to be "Unsuccessful" if there were insufficient numbers of high quality islets available to distribute to diabetes investigators. When this effort was initiated in July of 2011, we had set the criteria of a successful process to be >70% pure that had been achieved in 2010. However, that criteria was raised to the 80% level at the start of 2012 and carried on through 2016 during which time the average islet purity achieved 91.9% purity level. There were

242 Non-DM donor pancreases processed, with islets from 226 donors that were distributed which represents 93.4% of these pancreases processed. Of 35 T2D donor pancreases processed, islets from 29 donors were distributed which represents 82.8% of T2D pancreases processed. But, we have chosen to drop the T2D islet processing islet purity to below 80% to the 65-70% purity level with processing increased numbers of T2D donors in order to ship more of these rarely processed islets to investigators.

Table 2 Number of *HIR* distributed to diabetes investigators by Prodo and the SLRI.

Process Year	2007 6 mo	2008	2009	2010	2011*	2012*	2013*	2014*	2015*	2016*	Totals
# IEQ ($x10^6$) Distributed for Research	2.40	3.10	4.05	6.10	7.33	12.10	11.26	11.10	10.07	10.94	$78.45x10^6$ IEQ Total Shipped
# Pancreas Processed for Research	15	30	22	21	36	38	41	42	50	48	343 Total Processed
Ave IEQ / Donor Shipped per Year	160, 000	103, 000	337, 500	290, 476	293, 207	335, 961	296, 433	277, 458	218, 506	260, 431	280,332.7 Ave IEQ / Donor Shipped per Years of Study
Ave Islet Purity % Shipped per Year	-	68.5	67.5	78.0	88.1	88.4	86.3	87.3	88.6	91.9	88.4% Ave Purity Shipped Per Years of Study
# OPO's per Year	2	2	2	3	4	5	5	6	9	9	6.3 Ave # OPO's Per Years of Study

* Process Years Included in this Study

Table 3 Human pancreas processes from Non-DM donors and T2D donors with the percentage of successful islet processes.

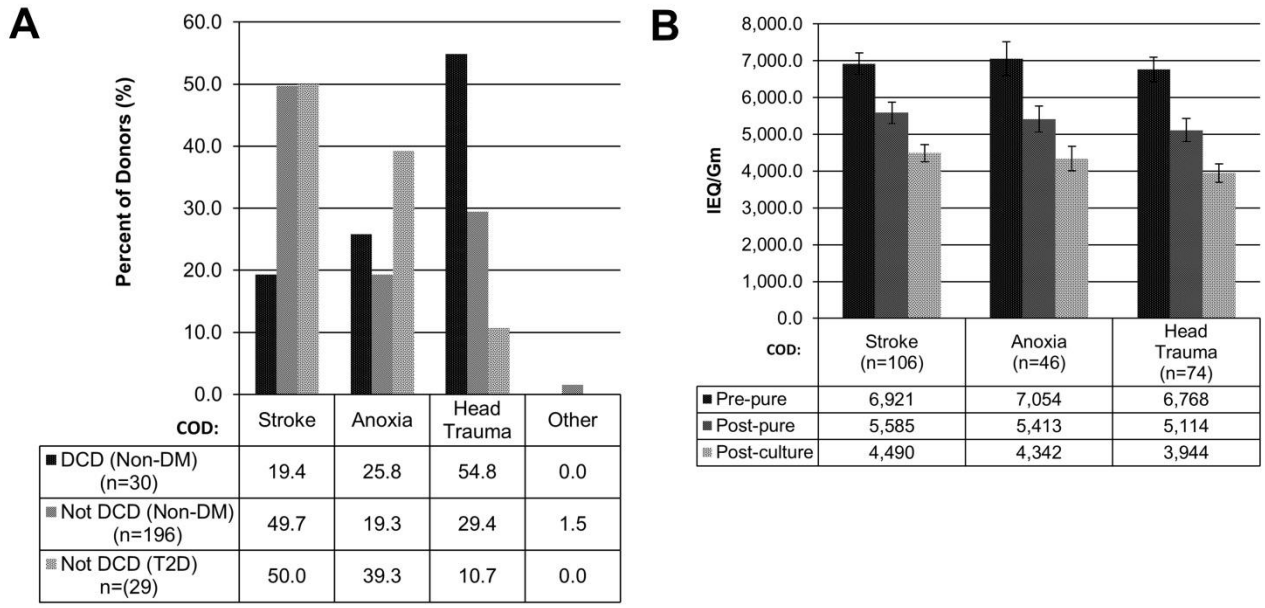
Year	Total Non-DM	Islets Shipped	% Success	Total T2D	Islets Shipped	% Success
2011	28	26	93	13	10	77
2012	41	36	88	2	2	100
2013	44	38	86	3	3	100
2014	43	40	93	2	2	100
2015	44	44	100	7	6	86
2016	42	42	100	8	6	75
SUM	242	226	93	35	29	83

Donor Demographics. The demographics of pancreas donors in this study are presented in Table 4 and compared to recent Clinical Islet Transplant Reports (CITR) [45, 46]. The average age of the CITR donors was 43.2±12.2 years for n=1,017 donors. For this study the Non-DM donors average age resulting in Successful processes (n=226) was 42.4±0.9 years while the Unsuccessful Non-DM donors (n=15) average age was 48.2±4.1 years. The average age of the T2D Successful donors (n=29) was 49.8±1.8 years while the average age of the T2D Unsuccessful donors (n=6) was 47.0±4.0 years. For the CITR study there were 55.5% males and 44.5% females. In this study, there were 140 (61.9%) male donors and 86 (38.1%) female donors in the Non-DM Successful group with 7 male donors and 8 female donors in the Unsuccessful group. For the T2D donors, there were 17 males (58.6%) and 12 females (41.4%) in the Successful group and 3 males and 3 females in the Unsuccessful group. In terms of the race of the donors, the CITR reported 90.9% as Non-Hispanic, 7.3% as Hispanic, and 1.9% as mixed race donors. For this study, the racial mix of donors was much broader with 124 (54.9%) Caucasian, 59 (26.1%) Hispanic/Latino Specific, 26 (11.5%) African American, and 17 (7.5%) Asia/Pacific Islander that provided pancreases for the Non-DM Successful processes. The CITR study only processed islets predominantly from Caucasians (90.9%) while this study processed islets from 45.1% of non-white donors. The other races in this study are generally smaller donors that produce smaller islets. For the T2D Successful Processes, there were 14 (48.3%) Caucasian donors, 13 (44.8%) Hispanic/Latino Specific donors, and 1 (3.4%) Asia/Pacific Islander donor and 1 (3.4%) African American donor for the T2D Successful processes.

Table 4 Demographics of human pancreas donors for research (2011-2016).

Donor	Categories	CITR [45, 46] Results n=1017	Prodo Non-DM Successful n=226	Prodo Non-DM Unsuccessful n=15	Prodo T2D Successful n=29	Prodo T2D Unsuccessful n=6
Age	Ave Age± SEM (yrs)	43.2±12.2	42.4±0.9	48.2±4.1	49.8±1.8	47.0±4.0
Sex	Male Female	55.5% 44.5%	61.9% (140) 38.1% (86)	46.7% (7) 53.3% (8)	58.6% (17) 41.4% (12)	50% (3) 50% (3)
Race	Caucasian/ Non-Hispanic	90.9%	54.9% (124)	53.3% (8)	48.3% (14)	16.7% (1)
	Hispanic / Latino Specific	7.3%	26.1% (59)	46.7% (7)	44.8% (13)	33.3% (2)
	African America	0%	11.5% (26)	0%	3.4% (1)	0%
	Asian/Pacific Islander	0%	7.5% (17)	0%	3.4% (1)	50% (3)
	Mixed Race	1.9%	0%	0%	0%	0%

Causes of Brain Death. There were significant differences in the primary causes of Brain Death in donors from the Non-DM, T2D, and DCD groups (Figure 2A). By way of comparison, the Cause of Death data reported in the CITR study of 2016 was at 55.9% for Stroke, 31.0% for Head Trauma, and 12.9% for Anoxia [5]. The Phase 3 trial [3, 4] reported the cause of death due to Stroke was 44.0%, Head Trauma was 45.3%, and Anoxia was 5.3% with another 5.3% as "Other". For this study, the Non-DM donors' primary cause of brain death was Stroke at 49.7% followed by Head Trauma at 29.4% and then Anoxia that includes respiratory and myocardial infarction deaths at 19.3%. The "Other" category of donors in Figure 2A includes those with brain tumors for the Non-DM donors (1.5%). The T2D donors' primary causes of brain death were divided between Stroke at 50.0%, Anoxia at 39.3%, and Head Trauma much lower at 10.7%. This increased incidence of both stroke and cardiac death in the T2D donors over the Non-DM donors would be expected since these are the two primary causes of death in diabetics that directly relate to their secondary complications of diabetes. In contrast in this study, the DCD donors' primary cause of death was Head Trauma at 54.8% followed by Anoxia at 25.8% and Stroke as the lowest of these three donor groups at 19.4%. This suggests that the Stroke cause of death is far more effective than Anoxia or Head Trauma in resulting in brain death, most likely as a result of intracranial hemorrhage that frequently is part of stroke and can significantly increase intracranial pressure readily causing brain stem herniation and brain death.



COD:	Stroke	Anoxia	Head Trauma	Other
DCD (Non-DM) (n=30)	19.4	25.8	54.8	0.0
Not DCD (Non-DM) (n=196)	49.7	19.3	29.4	1.5
Not DCD (T2D) n=(29)	50.0	39.3	10.7	0.0

COD:	Stroke (n=106)	Anoxia (n=46)	Head Trauma (n=74)
Pre-pure	6,921	7,054	6,768
Post-pure	5,585	5,413	5,114
Post-culture	4,490	4,342	3,944

Figure 2 Organ donor brain death. A: Causes of brain death in Non-DM, T2D, and DCD organ donors for research pancreases; B: Effect of cause of brain death on IEQ/Gm yields in Non-DM, T2D, and DCD donor.

A potential variable to examine affecting Islet yields and losses was the Cause of Death of the donors. This analysis combined all the causes for death together to determine any significant differences in islet yields based on Cause of Death of the donors. For the Pre-Purification IEQ/Gm yields and the Post-Purification IEG/Gm yields, there were no significant differences in islet yields. Analysis of the IEQ/Gm of islet yields (Figure 2B) by 2 tailed t tests only showed one combination that was significantly different in terms of islet yields in this study based on the causes of death in

the Post-Culture group. There were no statistically significant differences for Anoxia versus Stroke in islet yields (p=0.343), nor for Anoxia versus Head Trauma (p=0.156). However, for Stroke versus Head Trauma, the islet yields were significantly larger for Stroke over Head Trauma (p=0.044). This was the only combination that resulted in a significant difference in islet yields as IEQ/Gm of the three causes of death of the donors.

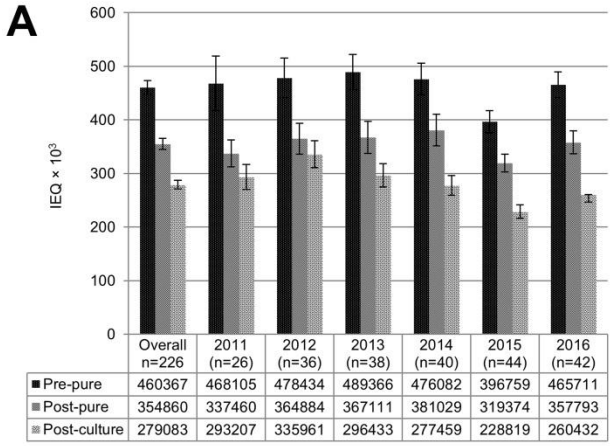
3.1.2 Prodo Labs Processing Islet Yields

Islet Yields from Non-DM and DCD Donors. The overall islet yields are represented as Islet Equivalents (IEQ) that is defined in the Methods section and reference [62]. These results are shown for the six processing years (2011-2016) for Non-DM pancreas donors expressed as Islet IEQ Yield per Pancreas Process (Figure 3A) and as Islet IEQ Yield per Gram (IEQ/Gm) of pancreas processed (Figure 3B) showing results at the three processing steps: Pre-Purification, Post-Purification, and Post-Culture for human islets. These results demonstrate the consistency of the pancreas processing over the six consecutive years, showing essentially the same islet yields for Pre-Purification, for Post-Purification, and for Post-Culture for each year. Expressed as Overall Islet IEQ Yields per process (Figure 3A), there were 460,367±12,732 IEQ Pre-Purification and 354,860±10,418 IEQ Post-Purification followed by 279,083±7,996 IEQ Post-Culture. These islet yields per pancreas process showed by linear regression analysis there were no significantly different islet yields per process identified at any of the three processing steps. However, expressing the islet yields as IEQ/Gm of pancreas processed showed different results (Figure 3B).

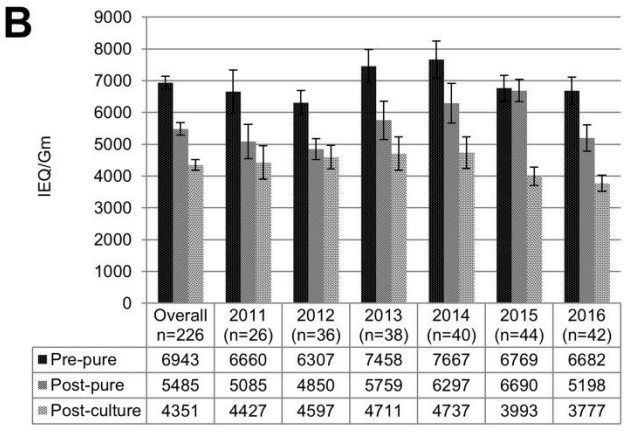
By comparison, expressing the overall islet yields as IEQ/Gram of pancreas (Figure 3B) processed rather than IEQ Yield per Process (Figure 3A), the Overall Results were 6,943±201 IEQ/Gram isolated Pre-Purification and 5,485±199 IEQ/Gram pancreas at Post-Purification with 4,351±167 IEQ/Gram of pancreas Post-Culture. Using two-tailed Student t tests (see Methods), for the Post-Culture, islet yields expressed as IEQ/Gm for all of the six years (2011 - 2016), showed several significantly different yields through these years with $p<0.050$). An explanation of these differences of expressing islet yields per process versus per Gm of pancreas was discovered when evaluating the range of pancreas weights of the 226 successful donors for these six processing years that varied from 40.3 gm to 194.5 gm with an average weight of 89.4gm. With such a wide range of pancreas weights observed, one has to be concerned about the accuracy of representing islets yields by using the IEQ yield per pancreas process to represent all of the results. Based on the islet yields from this study, the more accurate measurement of islet yields should be expressed as IEQ/Gram of pancreas processed that is utilized throughout the remainder of this report. Using this IEQ/Gm analysis also demonstrated process variables for Non-DM islet yields and showed statistically significant process success with shorter Cold Ischemia Times (CIT) (p=0.027), shorter Switch times (p=0.043), and greater percent of pancreas digested (p=0.006) than IEQ per process, but no significant difference in islet yields based on pancreas weight alone (p=0.319). Unfortunately, when reviewing the results of the clinical islet transplant trials in diabetic patients (Table 1), one discovers that these investigators predominantly express their results as total IEQ per pancreas processed rather than IEQ per Gram of pancreas processed. As we have now demonstrated in this publication, the markedly different pancreas weights of processed pancreases prevents the ability to demonstrate significant differences in the three processing steps in terms of expressing islet yields per process. Yet, there clearly are significant

differences in islet yields per process step if the results are expressed as IEQ/Gm of pancreas processed. It is possible that if the clinical islet transplant centers would consistently express their processing outcomes as IEQ/Gm of pancreas processed, in addition to their IEQ/Process, their islet processing outcome results may better clarify center to center clinical results. Clinical islet transplantation investigators are obviously interested in knowing the total number of islets implanted and will report that result as IEQ per Process, as they should. Yet, this use of using islet yields per process is primarily affected by the weight of the pancreases as will be detailed later in this report. So, in terms of analyzing islet processing success, in our opinion, reporting IEQ per gram of pancreas is the only effective way to directly compare islet processing effectiveness between processes and between centers. Thus, all additional islet yield results are expressed in this report only as IEQ/Gm of pancreas processed throughout the remainder of this report.

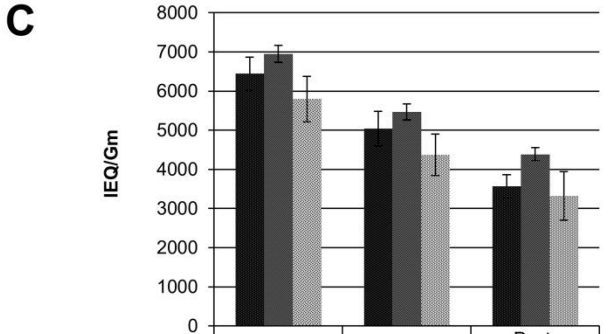
Included within these results of the Non-DM pancreas donors, there were a total of 31 pancreas donors that were designated as DCD (Donation after Cardiac Death) or non-heart beating organ donors included in the total number of pancreases processed from 2011-2016 that were included in the previous data in Figure 3A, Figure 3B, and Table 4. Based on our preliminary experiences prior to 2011, we had set the criteria for accepting a pancreas from a DCD donor for this study as: a) an initial Down Time of <15 minutes without Cardio Pulmonary Resuscitation (CPR) at the start of hospitalization and b) a DCD declaration of cardiac arrest within 30 minutes of disconnecting the artificial ventilation that starts the procedure to recover organs for transplant or research. Of the 34 DCD donors meeting these criteria, three were not utilized in these calculations. One was a Type 2 Diabetic and two of the Non-DM donors failed to have sufficient numbers of quality islets to enable shipping. All of the 31 DCD donors utilized in the study were Non-DM donors with their islet yields shown in Figure 3C. Islet yields in this figure also include the yields from T2D donors for quick reference with their complete results presented later in Section 3C. The DCD Pre-Purification yields were 6,444±425 IEQ/Gm compared to the 196 Non-DM donor average yields at this level of 6,949±215 IEQ/Gm (p=0.295). The Post-Purification results for the DCD donors yield were 5,042±444 IEQ/Gm compared to the Non-DM donor yields of 5,465±202 IEQ/Gm (p=0.391). The Post-Culture islet yield for the DCD donors was 3,567±299 IEQ/Gm that were significantly lower than the Non-DM donor islet yields of 4,384±167 IEQ/Gm (p=0.013). Thus, there is a significantly lower islet yield Post-Culture from Non-DM, DCD donors compared to Non-DM, Non-DCD donors. These results also confirm the importance of using the two defined, restrictive criteria for accepting a DCD donor for islet processing with 31 of 34 (91%) DCD donors providing high quality *HIR*. Even with this significantly lower islet yield Post-Culture in this DCD group, we chose to combine them with the Non-DM, Non-DCD donors as a single pool of donors for islet recovery for research for the remainder of this report.



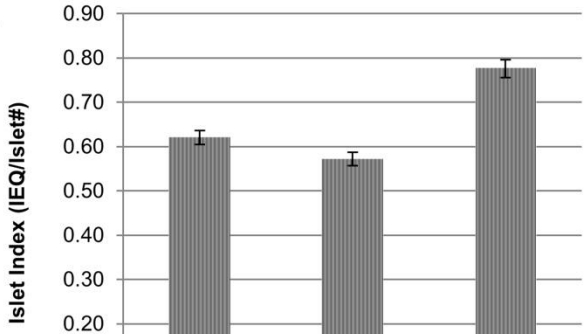
	Overall n=226	2011 (n=26)	2012 (n=36)	2013 (n=38)	2014 (n=40)	2015 (n=44)	2016 (n=42)
Pre-pure	460367	468105	478434	489366	476082	396759	465711
Post-pure	354860	337460	364884	367111	381029	319374	357793
Post-culture	279083	293207	335961	296433	277459	228819	260432



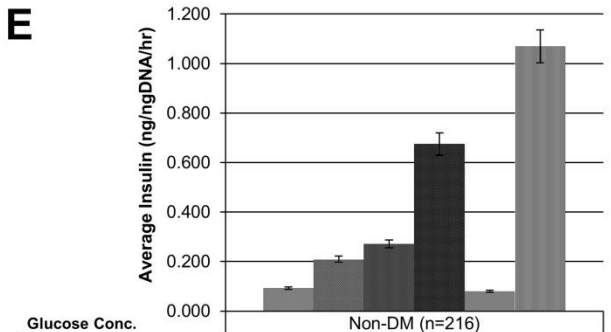
	Overall n=226	2011 (n=26)	2012 (n=36)	2013 (n=38)	2014 (n=40)	2015 (n=44)	2016 (n=42)
Pre-pure	6943	6660	6307	7458	7667	6769	6682
Post-pure	5485	5085	4850	5759	6297	6690	5198
Post-culture	4351	4427	4597	4711	4737	3993	3777



	Pre-Pure	Post-Pure	Post-Culture
DCD (Non-DM) (n=31)	6444	5042	3567
Not DCD (Non-DM) (n=196)	6949	5465	4384
Not DCD (T2D) n=(27)	5797	4371	3323



	Pre-pure	Post-pure	Post-culture
Islet Index	0.62	0.57	0.78



Glucose Conc.	Non-DM (n=216)
3mM (1st Basal)	0.093
12mM	0.209
20mM	0.272
20mM+IBMX	0.675
3mM (2nd Basal)	0.080
Total Stimulated Insulin	1.069

Figure 3 Processing human Non-DM pancreases into islets. A: Overall islet IEQ yields per "pancreas process" – Non-DM successful; B: Overall islet IEQ yields "per gram" (IEQ/Gm) of pancreas processed – Non-DM Successful; C: Comparison of islet IEQ/Gm yields between DCD, Non-DM, and T2D donors; D: Overall islet indices from islets processed from Non-DM donors; E: GSIR from successful Non-DM donors for 2011-2016.

For comparison, a publication in 2010 for all donors recovered for whole organ transplantation showed there is an increasing percentage of DCD donors for organ transplantation from 14.8% in 1998-2002, to 48.4% in 2003-2005, and to 60% in 2006-2008 [64]. Another recent publication in 2016 by the Edmonton group for clinical islet transplantation [65] compared islet yields from 418 Non-DM and 15 DCD donors with Pre-Purification, islet processing yields of $576x10^3$ IEQ/Process (Non-DM) and $608x10^3$ IEQ/Process (DCD) and Post-Purification yields of $386x10^3$ IEQ/Process (Non-DM) and $379x10^3$ IEQ/Process (DCD) without any significant difference in their yields. However, they did not report any Post-Culture islet yields nor did they report their islet yields in IEQ/Gm of pancreas processed. They also recommend the inclusion of DCD donors for clinical pancreatic islet transplantation. In contrast, the recently published Phase 3 Trial [3, 4] in 2016 excluded DCD donors without explanation, as their pancreas donors selected for clinical islet transplantation. It would seem appropriate with the DCD donor results reported in this study of islet processing that DCD donors with specific restrictions should be considered to provide human islets for clinical transplantation as they already are in use for whole pancreas transplants. In terms of Non-DM islets from DCD donors, concern about their quality should be minimal with our report comparing their cultured islet yields with Non-DM donor cultured islets, assuming the same restrictions for accepting DCD donors would be used.

Returning to the analyses of the Non-DM donors combined with the DCD donors overall results, the Islet Index is defined as the ratio of Islet IEQ divided by the Islet Number for each pancreas processed [62]. In most published clinical reports, the size of the islets reduces during the process and as well as following islet culture, even if that culture is longer than a couple of days using the basic islet culture medium (CMRL1066 (500ml), Human Serum albumin (10ml), and Lisofyllin (240uL) as described as the original "CIT Culture Medium" [44]). As shown in Figure 3D, for the Successful Non-DM processes in this study, the Islet Index for the Pre-Purification step was 0.62±0.02 with the Post-Purification Islet Index of 0.57±0.02, which are statistically not different. Yet, for the Post-Culture group, the Islet Index increased for these islets to 0.78±0.02 which is a significant increase from Pre-Purification to Post-Culture as well as from Post-Purification to Post-Culture islet yields. In fact, each of these are statistically significantly different from the other with Pre-Pure greater than Post-Pure ($p=0.044$), Post-Culture greater than Post-Pure ($p<0.001$), and Post-Culture greater than Pre-Pure ($p<0.001$). In contrast to the published clinical studies using CMRL culture media, this Post-Culture increased Islet Index appears to be due to the use of PIM(R)® supplemented islet culture medium that was designed to improve islet recovery post-processing. Its use over several days to recover islets from processing-induced damage also causes smaller islets to aggregate with each other or to become larger islets during the usual 3-7 days of islet culture prior to shipping to research investigators. This explains the significant increase in the human Islet Index Post-Culture. This effect from using PIM(R)® culture media is consistently observed throughout this report.

HIR functional analysis was obtained by Glucose Stimulated Insulin Release (GSIR) testing that was performed routinely on the 5^{th}-8^{th} day post-pancreas processing with results shown in Figure 3E (see Methods). Processed islets were already distributed to recipients prior to the GSIR testing on most all of the 226 human islet preparations in this study. GSIR results for the Non-DM donor processed islets given an average 3mM (54mg/dl) glucose stimulation resulted in the basal insulin release level of 0.093±0.005 ng insulin/ng DNA/Hr. The Glucose Stimulation Index (GSI) is the glucose stimulated insulin release value at given glucose concentrations divided by the insulin

release from the average basal (3mM) glucose concentration. The 12mM (216mg/dl) glucose stimulation released 0.209±0.012 ng insulin/ngDNA/Hr that was significantly larger than the 3 mM glucose ($p<0.001$). Dividing the 12mM glucose insulin release by the basal insulin release results in the 1st GSI of 2.652±0.105. The 20mM (360mg/dl) glucose stimulation released 0.272±0.016 ng insulin/ngDNA/Hr that was significantly higher than the basal insulin release ($p<0.001$) and significantly higher than the 12 mM glucose stimulation ($p<0.001$). These values resulted in the 2nd GSI of 3.535±0.156 ng insulin/ngDNA/Hr. The 1st GSI was significantly less than the 2nd GSI ($p<0.001$). The 20mM glucose+0.10mM IBMX stimulation released 0.675±0.045 ng insulin/ng DNA/Hr which was significantly higher than the basal insulin release ($p<0.001$), the insulin release from the 12mM glucose stimulation ($p<0.001$), and the insulin release from the 20mM glucose stimulation ($p<0.001$) that also resulted in the 3rd GSI of 8.789±0.405. This 3rd GSI was not only significantly higher than the 2nd GSI ($p<0.001$), but was also significantly higher than the 1st GSI ($p<0.001$). The Total Stimulated Insulin Release is calculated as the sum of the three insulin levels released in response to stimulating glucose concentrations as well as glucose+IBMX minus the average insulin levels released from the two basal glucose levels. The average of the Overall Total Stimulated Insulin Release for the Non-DM islets under GSIR testing was 1.069±0.067 ng insulin/ng DNA/Hr. Thus, these Non-DM human islets demonstrate significant increases in insulin release over basal and over each previous stimulating glucose concentration throughout the entire GSIR test which was performed after 5 to 8 days of islet culture in PIM(R)® supplemented culture medium.

Islet Yields from Non-DM and T2D Donors. With the results already presented for the Successful Non-DM donors, this section expands the study to add results for comparison to the T2D Successful processes, the T2D Unsuccessful processes, and the Non-DM Unsuccessful processes. To be included in this study, T2D donors had to meet the first and then one of the next two criteria for islet processing: a) a clinical diagnosis of T2D prior to death with a HgbA1c value <10.0%, and b) T2D treatment by diet and exercise alone, or c) T2D treatment by diet, exercise, and oral diabetes medications. All other T2D donors who had taken insulin injections prior to their terminal hospitalization were excluded from processing since our previous experience in those donors has been that very few DTZ positive islets were ever recovered by pancreas processing and islet purification, especially in those with HgbA1c values of 10% or greater.

The processing details for these four groups are presented in Table 5. In terms of Successful processes for both the Non-DM and T2D donors, the processing details are very similar. The Cold Ischemia time for the Non-DM donors was 10.4±0.2 hours and 10.4±0.6 hours for the T2D donors ($p=0.463$). The Switch Times were 7.1±0.1 minutes for the Non-DM donors and 7.8±0.5 minutes for the T2D donors ($p=0.070$). The digested pancreas weight for the Non-DM donors was 70.7±1.5 grams and 68.5±4.7 grams for the T2D donors ($p=0.319$). The Percent Pancreas Processed for the Non-DM donors was 80.1±0.8 percent that was significantly higher than for the T2D donors at 74.2±2.9 percent ($p=0.027$). So, except for the lower percent of the pancreas processed for the T2D donors both the Successful Non-DM donors and the Successful T2D donors, the processing results were essentially the same. Processing Results for Unsuccessful Non-DM had longer Cold Ischemia times, Switch Times, Pancreas Digested in both Weights and Percent Processed than Successful Non-DM pancreases. Processing results for T2D pancreases processed showed longer Switch Times, but shorter Cold Ischemia Times, Pancreas Weights, and Percent Pancreas Processed than recorded for Non-T2D donors. A major challenge in processing T2D donor

pancreases under these acceptance criteria is that 17.0% of these T2D pancreases simply did not digest well with only 42% of their pancreases digesting. At this point of our experience of processing T2D pancreases, we are not able to predict which processes will fail with similar characteristics. Since we have limited our acceptance criteria to only processing T2D pancreases with HgA1c values <10.0%, the islets recovered from these donors perform rather well as shown below.

Table 5 Pancreas processing details for *HIR.*

Processing Details	Non-DM Donors Successful (n=226)	Non-DM Donors Unsuccessful (n=15)	T2D Donors Successful (n=29)	T2D Donors UnSuccessful (n=6)
Cold Ischemia (Hr)	10.4±0.2	12.3±0.9	10.4±0.6	9.1±1.3
Switch Time (min)	7.1±0.1	9.2±0.6	7.8±0.5	13.8±2.0
Pancreas Digested (Gm)	70.7±1.5	92.4±6.5	68.5±4.7	39.7±12.7
Percent Pancreas Processed (%)	80.1±0.8	86.4±2.1	74.2±2.9	42.1±7.5

Figure 4 repeats the Overall results from Non-DM donors shown in Figure 3, but now provides a side-by-side comparison with the same analyses for the results of pancreas processing into islets from T2D organ donors. These components of Figure 4A present the overall results from the Unsuccessful processes for both Non-DM and T2D donors shown as IEQ/Gm per pancreas process. Comparing the Successful Non-DM donor results to the Successful T2D donor results shows a significantly decreased islet yield only at the Post-Culture step for the Successful T2D donors. At the Pre-Purification step, the Non-DM donors yielded 6,942.7±201 IEQ/Gm while the T2D donors yielded 5,797.0±734 IEQ/Gm, that were not significantly different (p=0.073). At the Post-Purification step, the Non-DM donors yielded 5,484.5 ± 199 IEQ/Gm while the T2D donors yielded 4,387.8±866 IEQ/Gm, that were not significantly different (p=0.057). Yet, after islet culture, the Non-DM donors yielded 4,351 ±167 IEQ/Gm while the T2D donors yielded 3,323.1±423 IEQ/Gm that showed a significantly greater Post-Culture loss of IEQ/Gm of islets from T2D donors after islet culture (p=0.014).

Turning to the analyses of the T2D Unsuccessful processes, the Unsuccessful Non-DM donor results at the Pre-Purification level were at 53.3%, at the Post-Purification level were at 31.3%, and at the Post-Culture level were at 8.4% of the Successful Non-DM donor yields as IEQ/Gm. In contrast, the Unsuccessful T2D donor results were at the Pre-Purification level at only 2.0%, with the Post-Purification level and the Post-Culture level were so low that their numbers were not definable. Thus, most of the islets were lost in the Unsuccessful Non-DM donors during the purification and culture steps while most of the islets were lost in the Unsuccessful T2D donors during the digestion process prior to purification.

The Islet Index values are shown in Figure 4B from T2D donors that have been added to those from Non-DM donors as previously presented in Figure 3B. For the Non-DM donors, the Islet Indices for the Pre-Purification Group was 0.62±0.20 while the Islet Indices for the T2D donors was 0.68±0.6 that was not significantly different (p=0.182). For the Post-Purification Group, the Non-DM Islet Index was 0.57±0.02 with the T2D Islet Index was 0.56±0.06 that was not significantly

different (p=0.341). The Post-Culture Group Non-DM Islet Index was 0.78±0.02 while the T2D islet index was 0.69±0.07 that were not significantly different than the Non-DM islet indices (p=0.150).

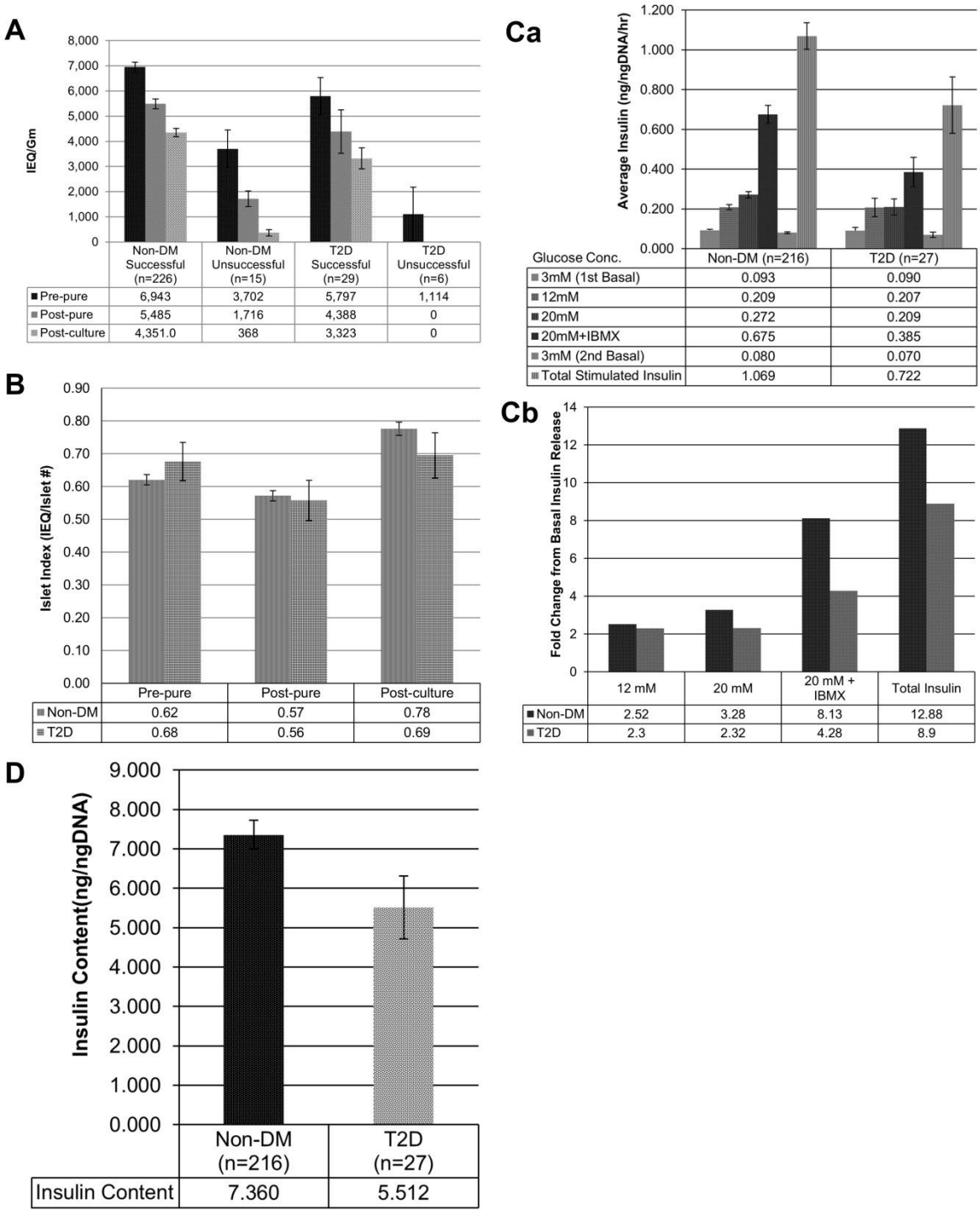


Figure 4 Comparing Non-DM and T2D donor islet processing. A: Islet yields as IEQ/Gm from Non-DM and T2D donors from successful and unsuccessful processes; B: Islet indices from both Non-DM and T2D donors; Ca: Islet GSIR from successful processes in Non-DM and T2D donors; Cb: Comparison of the fold change from basal insulin release by Non-DM islets and T2D islets; D: Total islet insulin content from Non-DM islets versus T2D islets.

Glucose stimulated insulin release (GSIR) assay results were presented in Figure 3E for the Non-DM donors and repeated here with the added T2D processed islets GSIR results in Figure 4Ca for ease of comparison. The Stimulated Insulin results are all reported as ng insulin/ng DNA/hr with the 3mM basal glucose stimulated 0.093±0.01 insulin for the Non-DM islets and 0.090±0.016 insulin for the T2D islets (p=0.872); the 12 mM glucose stimulated 0.209±0.012 insulin for the Non-DM islets and 0.207±0.046 insulin for the T2D islets (p=0.097); the 20mM glucose stimulated 0.272±0.016 insulin for the Non-DM islets and 0.209±0.041 insulin for the T2D islets (p=0.165), all of which are not significantly different. However, the 20mM glucose+0.10mM IBMX stimulated 0.675±0.045 insulin for the Non-DM islets that was significantly larger than the stimulated 0.385±0.074 insulin for the T2D islets (p<0.001). Then, the return to the 3 mM glucose stimulated 0.080±0.005 insulin for the Non-DM islets and 0.070±0.014 insulin for the T2D were not statistically different (p=0.484). It appears that islets from these T2D donors have significantly less ability to provide sufficient insulin release under elevated glucose levels.

The calculated Glucose Stimulation Indices (GSI) for each are also similar from these results. The 1st GSI for the Non-DM islets was 2.65±0.10 and for the T2D islets was 2.68±0.23 (p=0.463); the 2nd GSI for the Non-DM islets was 3.55±0.156 and for the T2D islets was 2.78±0.25 that is significantly lower (p=0.006); and the 3rd GSI for the Non-DM islet was 8.79±0.405 and for the T2D was 5.102±0.537 that is also significantly lower (p<0.001). So the T2D islets that were tested at the same times post-culture of 5-8 days functioned essentially the same as the Non-DM islets at the lowest glucose concentration (3, 12, and 20mM), but the Non-DM islets stimulated significantly more insulin release with 20mM glucose+10mM IBMX. Both the 2nd GSI and the 3rd GSI were significantly higher with the Non-DM than with the T2D GSIR's. The Total Stimulated Insulin expressed as ng insulin/ng DNA for each was 1.069±0.067 for Non-DM islets and 0.722±0.142 for T2D islets and were significantly different with (p=0.032). The protocol for this study was designed to only test by GSIR at the same 5-8 days post isolation and culture for these two groups. Whether the T2D stimulated insulin response could have been significantly less in glucose responsiveness immediately after islet processing rather than after a week of culture was not tested. The role of PIM(R)® culture medium that is designed to rejuvenate isolated islets was not specifically tested for there being a potential of its use to have rejuvenated expected significantly lower GSIR results for T2D donor islets. Thus, the T2D isolated islets only release insulin at the same level as the Non-DM islets after stimulation by low glucose levels of 12mM glucose. With the higher glucose stimulation levels, the T2D islets release significantly less insulin and are not able to store as much insulin compared to the Non-DM islets, as shown below in Figure 4D.

The reduced capability of T2D islets to release glucose stimulated insulin compared to Non-DM islets is further defined by a second evaluation. This is shown in Figure 4Cb by calculating the glucose stimulated Fold Increase in insulin release to these different GSIR stimulants. Compared to basal glucose levels, stimulating these islets by 12 mM Glucose resulted in an increased release of insulin for Non-DM islets by 2.52 Fold and for T2D islets by 2.30 Fold that were essentially the same. Increasing glucose stimulation to 20 mM increased the insulin release for Non-DM islets up to 3.28 Fold, but for the T2D islets, only a 2.32 Fold increase was observed that was essentially unchanged from the 12 mM glucose stimulation. Adding 0.1 mM IBMX to the 20 mM glucose levels increased the insulin release by Non-DM islets up to a 8.13 Fold increase but the T2D islets stimulated insulin release only increased to a 4.28 Fold increase. Evaluating the Fold increase in Total Stimulated insulin release to glucose challenge resulted in a 12.88 Fold increase for Non-DM

islets but only an 8.9 Fold increase in Total Stimulated insulin released by the T2D islets. Even though these T2D islets had been cultured in the PIM(R)® for 5-8 days after processing at the time of the GSIR testing, this major difference in capacity of glucose stimulated insulin release by T2D islets compared to Non-DM islets clearly presents a significant reduction in capability of T2D islets for glucose stimulated insulin release at the higher glucose concentrations.

As the GSIR functional insulin release was presented in Figure 4C, similarly the insulin content (Figure 4D) for the Non-DM islets was 7.360±0.365 ng insulin/ng DNA that was a significantly higher value of insulin content than in the T2D islets of 5.512±0.800 ng insulin/ng DNA (p=0.025). Thus, T2D islets remained with significantly lowered insulin content when compared to Non-DM islets after culture.

Effect of Donor Hemoglobin A1c Values. To potentially improve the understanding of the difference in T2D IEQ/Gm yields observed in the Successful T2D donors, we examined the potential effect of donor Hemoglobin A1c values on IEQ/Gm yields for all T2D donors (Figure 5A). Using the criteria for including T2D donors into this study meant that some of the T2D donors could have had HgbA1c levels of less the 6.5% due to their non-insulin treatments and others could have had levels >10.0% due to insufficient oral diabetes medication. Thus, the T2D donors were arbitrarily grouped by HgbA1c levels into three groups: <6.5%, from 6.5%-10.0%, and >10.0%. The results in Figure 4A appear to show a major effect on IEQ/Gm yields from pancreas processed from these three T2D groups based on the HgbA1c values. Yet, the small numbers of donors per group and the high SEM prevent the declaration of statistical difference to be made. Those T2D donors with HgbA1c values <6.5 % had very high islet yields on average of 8,453±3,143 IEQ/Gm pancreas processed initially with 6,155±1,578 IEQ/Gm remaining Post-Culture, compared to those with HgbA1c values from 6.5% to 10.0% that yielded only 5,353±499 IEQ/Gm initially which reduced to 2,895±265 IEQ/Gm Post-Culture. Those T2D donors with HgbA1c values of >10.0% yielded fewer islets at 4,933±1,289 IEQ/Gm initially and only 2,378±1,223 IEQ/Gm after culture. Attempting a linear regression analysis of hemoglobin A1c values versus the processing steps did not show statistical significance with Pre-Purification (p=0.480), Post-Purification (p=0.128), and Post-Culture (p= 0.143). Adding additional donors to these HgbA1c values from the T2D donor pool, may in the future demonstrate significant differences in islets yields. However, due to finding these low islet yields associated with high HgbA1c levels that were demonstrated during this study, even though not statistically significant, we have established new Exclusion Criteria for processing T2D donors where we have chosen to eliminate those T2D donors with Hemoglobin A1c levels >10.0% at the time of brain death.

GSIR stimulated insulin results are shown in Figure 5B comparing Successful Non-DM stimulated insulin release with those of Successful T2D islets from donors with different Hemoglobin A1c values. Compared to the Non-DM results, it appears the T2D donors with HgbA1c values <6.5% may be stimulated to secrete more insulin by GSIR than Non-DM donors. The Total Stimulated Insulin released from islets from Non-DM donors was 1.069±0.067 ng/ngDNA/hr while the T2D donors with HgbA1c levels <6.5% released total insulin levels of 1.412±0.667 ng/ngDNA/hr. The T2D donor group with HgbA1c levels of 6.5-10% released less total insulin at 0.510 ±0.104 ng insulin/ng DNA/Hr than the <6.5% HgbA1c group T2D islets. The >10% HgbA1c T2D group also released a low amount of total insulin in response to GSIR testing at 0.633±0.26 ng insulin/ng DNA/Hr. Linear regression analysis failed to show any significant difference by HgbA1c values with Total Stimulated Insulin Release (p=0.413). This study of HgbA1c effects will continue

adding more process results to determine if a significant difference can be achieved with additional testing from T2D islet donors.

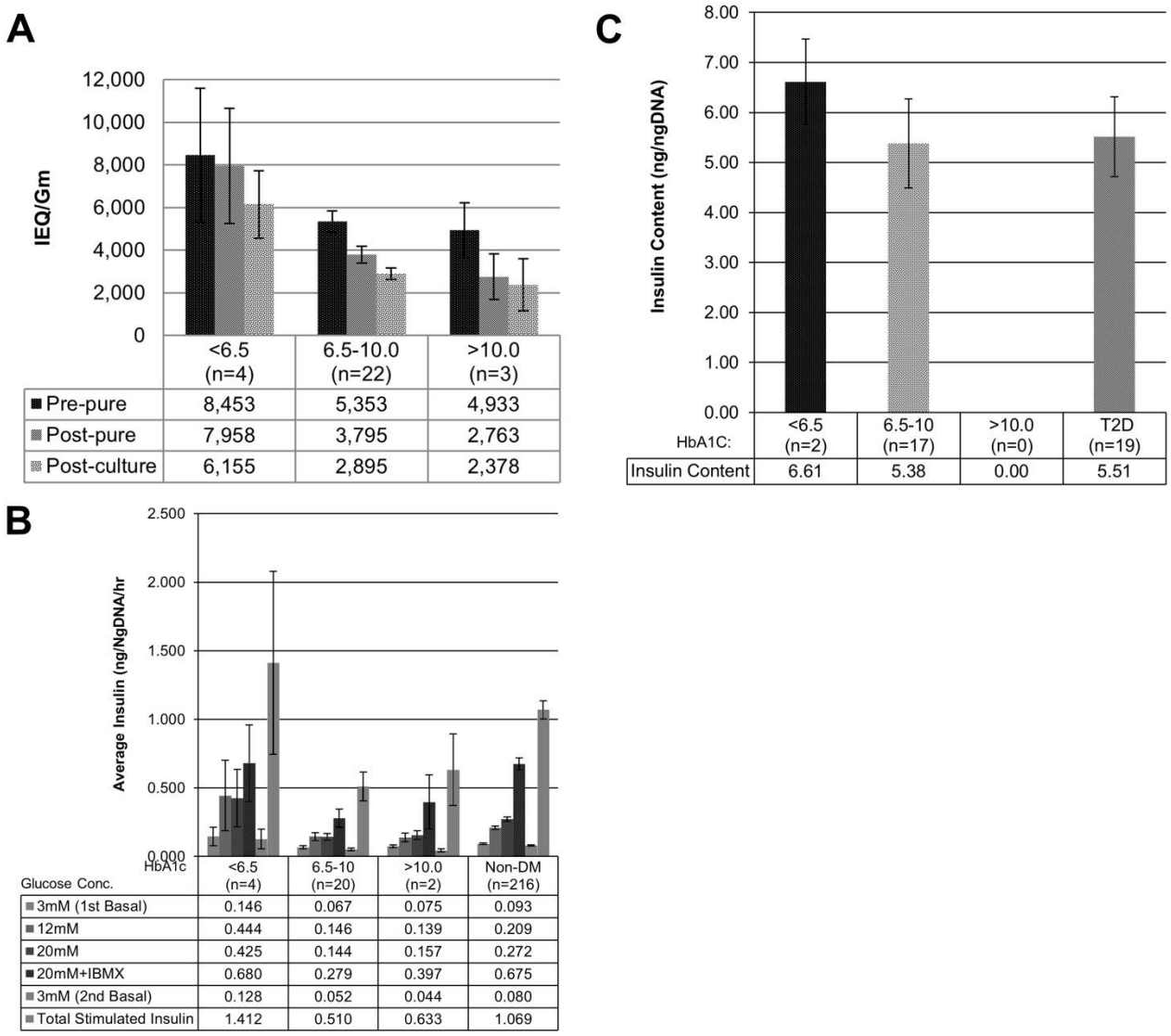


Figure 5 Effect of HgbA1c levels on T2D islet processing. A: T2D IEQ/Gm yields vs HgbA1c levels at time of donation; B: The effect of hemoglobin A1c levels on GSIR results; C: Total islet insulin content levels for T2D donors vs. hemoglobin A1c levels.

Evaluation of the islet insulin content for T2D donors is incomplete in this HgbA1c analysis since insulin content was not determined in all of 2011 T2D donors when some of these donors were processed into islets. Of the data available, Figure 5C shows there may be no difference in the insulin content of the majority of these donors with HgbA1c levels between 6.5% and 10.0%. There are only 2 of 3 T2D donors with HgbA1c values <6.5% with insulin content data available from their GSIR with those values being much higher at 6.61 ng insulin/ng DNA than the average. However, there were 17 T2D donors with HgbA1c values measured that had an average insulin content of 5.38±0.888 ng insulin/ng DNA. Unfortunately, none of the three T2D donors with HgbA1c levels >10.0% levels had insulin content measured for comparison. So additional studies of the T2D islets are required to collect additional results for future analysis.

Recent Evidence of Reversible and Irreversible Human Beta Cell Destruction from Fatty Acid Exposure Related to Mechanisms of T2D. There is clear evidence in the literature (70-75), that human beta cell early exposure to high levels of fatty acids, initially increase insulin secretion. But, more chronic exposure to fatty acids reduce the beta cell's ability to function normally and if prolonged will lead to the loss of islets and their function resulting in T2D islets. The very recent publication by Sargsyan et al [70] utilizing normal human islets from Prodo showed that acute exposure to high concentrations of fatty acids initially increased insulin secretion while chronic exposure led to induction of early T2D responses from these islets from Non-DM donors. Others had suggested this type of fatty acid exposure would lead to human beta cell dysfunction [71-75], but this latest paper is focused on understanding the early mechanisms involved so that one can possibly identify the earliest changes to prevent further islet disorder and loss of function in those early T2D patients. The very premature results in this publication suggest organ donors with a history of T2D with HgbA1c levels <6.5% have increased glucose stimulated insulin release (GSIR) of 1.4 ng insulin/ng DNA/Hr (Non-DM 1.1 ng insulin/ng DNA/Hr and T2D average of 0.5 ng insulin/ ng DNA/Hr) and insulin content of 6.6 ng insulin/ng DNA (Non-DM 7.4 ng insulin/ ng DNA with T2D average of 5.5 ng insulin/ ng DNA). With Prodo's commitment to continue to supply islets from T2D donors as well as from Non-DM donors for research, it is hoped that additional investigators will take advantage of the availability of these types of islets to further the advancement of T2D understanding that can lead to improved treatments.

Effect of Tissue Culture Media on Long-Term Islet Function. This portion of the study was designed to place human islets under the effect of different islet culture media for the purpose of defining what types of islet culture media would be optimal for the potential use in maintaining human islets with *in vitro* functional survival prior to islet allograft implantation [66]. Since there currently is not a direct comparison of these reagents for islet culture in the literature, this study was designed with the outcomes added to this report. The traditional clinical method of maintaining islets in tissue culture prior to islet allograft transplantation into patients is by the use of culture medium CMRL-1066 with 10% human serum albumen (HuSA) and 260ul of Lisofylline that was called the original "CIT Culture Medium" [44]. But, the latest "CIT Culture Medium" since 2016 [3, 4] replaced lysofilline with Insulin Growth Factor-1 that is a primary mediator of growth hormone. It needs to be clear that the currently published clinical protocol use of CIT Culture Medium only cultures high purity islets at 37^{o}C for 12-24 hours then places them at 22^{o}C until the islets are implanted which is usually within a few days [3, 4]. The middle and the low purity islets are never cultured at 37^{o}C and are only cultured in this culture medium at 22^{o}C for the same time period. To compare the results of using this current "CIT Culture Medium" to other media combinations, we processed human islets from a single donor and placed them into 3 separate aliquots of culture medium: a) PIM(R)$^{®}$ - Prodo Recovery Culture Medium supplemented with PIM(ABS)$^{®}$ (human AB serum) + PIM(G)$^{®}$ (glutathione and glutamine), b) CMRL-1066 + PIM(ABS)$^{®}$, and c) CMRL-1066 + HuSA (human serum albumen), also called the "CIT Culture Medium" [3, 4]. It also should be noted that PIM(R)$^{®}$ with PIM(ABS)$^{®}$ and PIM(G)$^{®}$ actually promote acinar cells to survive along with the islets in 37^{o}C tissue culture rather than the observed CIT medium's acinar cell damage that promotes acinar cell death in the first 48 hours of tissue culture. All islet cultures were maintained at 37^{o}C for the 4 weeks with culture media changed at the 50% level every 3-4 days. For each of the 4 weeks of culture, human islets were taken from the flasks and subjected to GSIR [63] with a protocol that included initial 3mM glucose control media change twice, and then

exposure to increasing glucose stimulation every hour using 3mM glucose as basal, then 15mM glucose, then 28mM, then 28mM + 0.1mM IBMX, and followed by basal 3mM glucose. A Total Stimulated Insulin Release was calculated for each culture media tested.

The first week's results are shown in Figure 6A that demonstrates significantly different insulin responses to the increasing glucose concentrations over the basal levels (2-tailed T tests) for the islets kept in the three different culture medium. For the first week results, the PIM(R)® media results were significantly greater than the CMRL+HuSA ($p=0.025$) but not CMRL+PIM(ABS)® ($p=0.084$). Also, the islets cultured in CMRL+PIM(ABS)® insulin release were greater than those cultured in CMRL+HuSA ($p<0.001$). For the first week, the Total Stimulated Insulin Release as ng insulin/ng DNA/Hr for PIM(R)® was 2.236±0.738, for CMRL+PIM(ABS)® was 0.765±0.104, and for CMRL+HuSA was 1.319±0.043.

The second week results of GSIR testing presented in Figure 6B showed the maximal stimulation of 28mM glucose + IBMX for PIM(R)® was 0.942 ngInsulin/ngDNA/Hr, for CMRL+ABS was 0.217 ngInsulin/ngDNA/Hr, and for CMRL+HuSA was 0.188 ngInsulin/ngDNA/Hr. Total Insulin Stimulation Release for the islets kept in PIM(R)® had significantly greater insulin release over CMRL+PIM(ABS)® ($p=0.024$) and also had significantly greater insulin release over CMRL+HuSA ($p=0.008$).

For the third week of this study, the maximal stimulation by 28mM glucose + IBMX was 0.585 ngInsulin/ngDNA/Hr for PIM(R)®, 0.147 ngInsulin/ngDNA/Hr for CMRL+PIM(ABS)®, and 0.061 ngInsulin/ngDNA/Hr for CMRL+HuSA. GSIR testing presented in Figure 6C showed the Total Insulin Stimulation Release for the islets kept in PIM(R)® had significantly greater insulin release over CMRL+PIM(ABS)® ($p=0.022$) and also had significantly greater insulin release over CMRL+HuSA ($p=0.009$). In addition, CMRL+PIM(ABS)® had significantly greater insulin release over CMRL+HuSA ($p=0.002$).

For the final fourth week of this study, maximal insulin release from PIM(R)® was 0.649 ngInsulin/ngDNA/Hr, from CMRL+PIM(ABS)® was 0.152 ngInsulin/ngDNA/Hr, and from CMRL+HuSA was 0.042 ngInsulin/ngDNA/Hr. GSIR testing presented in Figure 6D showed the Total Insulin Stimulation Release for the islets kept in PIM(R)® did have significantly greater insulin release over CMRL+PIM(ABS)® ($p=0.050$) and did have significantly greater insulin release over CMRL+HuSA ($p=0.007$). In addition, CMRL+PIM(ABS)® had significantly greater insulin release over CMRL+HuSA ($p<0.001$) for the first week, the second week ($p=0.016$), and the third week ($p=0.002$), but not the fourth week ($p=0.262$).

The superiority of the PIM(R)® tissue culture group on islet function at all observed times is confirmed by recording the total insulin released by islets each week of culture. It should be noted that the "CIT Culture Medium" gave the lowest ability to produce an insulin response after the first week of culture of the three media and had a significantly lower total insulin response than the PIM(R)® culture medium at week one. Thus, on a functional basis, the PIM(R)® culture medium was optimal over the two CMRL media combinations at all times tested after 1 through 4 weeks of islet culture (Figure 6E).

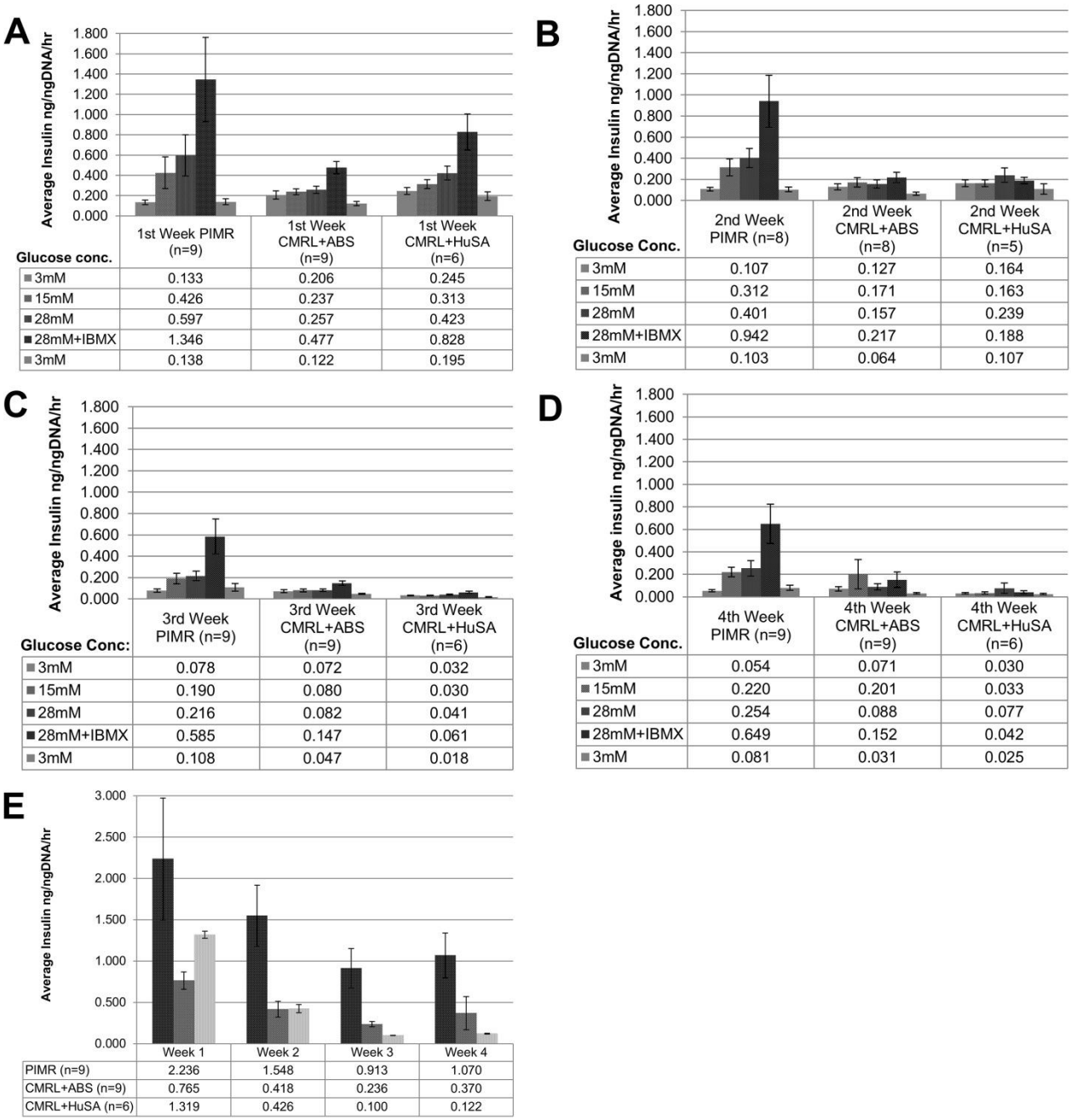


Figure 6 Effect of islet culture media on long term islet function. A: First Week GSIR results of long term cultured human islets; B: Second week GSIR results of long term cultured human islets; C: Third week GSIR results of long term cultured human islets; D: Fourth week GSIR results of long term cultured human islets; E: Total stimulated insulin release by GSIR.

It should be noted that the ongoing clinical choice of CMRL 1066 + HuSA, "CIT Culture Medium", is continuing to be favored as the primary culture medium for definitive clinical human islet transplantation trials [3, 4], with other improved media choices readily available today. As shown in Table 6, the CMRL 1066 medium was developed in 1960 at Connaught Medical Research Laboratories with its primary use for mouse L-cells as a less complex culture medium modified from Medium 199. Medium 199 was formulated in 1950 and is considered to have had a variety of

cell types responding to its formulation in culture. Medium RPMI 1640 is even more primitive and was developed in 1966 for the culture of white blood cells, but requires increased Ca^{++} ion addition for *in vitro* human islet culture and function. While early islet culture studies began with CMRL 1066, many studies since have shown other media to be optimal. Yet, it still remains the current choice for the CIT studies forcing modified culture methods for its use such as decreasing cell culture temperatures to room temperature in order to prolong islet *in vitro* survival. Prodo developed PIM(R)® in 2006 as a proprietary, human islet specific culture medium since the previous three culture media when studied for human islets were all unable to sustain meaningful cultured human islet qualities for many days, let alone weeks, as shown in Figure 6, above. These PIM islet culture media products are continuing to be distributed throughout the islet research community since their introduction. The proprietary PIM(R)® islet culture media formulation has been submitted to the FDA as a Biological Master File. The general components of these different media being used for islet culture are presented in Table 6. It is clear that the PIM(R)® culture medium with >85 components has the largest number of individual components than any of the other three culture media that have been used for islets and readily maintains human islet survival and function for several weeks in tissue culture at 37°C as shown above. While the PIM(R)® medium can be used for islet culture prior to implantation for clinical trials, it has not been approved as the culture medium used to actually inject the islets into patients with diabetes.

Table 6 Commonly used human islet culture media.

Media	Year Developed	Number of Components[#]	Inorganic Salts	Amino Acids	Vitamins	Other
Medium 199	1950	60	7	21	17	15
CMRL 1066	1960	58	7	21	9	21
RPMI 1640	1966	42	6	20	11	5
*PIM(R)®	2006	>85	>10	>20	>15	>35

[#] Components of culture media only, not including serum additive or antibiotics

* PIM(R)® has a submitted Biological Master File with the FDA defining and justifying the proprietary human islet culture components.

3.2 Processing Variables for Optimal Islet Yields from Non-DM Donors

3.2.1 Donor Age

There are some significant differences in Islet Yields based on Donor Age in this study as shown in Figure 7A. In the Pre-Purification group of islets, there does not seem to be any major difference in islets released between the arbitrary 6 different age groups (18-19 years, 20-29 years, 30-39 years, 40-49 years, 50-59 years, and 60-69 years). This was confirmed by Linear Regression Analysis of the Pre-Purification group (p=0.920). Examining Islets IEQ/Gm recovered Post-Purification, there is clearly a significant difference of Islet IEQ/Gm by age differences. There were significantly less IEQ/Gm islets recovered after purification in the 18-19 age (4,192±757 IEQ/Gm)

and the 20-29 age groups (4,486±305 IEQ/Gm) than any of the other age groups (5,392±360 IEQ/Gm (30-39 years) to 6,721±804 IEQ/Gm (60-69 years). Statistical Analysis for the Post-Purification group by Linear Regression Analysis showed a significant positive correlation as a linear trend for increasing age improving islet yields ($p<0.001$). The Post-Culture group showed similar results as the Post-Purification group regarding age differences with the 18-19 age (3,389±768 IEQ/Gm) and the 20-29 age (3,587±243 IEQ/Gm) groups at the lowest yields with progressively higher yields out to the 60-69 age (5,571±625 IEQ/Gm). The Post-Culture islet yields as IEQ/Gm were increased significantly on this Post-Culture group ($p<0.001$).

Thus, while Pre-Purification results based on age were not significantly different, the Post-Purification and the Post-Culture islet yields clearly show a significant increase in islet yields with increasing age. Linear regression analysis comparing donor age and percent of embedded islets implies a significant decrease in islets embeddedness as age increases ($p<0.001$) Thus, the primary reason for the lower Post-Purification and Post-Culture IEQ/Gm yields and the higher islet loss in the 18-19 and the 20-29 age groups was that these younger donors had a higher incidence of embedded islets prior to islet purification as shown in Figure 7Ba and 7Bb.

Figure 7Ba shows the percent of embedded islets in the 18-19 year old group was 28% and in the 20-29 year old group was 24%. The next three age groups, 30-39 years, 40-49 years, and 50-59 years, respectively, had a range of embedded islets of 16.5% to 15.6%. The oldest group, 60-69 years had the lowest degree of embedded islets at 7.9%. Linear Regression Analysis showed younger donors have higher levels of islet embeddedness than older donors ($p<0.001$). When islets remain embedded, they become entrapped on lower levels in the purification gradients since they are held further down into the gradient by the attached, more dense, acinar tissue. Including these embedded islets in the final preparation for research would result in unacceptably low islet purities. Anecdotal experience with organ donors aged 15-18 show embedded islets up to 50% and higher, making islet isolation with duct distension very inefficient for these young donors. A possible explanation for this finding is that the pancreatic duct tissue in young donors appears to be too weak to hold the pressure and activity of the injected enzymes and leaks the enzymes out of the ducts prior to their effectively reaching the acinar/islet interface. The older donors' pancreatic ducts appear stronger and hold the injected ductal pressure longer, permitting greater separation of islets at the islet/acinar interface, resulting in significantly higher islet yields and islet purity due to the loss of acinar cell surface embedding of islets.

An additional evaluation of Age effects on islet yields is to document the percent of the islet losses at these different ages as shown in Figure 7Bb. The data were calculated by dividing the following groups: the Purification Loss as the Post-Pure islet loss divided by the Pre-Pure value, the Post-Culture islet loss divided by the Post-Pure value, and the addition of these two losses for the Total percent loss of islets. Focusing on the Total % Loss, and ignoring the 18-19 age group (n=3), the 20-29 age group lost 56.9% of the islets processed due to islet embeddedness and purification while the 30-39 age group lost 46.2% of the islets lost due to islet embeddedness and purification. The next age groups had reduced embeddedness loss with the 40-49 age group total loss of 35.6%, the 50-59 age group total loss of 34.2%, and the 60-69 age group total loss of 24.8% loss. The Purification percent Losses were fairly constant across all the age groups ranging from 19.9% loss to 21.8% loss. Thus, the primary loss of islets based on age is the problem of islet embeddedness that occurs in the younger age groups. Finding a way to eliminate islet embeddedness during the pancreas processing and islet purification in the younger donors would provide significantly

increased numbers of viable islets available for both transplantation as well as research efforts. However, eliminating embeddedness in younger pancreas donors has not yet been achieved and should become an important research target.

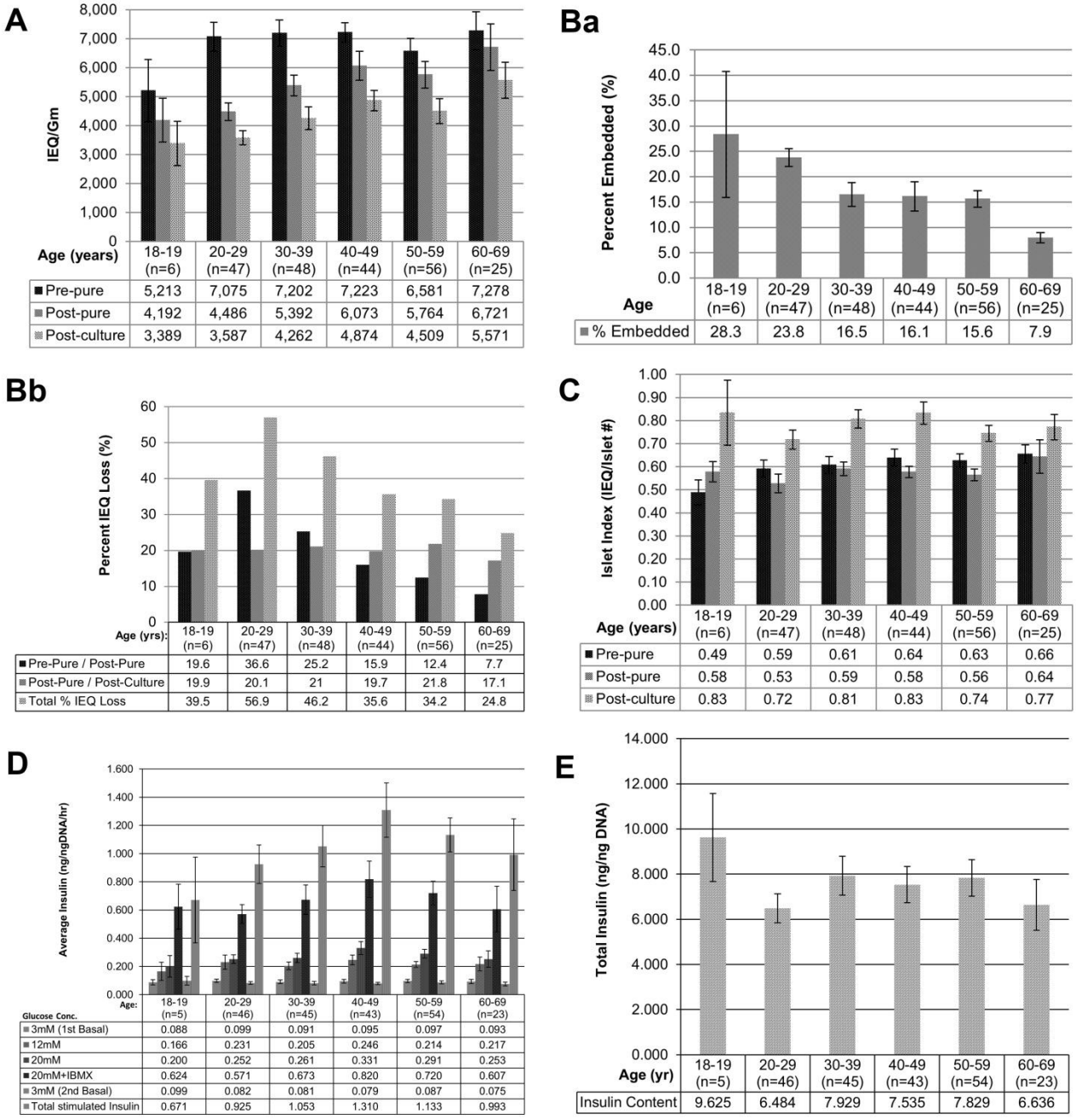


Figure 7 Effect of donor age on human islet processing. A: The effect of donor age on islet yields as IEQ/Gm; Ba: The degree of embedded human islets post-purification by age; Bb: Percent islet losses at the three digestion steps based on age; C: The effect of donor age on islet indices; D: The effect of donor age on GSIR; E: The effect of donor age on islet insulin content.

The effect of donor age on Islet Index seems to show a trend of higher islet indices after tissue culture than after islet purification in Figure 7C, as has been observed several times in this report

to be a probable effect of culturing islets in PIM(R)®. Comparing the Pre-Purification Islet Indices with the Post-Culture Islet Indices shows the 18-19 year old group Islet Index was 0.49±0.05 to 0.83±0.14, the 20-29 year old group was 0.59±0.04 to 0.72±0.04, the 30-39 year old group was 0.61±0.04 to 0.81±0.04, the 40-49 year old group was 0.64±0.04 to 0.83±0.05, the 50-59 year old group was 0.63±0.03 to 0.74±0.03, and the 60-69 year old group was 0.66±0.004 to 0.77±0.06. The Linear regression on donor age associated with Islet Indices of Pre-Purification confirms Islet Index were not significantly different (p=0.958). As previously discussed, the Post-Culture Islet Index increases observed may be explained by the effect of the supplemented PIM(R)® culture medium on small islets and islet fragments that make then tend to stick together while recovering from islet processing damage, that can result in a higher Islet Index after culture. Uniquely, the 18-19 age group, has an exaggerated finding. The Islet Index for these donors is lower than any other group in the Pre-Purification at 0.49±0.05 while the Post-Culture Islet Index is one of the highest at 0.83±0.14. This suggests these islets from younger donors may be more likely to aggregate together to form larger islet aggregates in culture with the supplemented islet culture medium than the older age groups after islet processing. This interesting observation needs further evaluation to statistically confirm this finding and determine the reasons for any increased aggregation activity in young donor islets.

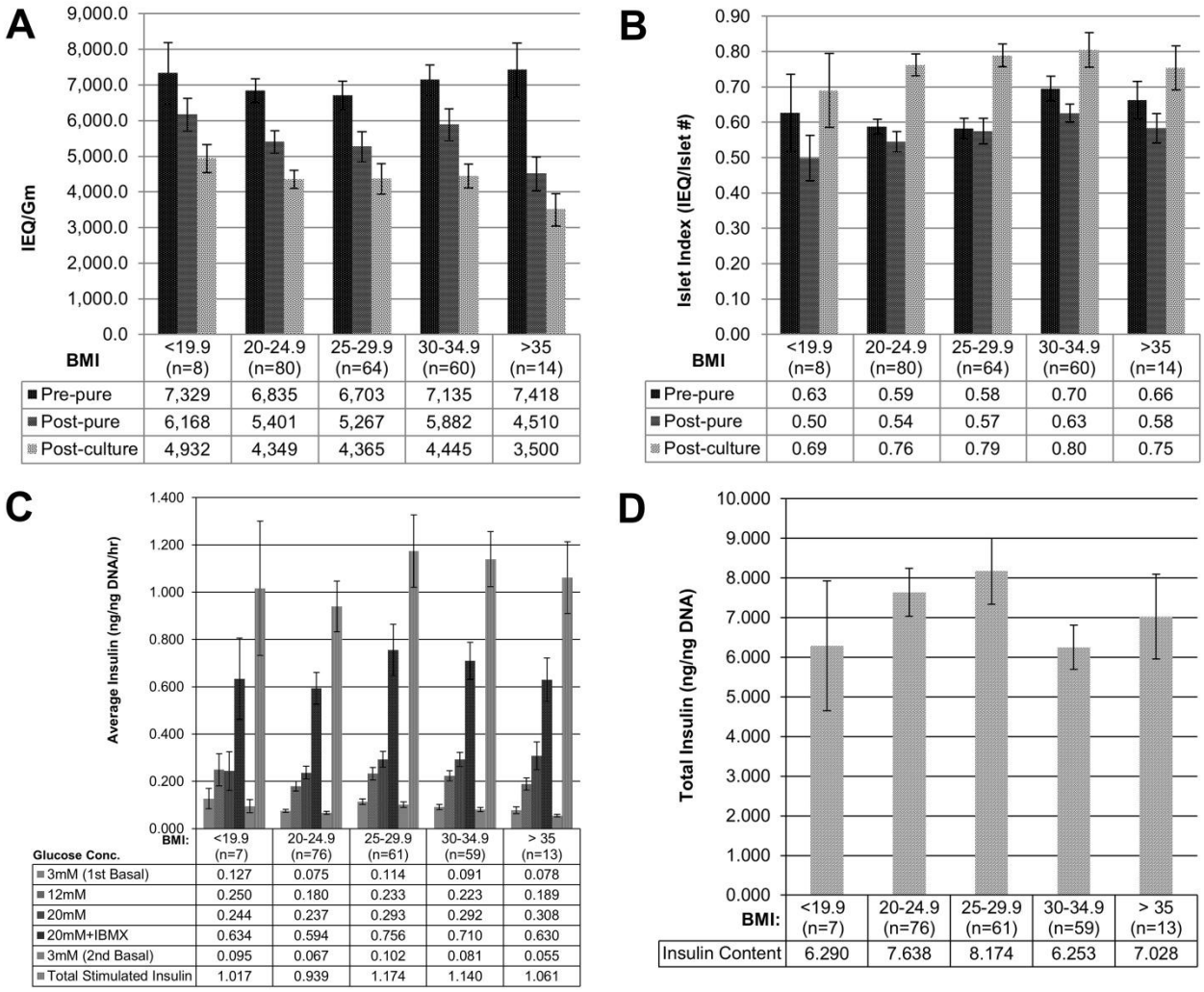
It appears that all of the Non-DM islets processed and cultured from different age groups demonstrate similar GSIR results regardless of donor age, as shown in Figure 7D. The highest three Total Stimulated Insulin Release results were recorded in the 40-49 year old group (1.31±0.19 ng insulin/ngDNA/Hr), the 50-59 year old group(1.133±0.12), and the 30-39 year old group (1.053±0.15). While the 60-69 year old and the 18-19 year old and the 20-29 year old had lower Total Stimulated Insulin Release results, the Linear regression of the Total Stimulated Insulin Release in terms of age were not significantly different (p=0.275). So islets successfully processed and cultured show clear ability to respond to glucose stimulated insulin release regardless of donor age.

As shown in Figure 7E, there is essentially no significant difference in insulin content based on age with a range from 6.484±0.64 to 9.625±1.95 ng/ng DNA shown by regression analysis (p=0.275). While the 18-19 year old group (n=5) had the highest value (9.625±1.95 ng/ngDNA) than any of the other ages, its significance could not be determined due to the small sample size.

3.2.2 Donor Body Mass Index (BMI)

In addition to Donor Age, potential BMI effects on human islets may make it a candidate as a major variable in islet processing. Most islet processing groups believe that the larger the donor BMI, the larger the islets will be recovered and the greater the islet yield. Unlike many clinical islet implant studies, we elected in this study to not process islets from any donors with BMI >40 since our previous studies had demonstrated that these islets were significantly damaged during digestion with larger losses Post-Purification. These increased losses seem to be the result of the increased pancreatic fat content which when released during the process may lead to a more toxic preparation from free fatty acids potentially reducing islet integrity and viability. There appears to be evidence in this study's donor group with the largest BMI group (>35) that the IEQ/Gm yields are less after purification and after islet culture compared to the other arbitrary BMI groups, as shown in Figure 8A. The Pre-Purification IEQ/Gm (7,417.6±765) compared to the Post-Culture

IEQ/Gm (3,499.8±453) values for the BMI>35 group IEQ/Gm are the lowest of the BMI groups. The three middle BMI Groups (20.0-24.9, 25.0-29.9, and 30.0-34.9) Post-Culture Islet quantities are respectively 4,349.0±254, 4,365.4±425, and 4,445.1±336 IEQ/Gm with the <19.9 BMI group at 4,932.5±395 IEQ/Gm. The linear Regression Analysis of BMI and Islet Yield show a negative correlation, but it is not significant.



BMI	<19.9 (n=8)	20-24.9 (n=80)	25-29.9 (n=64)	30-34.9 (n=60)	>35 (n=14)
Pre-pure	7,329	6,835	6,703	7,135	7,418
Post-pure	6,168	5,401	5,267	5,882	4,510
Post-culture	4,932	4,349	4,365	4,445	3,500

BMI	<19.9 (n=8)	20-24.9 (n=80)	25-29.9 (n=64)	30-34.9 (n=60)	>35 (n=14)
Pre-pure	0.63	0.59	0.58	0.70	0.66
Post-pure	0.50	0.54	0.57	0.63	0.58
Post-culture	0.69	0.76	0.79	0.80	0.75

Glucose Conc. / BMI:	<19.9 (n=7)	20-24.9 (n=76)	25-29.9 (n=61)	30-34.9 (n=59)	> 35 (n=13)
3mM (1st Basal)	0.127	0.075	0.114	0.091	0.078
12mM	0.250	0.180	0.233	0.223	0.189
20mM	0.244	0.237	0.293	0.292	0.308
20mM+IBMX	0.634	0.594	0.756	0.710	0.630
3mM (2nd Basal)	0.095	0.067	0.102	0.081	0.055
Total Stimulated Insulin	1.017	0.939	1.174	1.140	1.061

BMI:	<19.9 (n=7)	20-24.9 (n=76)	25-29.9 (n=61)	30-34.9 (n=59)	> 35 (n=13)
Insulin Content	6.290	7.638	8.174	6.253	7.028

Figure 8 Effect of BMI on islet processing. A: The effect of BMI on islet yields as IEQ/Gm; B: The effect of BMI on islet indices; C: Effect of BMI on GSIR; D: The effect of BMI on islet insulin content.

Experienced investigators would expect the Islet Indices to go up with increasing BMI values. But, since this study restricted pancreas donors to have BMI's of <40, the effects of larger BMI's up to 50 or higher islet mass were not evaluated. Therefore, according to Linear Regression Analysis, the Post-Culture Islet Index and the BMI do not have a positive correlation (p=0.290) when restricting the BMI range to <40 (Figure 8B).

There is not any significant effect of BMI on GSIR results since islets from every BMI group appear to have a similar response, as shown in Figure 8C. Focusing on the Total Stimulated Insulin Release in these different BMI groups, one observes that the <19.9 BMI group had 1.017±0.116 ng insulin/ ng DNA, the 20-24.9 BMI group had 0.939±0.107 ng insulin/ng DNA, the 25-29.9 BMI

group had 1.174±0.154 ng insulin/ng DNA, the 30-34.9 group had 1.140±0.116 ng insulin/ng DNA, while the >35 BMI group had 1.061±0.152 ng insulin/ng DNA. Regression analysis on the Total Stimulated Insulin Release effect by BMI confirmed the lack of any significant differences (p=0.215).

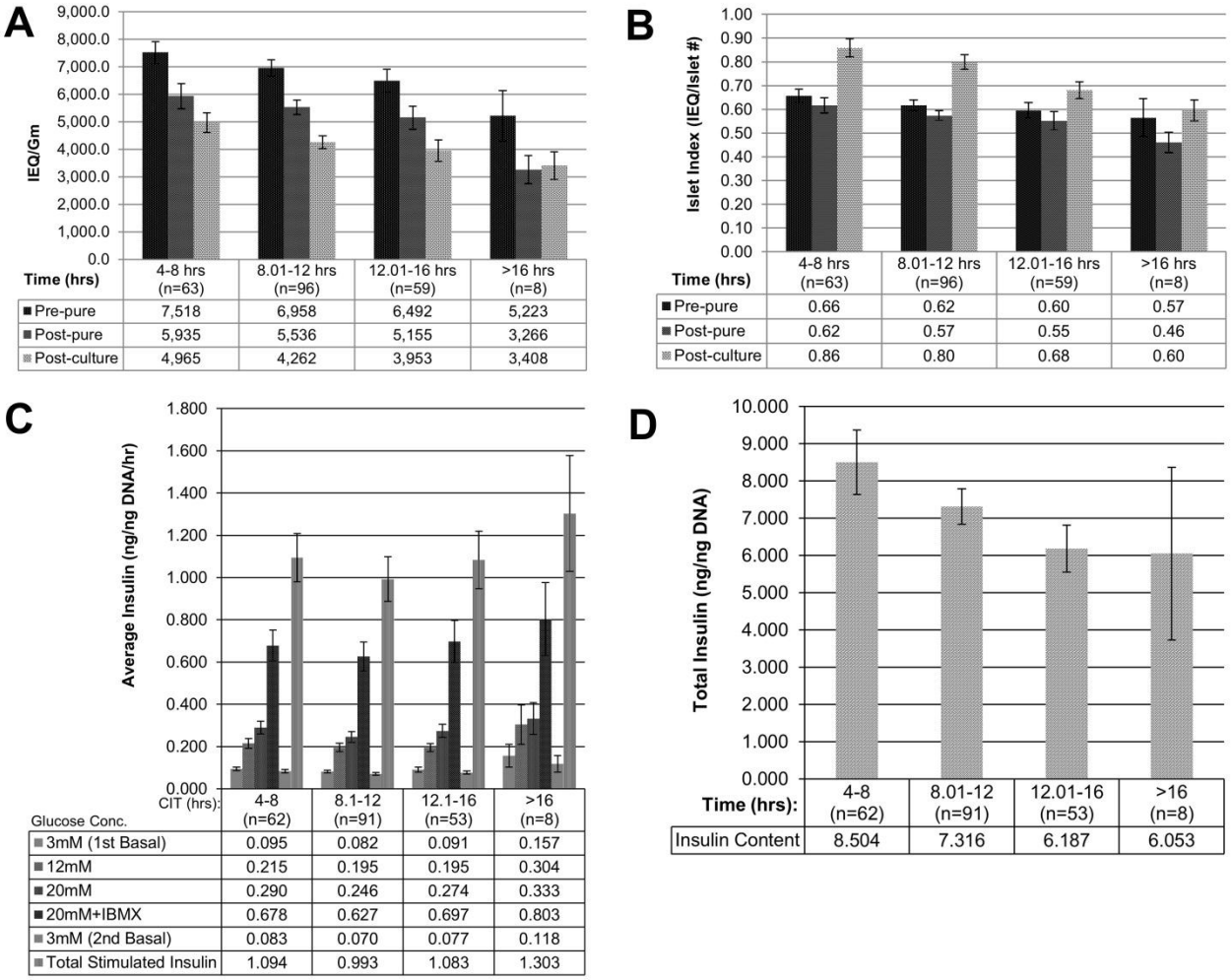
With the GSIR results of the different BMI groups not showing any significant difference, neither were there any significant differences of BMI on Insulin Content as shown in Figure 8D. For the <19.9 BMI group, the insulin content was 6.290±1.63 ng/ng DNA, for 20.0 to 24.9 group the content was 7.638±1.067, for the 25.0-29.9 group the content was 8.174±834, for the 30.0-34.9 group the content was 6.253±0.562, and for the 35.0-39.9 group the content was 7.028±1.067 ng/ng DNA (p=0.312).

3.2.3 Pancreas Cold Ischemia Time (CIT)

The pancreas CIT starts with the aortic cross clamp in the donor and ends with the first injection of collagenase into the pancreatic duct. The general consensus within the islet processing centers is that longer cold ischemia times, especially beyond 12 hours, are quite detrimental to successful islet isolations. Our previous publication in 2010 (n=21 donors) with relatively high islet yields for *HIR* was published with an average of 14 hours of CIT (1). This longer duration was due to our having at that time, limited access to local pancreases to process with lower cold ischemia times. Now at the time of this study, our average cold ischemia has significantly dropped since we have increased the number of pancreases procured predominantly from local centers (2016, n=48 donors), Table 2). Figure 9A shows the results with 4 arbitrary groups of CIT: 4-8 hours, 8.1-12 hours, 12.1-16 hours, and >16 hours. The Pre-Purification islet yields show a progressive and significant drop from 4-8 hours (7,517.8±394 IEQ/Gm), to the 8.1-12 hour group (6,957.9±298 IEQ/Gm), to the 12.1 – 16 hour group (6,491.9±422 IEQ/Gm) and to the >16 hour group (5,233±919 IEQ/gm) by Linear Regression (p=0.032). Similarly, the Post-Purification islet yields also show a significant drop from 4-8 hours CIT (5,934.6±456 IEQ/Gm), to the 8.1-12 hours CIT (5,536.3±259 IEQ/Gm, to the 12.1-16 hours CIT (5,154.5± 417 IEQ/Gm), and to the >16 hours CIT (3,266± 505 IEQ/Gm) by Linear Regression (p=0.022). Additionally, the Post-Culture islet yields show a significant drop from 4-8 hours CIT (4,964.8±359 IEQ/Gm), to the 8.1-12 hours CIT (4,262.3±236 IEQ/Gm), to the 12.1-16 hours CIT (3,953.4±304 IEQ/Gm), to the >16 hours CIT (3,407.7±500 IEQ/Gm) by Linear Regression (p=0.018). Statistical analysis showed that the islet yields Post-Culture from 4-8 hours were statistically significantly greater than the 8.1-12 hours yields (p=0.040), were greater than the 12.1-16 hours yields (p=0.024), and were greater than the >16 hour yields (p=0.012). So regardless of the amount of increasing CIT >4-8 hours, islet yields from Pre-Purification, Post-Purification, and Post-Culture all dropped significantly over the 4 consecutive 4 hour groups to >16 hours CIT.

The CIT results in this study from 4-8 hours out to > 16 hours on Post-Culture Islet Indices showed a significant inverse relationship with time by Linear Regression (p<0.001) as shown in Figure 9B. The Pre-Purification Islet Indices for 4-8 hours was 0.66±0.117, for 8.1-12 hours was 0.62±0.02, for 12.1-16 hours was 0.60±0.03, and for > 16 hours cold ischemia time was 0.57±0.08 and was not significantly different by Linear Regression (p=0.117). For the Post-Purification Islet Indices, the 4-8 hours was 0.62±0.03, the 8.1-12 hours was 0.57±0.02, the 12.1-16 hours was 0.55±0.04, and the >16 hour Islet Index of 0.46±0.04 that also was not significantly different by

Linear Regression (p=0.078). Yet, the Post-Culture results of Islet Indices for 4-8 hours Islet Index were 0.86±0.04, the 8.1-12 hours was 0.80±0.03, the 12.1-16 hours was 0.68±0.03, and the >16 hour Islet Index was 0.60±0.04 and by Linear Regression was significantly different (p<0.001). There was a significant trend for decreasing Islet Indices with increasing CIT at the Post-Culture level of processing which again suggests ongoing islet damage occuring during these longer cold ischemia times. Again, the Post-Culture Islet Indices were increased throughout the post-culture, when compared to Pre-Purification and Post-Purification results, presumbably from the use of PIM(R)® Culture Medium.



Time (hrs)	4-8 hrs (n=63)	8.01-12 hrs (n=96)	12.01-16 hrs (n=59)	>16 hrs (n=8)
Pre-pure	7,518	6,958	6,492	5,223
Post-pure	5,935	5,536	5,155	3,266
Post-culture	4,965	4,262	3,953	3,408

Time (hrs)	4-8 hrs (n=63)	8.01-12 hrs (n=96)	12.01-16 hrs (n=59)	>16 hrs (n=8)
Pre-pure	0.66	0.62	0.60	0.57
Post-pure	0.62	0.57	0.55	0.46
Post-culture	0.86	0.80	0.68	0.60

CIT (hrs): Glucose Conc.	4-8 (n=62)	8.1-12 (n=91)	12.1-16 (n=53)	>16 (n=8)
3mM (1st Basal)	0.095	0.082	0.091	0.157
12mM	0.215	0.195	0.195	0.304
20mM	0.290	0.246	0.274	0.333
20mM+IBMX	0.678	0.627	0.697	0.803
3mM (2nd Basal)	0.083	0.070	0.077	0.118
Total Stimulated Insulin	1.094	0.993	1.083	1.303

Time (hrs):	4-8 (n=62)	8.01-12 (n=91)	12.01-16 (n=53)	>16 (n=8)
Insulin Content	8.504	7.316	6.187	6.053

Figure 9 Effect of CIT on islet processing. A: The Effect of CIT on islet yield as IEQ/Gm; B: The effect of CIT on islet indices; C: The effect of CIT on GSIR; D: The effect of CIT on islet insulin content.

The GSIR results versus the different CIT have similar functional values regardless of the duration as shown in Figure 9C. The Total Stimulated Insulin values (ng insulin/ng DNA/Hr) for 4-8 hrs was 1.094±0.115, for 8.1-12 hours was 0.993 ±0.106, for 12.1-16 hours was 1.083 ±0.136, and for > 16 hours was 1.303±0.274 with no trend with respects to CIT according to Linear Regression analysis (p=0.349). This suggests that whatever effect the CIT may have had on the islets to reduce their Post-Culture islet function, the islets that survived the processes were essentially capable of

normal function after 5-8 days of islet culture with little change in Total Stimulated Insulin release post-culture using PIM® culture medium.

The Islet Insulin Content for different CIT are shown in Figure 9D. The 4-8 hour and the 8.1-12 hour Cold Ischemia Groups had the highest Insulin Content levels at 8.504±0.860 ng Insulin/ ng DNA and at 7.316±0.478 ng Insulin/ng DNA, respectively. The 12.1-16 hours group dropped to 6.187±0.629 ng/ng DNA with the >16 hour group with the lowest Insulin Content at 6.053±2.319 ng insulin/ ng DNA. The Linear Regression analysis showed a significant decrease of insulin content by these increasing CIT ($p=0.021$). While the GSIR results were not significantly affected by increasing CIT, the islet insulin content was progressively and significantly decreased following increasing cold ischemia times.

3.2.4 Process Switch Time from Recycling to Collection Modes

There are major significant differences in the process Switch Time effects on different islet outcomes in this study as shown in Figure 10A. The islet yields are significantly different according to the Switch Time as shown in Figure 10A with the maximal islet yields delivered by the shortest Switch Time and the lowest islet yields delivered by the longest Switch Time. The islet yields for the shortest Switch Time, the 3-8 minute group, show Pre-Purification yields at 7,190.3±237 IEQ/Gm, Post-Purification at 5,957.0±241 IEQ/Gm, and Post-Culture islet yields at 4,749.5±203 IEQ/Gm. There is a reduction in islet yields with increasing Switch Times as shown by the second Switch Time group for 8-10 minutes with the Pre-Purification yield at 6,570.3±416 IEQ/Gm, the Post-Purification yield at 4,565.8±459 IEQ/Gm, and the Post-Culture group yield at 3,369.1±386 IEQ/Gm. However, the largest decrease in islet yields was in the >10 min Switch Time to 4,410.9±392 IEQ/Gm for the Pre-Purification group, 3,505.9±485 IEQ/Gm for the Post-Purification group, and 2,617.2±366 IEQ/Gm for the Post-Culture group.

Thus, by linear regression analyses, the process Switch Times from the recycling to the collection mode is shown to be a very significant variable in this study that affects islet yields as IEQ/Gm. Statistical Analysis by Linear Regression of the Post-Culture yields clearly show there is a significant inverse relationship between Switch Time and Post-Culture islet yields ($p<0.001$). The yields between 3-8 minutes and 8-10 minutes Switch are significantly different as well ($p<0.001$) with the islet yields between the 3-8 minutes and >10minutes Switch also significantly different ($p<0.001$). But the yields of the 8-10 minutes Switch were not statistically significantly different from the >10.0 minute Switch ($p=0.051$, two tailed Student t Tests). Note that 164 (72.6%) processes were switched by 8 minutes of digestion, 41 (18.1%) processes were switched between 8 and 10 minutes, and 21 (9.3%) processes were switched at >10.0 minutes for this entire study of 226 processed human pancreases. Returning to Table 1 where data from all of the recent clinical islet transplantation processing is summarized, the average Switch Time recorded for 21 published reports of clinical islet processing is 17.2 minutes, that is a time that is basically off the chart for Figure 10A as shown here. So this Switch Time for processing human islets reported in this study are obviously quite different from the longer processing times used to produce human islets for research and for clinical transplantation at the other centers. It is probably the most important processing difference that permits *Human Pancreases for Research* to achieve islet yields within those from processing *Human Pancreases for Transplant*.

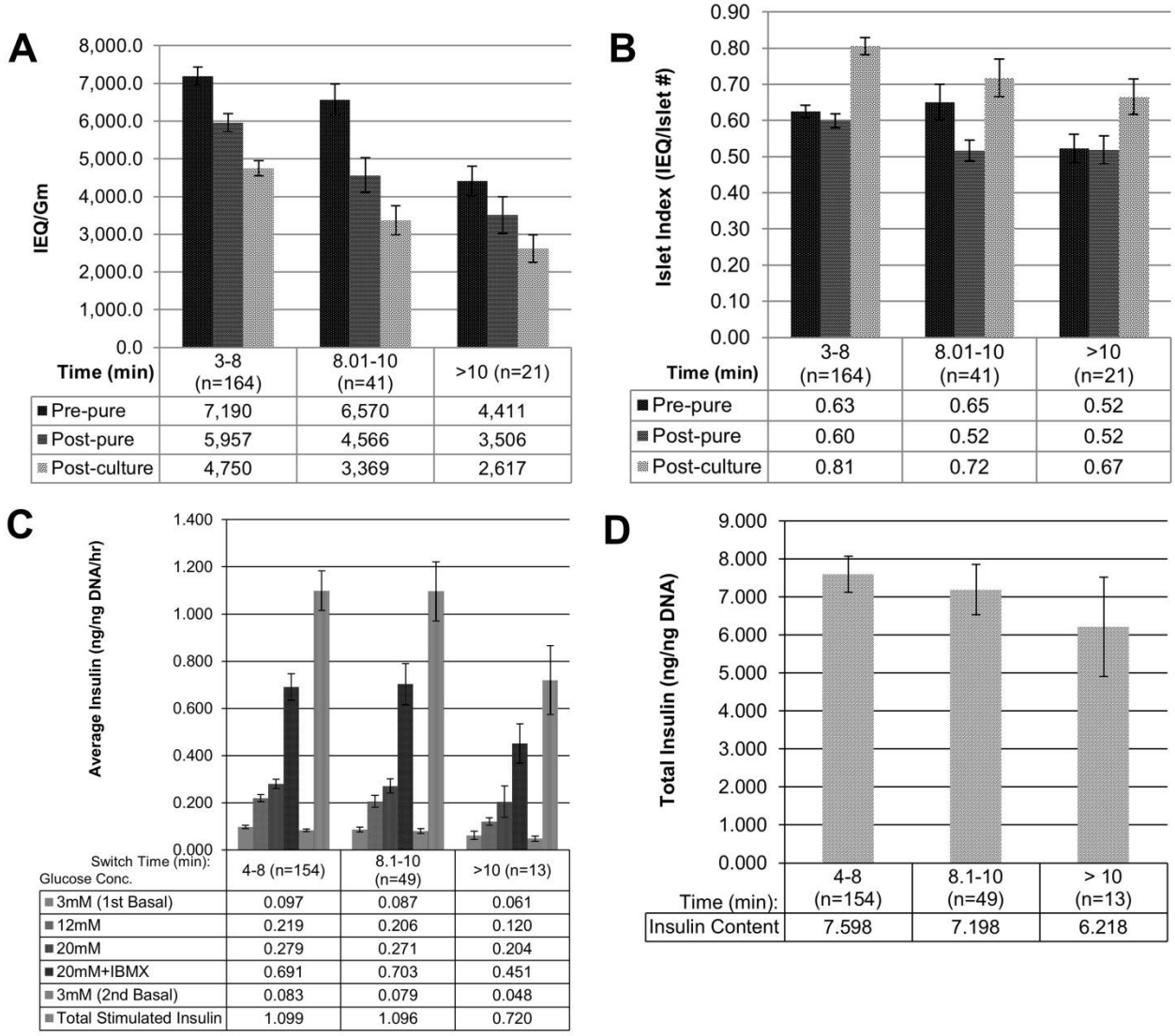


Figure 10 Effect of Switch Time on islet processing. A: The effect of switch time on islet yields as IEQ/Gm; B: The effect of switch time on islet indices; C: The effect of switch time on GSIR; D: The effect of switch time on islet insulin content.

The changes in Islet Index resulting from different Switch Times as shown in Figure 10B based on Pre-Purification ($p=0.078$) and Post-Culture ($p=0.076$) do not show a significant positive correlation between Switch Time and Islet Index. However, for the Post-Purification group, the Islet Index difference specifically for 3-8 minute Switch Time and the >10 min Switch Time are significantly different ($p=0.005$), while the other combinations of Switch Time are not significantly different at the Post-Culture group. For the 3-8 min Switch Time, the Pre-Purification Islet Index value was 0.63±0.02, the Post-Purification Islet Index was 0.60±0.02, and the Post-Culture Index was 0.81±0.02. For the 8.1-10 minute Switch time, the Pre-Purification Index was 0.65±0.05, the Post-Purification Islet Index value was 0.52±0.03, and the Post-Culture Index was 0.72±0.05. For the >10 minute Switch time, the Pre-Purification Index was 0.52±0.05, the Post-Purification Index was 0.52±0.04, and the Post-Culture Index was 0.67±0.05. Thus, increasing Switch Times did show a significant reduction in Islet Index values as the Switch times increased in the Post-Culture Index. In addition, all of the Post-Culture Islet Indices were significantly higher for each Switch Time

group than their Post-Purification Islet Indices as has been reported for most of these studies, due to the PIM(R)® culture media.

The GSIR results show a major loss of islet function when testing islets that had Switch Times of >10.0 minutes as shown in Figure 10C. While the average Total Stimulated Insulin Release for the 3-8 min (n=154) and the 8.1-10 min (n=49) groups were nearly identical with 1.099±0.084 and 1.096±0.125 ng insulin/ng DNA/Hr respectively, the >10 min (n=13) group values fell to 0.720±0.146 ng insulin /ng DNA/Hr. This 34.3% drop in islet function in this >10 min group is more severe than those resulting from the other islet processing variables affecting islet processing results. Also, the maximal stimulus of 20mM glucose + 0.1mM IBMX for this >10 min groups is also the lowest response for the three different Switch Times. Using a Switch Time in this system of islet processing that is prolonged not only adversely affects islet yields but also severely limits their function.

The insulin content results relating to different process Switch times, as shown in Figure 10D, mimic those already observed in the GSIR presented above. The insulin content for Switch Time for the 3-8 minutes group is 7.598±0.478 ng insulin/ng DNA and 7.198±0.661 ng insulin/ng DNA for the 8.1-10 minutes group. However, the Switch Time for the >10 minutes group insulin content dropped to 6.218±1.309 ng insulin/ng DNA. This suggests the ongoing digestion in the D/FC beyond 10 minutes is detrimental to not only to islet yield and islet function but also to islet insulin content. Yet, the linear regression was p=0.464, most likely due to the marked variances in the >10 minute group with a smaller sample size (n=13).

3.2.5 Organ Procurement Organizations (OPO's) and Organ Preservation Solutions (OPS's)

Another set of variables that can relate to CIT are the different OPO's and their location with different distances from the Prodo processing center in Aliso Viejo, CA. The Organ Procurement and Transplantation Network (OPTN) has defined 11 regions within the USA. Our center is in Region 5 that includes Arizona, California, Nevada, New Mexico, and Utah that is where the majority of pancreases for research for our program are procured by the OPO's. A second region, Region 4, includes Oklahoma and Texas where we also receive pancreases for research through 2 of the 3 Texas OPO's. There are very major differences in the numbers of pancreases processed during 2011-2016 per each OPO, as shown in Figure 11A. The California OPO's provided the bulk of the pancreases with One Legacy at 132 pancreases with Pre-Purification average islet yields of 7,288±247 IEQ/Gm, California Donor Transplant Network (CDTN) now named Donor Network West (DNW) at 23 pancreases with 7,640±904 IEQ/Gm, the Nevada Donor Network (NDN) with 7 pancreases with 7,672 IEQ/Gm, and the Sierra Donor Service at Sacramento (SDS) at 4 pancreases with 5,333±789 IEQ/Gm, for a total of 166 pancreases processed from Region 5. The total Region 4 OPO pancreases were 64 divided between LifeGift (Houston,TX) at 23 pancreases with 5,851±464 IEQ/Gm, Texas Organ Sharing Association (TOSA) (San Antonio, TX) at 20 pancreases with 5,412±482 IEQ/Gm, and the International Institute for the Advancement of Medicine (IIAM) had 17 pancreases with 6,474±642 IEQ/Gm (multiple western state OPO's). In terms of OPS's utilized, the Texas OPO's predominantly used Histidine-tryptophan-ketoglutarate solution (HTK) and Static Preservation Solution (SPS-1) and had longer cold ischemia times due to the greater distance from our processing center. The results from One Legacy had the largest numbers of pancreases processed, were the closest to our processing site, and only utilized University of Wisconsin

preservation solution (UW) with the best results in the study. DNW had fewer processes but results quite similar to One Legacy. SDS in California and NDN had few processes in the study. The two Texas OPO's had lower outcomes but their choice of OPS's combined with the longer storage times may explain these differences. There are no specific patterns or trends in the islet yields as the OPO potential effect is complicated due to local versus distant states as well as different types of OPS's utilized by different OPO's.

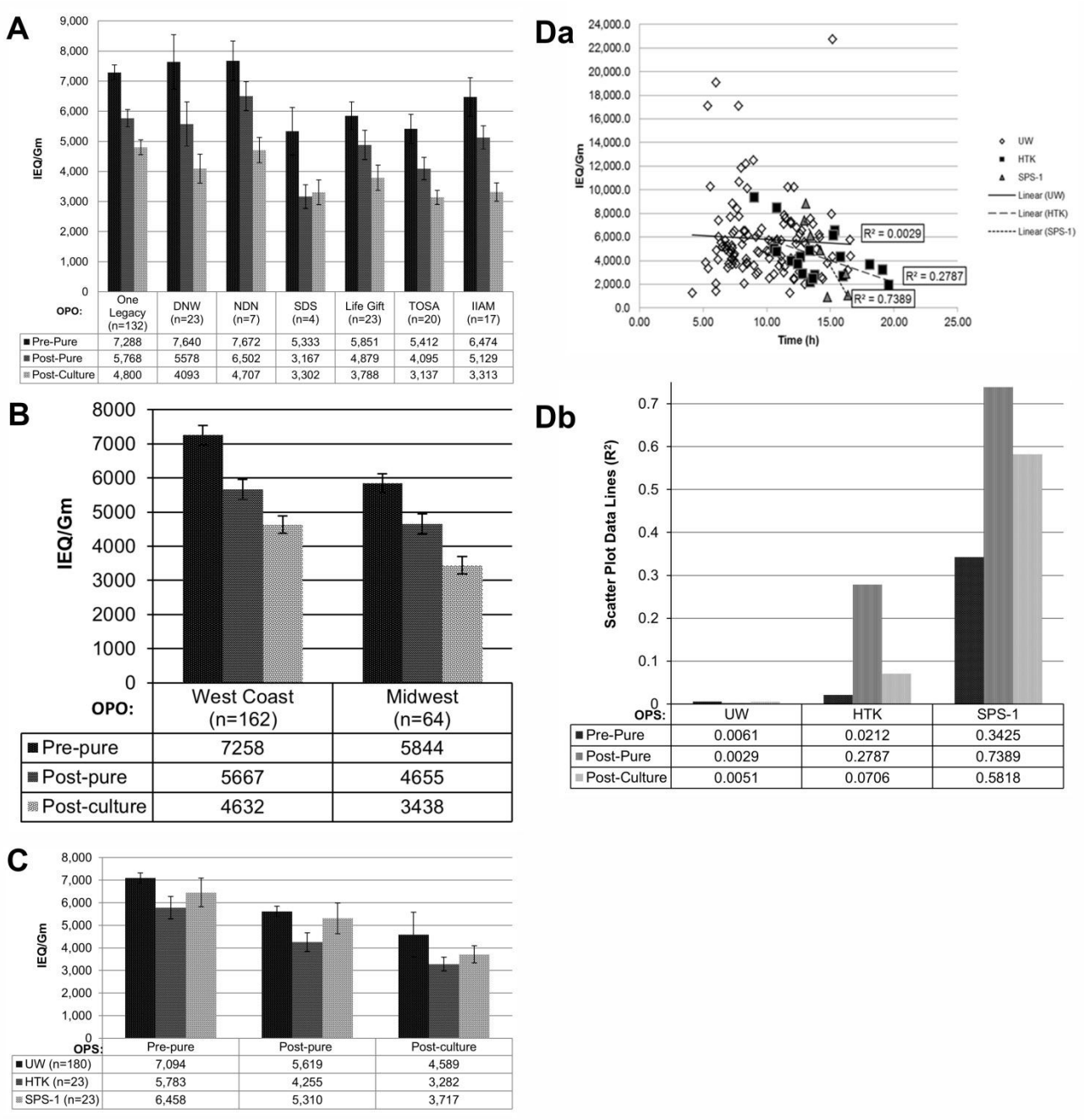


Figure 11 Effect of OPO's on islet processing. A: The effect of individual OPO's on islet yields as IEQ/Gm; B: West coast region 5 versus mid-west region 4 OPO's effect on islet yields; C: The effect of the type of OPS on islet yields as IEQ/Gm; Da: Scatter Plot of islet yields versus CIT's for UW, HTK, and SPS-1 OPS's; Db: Regression line (R2) values from scatter plots for OPS's UW, HTK, and SPS-1 for protecting islet yields.

However, if these OPO's are grouped according to region with the West Coast (Region 5) OPO's being One Legacy, DNW, NDN, and SDS (n=166) and the Mid-West (Region 4) OPO's being limited to Life Gift, TOSA, and IIAM (n=64), there emerges a significant difference between these two regional groups at all three processing levels of islet production as shown in Figure 11B. The Pre-Purification results of 7,258±255 IEQ/Gm for the West Coast are significantly higher than the Mid-West yields at 5,844±292 IEQ/Gm ($p<0.001$). Similarly, the Post-Purification islet yields are significantly higher from West Coast processing with 5,667±264 IEQ/Gm than the Mid-West processing with 4,655±250 IEQ/Gm ($p=0.002$). The Post-Culture islet yields have the highest significant difference with the West Coast yield of 4,632±277 IEQ/Gm and the Mid-West yield of 3,348±193 IEQ/Gm ($p<0.001$). With these significantly lower islet yields from pancreases procured in the Mid-West, Region 4, rather than the West Region, Region 5, for islet processing, the current question now becomes whether this significant difference in islet yields is due to effects from longer CIT or the type of OPS's utilized or a combination of both.

The types of OPS's used at different centers is becoming controversial in the published literature as to whether the lower-priced formulations are as protective as the standard UW Solution for organs being transplanted with longer cold ischemia times [67, 68, 69]. The use of these lower priced OPS's is not universal since their use is based on individual OPO and transplant surgeon preference. The primary OPO for this report over the last six years has been One Legacy, in CA, that only utilizes UW Solution. A few of our other contract centers have used HTK or SPS-1 at different times. So evaluation of these is limited in number in this study. Nevertheless, their evaluation on islet processing is interesting. The first comparison is shown in Figure 11C that presents results on islet yields as IEQ/Gm for the use of these different OPS's. The UW solution shows a Pre-Purification islet yield of 7,094±226 IEQ/GM, a Post-Purification yield of 5,619±229 IEQ/Gm, and a Post-Culture islet yield of 4,589±992 IEQ/Gm that represent the highest yields for the three preservation solutions utilized in this study. The HTK solution shows a Pre-Purification yield of 5,783±496 IEQ/Gm, a Post-Purification yield of 4,255±409 IEQ/Gm, and a Post-Culture yield of 3,282±307 IEQ/Gm. The SPS-1 solution shows a Pre-Purification yield of 6,458±635 IEQ/Gm, the Post-Purification yield of 5,310±677 IEQ/Gm, and the Post-Culture yield of 3,717±380 IEQ/Gm. Using 2 tailed t tests to compare UW (n=180) preservation with HTK (n=23) preservation showed the UW yields at all the three process steps were significantly higher (Pre-Purification ($p=0.022$), Post-Purification ($p=0.006$), and Post-Culture ($p<0.001$)). Comparing HTK (n=23) with SPS-1 (n=12) showed there were no significantly different yields with these two preservation solutions (Pre-Purification ($p=0.413$), Post-Purification ($p=0.182$), and Post-Culture ($p=0.378$)). While statistical analysis of UW (n=180) versus SPS-1 (n=12) was not able to demonstrate a significant difference with Pre-Purification islet yields ($p=0.182$) and Post-Purification yields ($p=0.378$), however, the Post-Culture islet yields were significantly greater with UW than with SPS-1 ($p=0.006$). Thus, the UW solution clearly shows statistically higher yields than HTK preservation or SPS-1 preservation. The choice of OPS's may also be the primary contributor to the lower yields observed in the Mid-West processes.

A more specific analysis for evaluating the protection of human islets with longer cold ischemia times is seen by the use of a scatter plot comparing the use of differing OPS's: UW, HTK, and SPS-1, as provided in Figure 11D. The scatter plot is shown in Figure 11Da below that plots the islet yields in IEQ/Gm versus the specific OPS's utilized in hours and calculates the linear regression line for each preservation solution. For UW preservation solution, the R^2 value of the linear line is R^2 =

0.0029 that is a minor sloping negative line suggesting excellent pancreas protection from cold ischemia. For HTK preservation, the linear line is R^2 = 0.2787 that is a far more negative line than observed with UW preservation suggesting less pancreas preservation capability than UW. For SPS-1 preservation, the linear line is steeply negative with R^2 = 0.7389 that suggests very poor protection with increasing cold preservation times. Since all of these human pancreases were procured with the intention of clinical transplanting either the pancreas or the isolated islets, there was no opportunity to compare these results to those organs that did not receive OPS's in storage.

Another way to plot these same analyses is to simply plot the R^2 values for each of the OPS's that is shown in Figure 11Db. This plot emphasizes the major differences in these three OPS's regression line values for each of the three islet processing steps. The optimal protection by the use of UW solution is far superior in these plots than the use of the other two options.

Analysis of these scatter plot results clearly suggest UW preservation solution is optimal for protecting human islets from the effects of longer cold ischemia times. HTK results show less protection capability compared with UW solution. The use of SPS-1 suggests limited ability to protect human pancreases and isolated islets from the damaging effects of increasing cold ischemia times.

3.2.6 Non-Significant Process Variables

Several additional variables were tested that did not offer any statistically significant different results in terms of islet yields or islet losses at any of the three process times, nor in islet function. These variables included minor surgical damage to the pancreas that could be controlled by tissue clamping during distension, minor superficial hemorrhage, mild pancreatic inter-lobular edema but not intra-lobular edema, breaks in sterile pancreas dissection by release of duodenal content followed by 2% chloro-hexadine rinse of the contaminated pancreas, and fatty infiltration of the pancreas outer surface.

4. Summaries and Discussions

4.1 Processing Outcomes

4.1.1 Non-DM Donors

a) While *there are differences in the three primary causes of death (Stroke, Anoxia, and Head Tauma) in different types of organ donors, the only significant difference in islet yields was at the Post-Culture step that showed Stroke donors providing higher islet IEQ/Gm yields than Head Trauma donors.*

b) While expression of Islet Yields per Pancreas process failed to provide significantly different outcomes due to the large variations in pancreas weights, expressing Islet Yields per Gram (IEQ/Gm) of pancreas processed did demonstrate significantly different results.

c) Islet Indices Pre-Purification and Post-Purification are statistically similar, but both are significantly lower than Post-Culture Islet Indices due to the use of PIM(R)® islet specific, recovery culture medium, stimulating islet recovery and aggregation.

d) GSIR results from purified human islets show that with each increase in the glucose concentration as well with IBMX has resulted in significantly increased insulin responses. In addition, each of the three GSI generated by the GSIR assay is significantly increased over the previous one due to their increased insulin responses to the increasing glucose concentrations.

One of *the* challenges for processing human pancreases into *HIR* is that the optimal pancreases are appropriately reserved for clinical pancreas transplantation or clinical islet transplantation. All of the pancreases that became available for this study for research originated from organ donors that had been consented for clinical transplant and had required at least one organ transplant other than the pancreas to have been accomplished. Less frequently, transplant quality pancreases do become available for research applications as when a late, failed cross match is determined. Many investigators assume the results of processing human pancreas for research into *HIR* are expected to provide lower quality and quantity of human islets for their research activities. However, with this review of clinical islet transplantation studies (Table 1), it becomes apparent that center to center differences in the results of processing human pancreases into islets followed by islet culture result in significant differences in the quantities of islets that become available for implantation, even using the optimal *HPT* donors. In terms of islets processed for research, the IIDP of the NIH distributing human islets for research to its funded investigators also struggle with major discrepancies in the consistent quality of human islets between different pancreas processing centers and within some centers [43]. When Prodo initiated its efforts to provide *HIR* in 2007 as a service business, it also had problems providing reproducibly high quality human islets from process to process. Therefore, a thorough review and identification of all the known variables involved in this process was done to bring as many process variables as possible under control. The consistent results from this 2011-2016 study described in this publication are the result of this effort as well as turning human pancreas processing methods into manufacturing processes. This has been achieved by defining and controlling the known process variables and locking them into narrow process limits that are not altered. Also, as the demand for high quality islets increased, Prodo was able to increase the number of pancreases processed by expanding the number of OPO's providing research pancreases.

Table 7 directly compares the average results of islet processing in the Clinical Islet Transplant Centers (Table 1) with the average results from this Prodo study. It must be emphasized that the primary focus of these clinical islet transplant reports were appropriately on islet transplant outcomes and not on the specifics of clinical islet processing. Thus, there are a number of centers that only reported partial islet processing outcomes as documented in Table 1. The "Clinical Islet Transplant Centers Groups" islet yields from Table 1 are reported in Table 7 as IEQ per Process with the results presented in three groups: a) Pre-Purification at 503,634 IEQ per process, b) Post-Purification at 356,307 IEQ per process, and c) Post-Culture at 411,462 IEQ per process with the discrepancies in these data due to unequal numbers of centers reporting their results at each step. The Clinical Centers Groups reported their IEQ/Gm process islet yields results as well: a) Pre-Purification at 5,680 IEQ/Gm, b) Post-Purification at 4,101 IEQ/Gm, and c) Post-Culture at 3,599 IEQ/Gm with a 59.2% islet purity, again with discrepancies in unequal numbers of centers reporting. This Prodo current study provides their islets yields: a) Pre-Purification at 6,943±201 IEQ/Gm, Post-Purification at 5,484±199 IEQ/Gm, and Post-Culture at 4,351±167 IEQ/Gm with a 90.0% islet purity. This initial analysis suggests the clinical islet centers islet processing results

were not as efficient in their islet processing methods as the Prodo results presented in this current report. However, there were many earlier years in the Clinical Islet Centers' averaged results from 2003 to 2016 than reported in this Prodo study that was from 2011 to 2016. Table 7 includes a report of Historic Results showing much lower human islet yields from initial human islet processing efforts from 1988. Instead of just comparing the combined clinical islet processing results from several years of processing from 2003 - 2016 to those of this more recent six-year study by Prodo, it may be more appropriate to directly compare the latest Phase 3 CIT study [3,4] published in 2016 to this current Prodo study. The Phase 3 CIT study published: a) Pre-Purification results at 6,813 IEQ/gm, b) Post-Purification results at 5,471 IEQ/Gm, and c) Post-Culture results at 4,730 IEQ/Gm. This last comparison between the Phase 3 CIT study islet processing results and Prodo's islet processing results expressed as IEQ/Gm are essentially in the same ranges of islet yields at each of the three processing steps. Statistical analysis is not possible since the complete data base is not available for the CIT study. However, it is also interesting to note that there is a major discrepancy in expressing the islet yields per process results between the Phase 3 CIT trial and Prodo's yields when expressed as IEQ/Process. The islet yields per process for the Phase 3 CIT study were: a) Pre-Purification at 708,479 IEQ/process, b) Post-Purification at 582,370 IEQ/process, and c) Post-Culture at 490,174 IEQ/process. The Prodo islet yields were by comparison: a) Pre-Purification at 460,367 IEQ/process, b) Post-Purification at 354,860 IEQ/process, and c) Post-Culture at 279,083 IEQ/process. So, there are major differences in islet yields expressed as IEQ/process between the Phase 3 study and this current Prodo study. However, the islet yields expressed as IEQ/Gm show essentially very little difference in these islet yields between the Phase 3 study and this Prodo study. Is there an explanation for these discrepancies?

The primary explanation for the discrepancy noted in islet yields between these two reports is found by focusing on the weights of the pancreases processed. This Prodo study reports the total number of processes at 226 Non-DM donors with a range of pancreas weights from 40 grams to 194.5 grams with an average weight of 89.4 grams as shown in Figure 12A. The results shown in this figure approach a Gaussian distribution of human pancreas processed weights that would be expected from the random human pancreas weight choices in processing pancreases into islets for these efforts. For the Phase 3 trial, their selected optimal donors for processing pancreases for their clinical trial was to only process islets from the largest pancreas donors available for their implants since these largest pancreases would produce more islets per pancreas, meeting their goal to only use one donor per recipient. As shown in Figure 12B, the Phase 3 study of 75 donors ranged in the eight centers from 93 grams to 124 grams with their average weight of 106 grams [3,4]. Yet, this Phase 3 study also reported they had to prepare 324 islet lots in order to implant their optimal 170 islet lots into these 75 patients [3, 4]. In other words, their human islet processing in terms of transplantable human islet lots was only 52.5% effective that means even more pancreas donors may be required going forward for the BLA. It is important to note that their choice of these larger pancreases to process for clinical trials only represents 18.1% of the pancreas donors that are available, if one accepts the Prodo distribution of human pancreas weights as being representative (Figure 12A). With the assumption that the IEQ/Gm process results between this Prodo report and their publications were close in pancreas numbers, then increasing the size of the pancreases being processed, one would be able to increase the numbers of islets available, even with the same IEQ/Gm yield.

Table 7 Comparison of results from clinical islet transplant isolations to this Prodo study of human islets for research.

Centers	Years	IEQ + IEQ/Gm Pre-Pure	IEQ + IEQ/Gm Post-Pure	IEQ + IEQ/Gm Post-Culture	IEQ/Kg BW Infused/ % Pure	Switch Time / Length	GSIR Stim Index	Donor Age (Yrs)	Donor BMI	Cold Ischemia Time
*Clinical Islet Trans- plant Centers Groups [3,4,25-49]	2003 - 2016 12 single & 6 multi-center	503,634 IEQ 5,680 IEQ/Gm	356,307 IEQ 4,101 IEQ/Gm	411,462 IEQ 3,599 IEQ/Gm	9,286 IEQ/kg BW / 59.2% pure	17.2 min / 47.6 min total	1st SI 2.8	42.3	29.7	7.7 hrs
*Phase 3 CIT-07 8 Centers [3,4]	2016 n=48 islet trans-plants	708,479 IEQ 6,813 IEQ/Gm	582,370 IEQ 5,471 IEQ/Gm	490,174 IEQ 4,730 IEQ/Gm	6,694 IEQ/kg BW / 61.9%	14 min / 48.0 min total	1st SI 2.1	42	33.4	7.7 hrs
$Prodo Labs Research Pancreas (This Study)	2011 to 2016 n=226	460,367 IEQ 6,943 IEQ/Gm	354,860 IEQ 5,484 IEQ/Gm	279,083 IEQ 4,351 IEQ/Gm	na / 90.0% pure	6.3 min / 34 min total	1st SI 2.6, 2nd SI 3.5, 3rd SI 8.8	42.4	27.2	10.1 hrs
Historical Results Washing-ton University [17]	1988 n=12	275,800 IEQ 3,917 IEQ/Gm	164,600 IEQ 2,279 IEQ/Gm	na	na / 79.0% pure	na	3.5 peri-fusion 300 G mg/dl	36.2	na	14.7 hrs

- Taken from Table 1 "Clinical Islet Transplant Center Results"

$ Taken from Figure's 3A, 3B, 3E, Tables 4 & 6

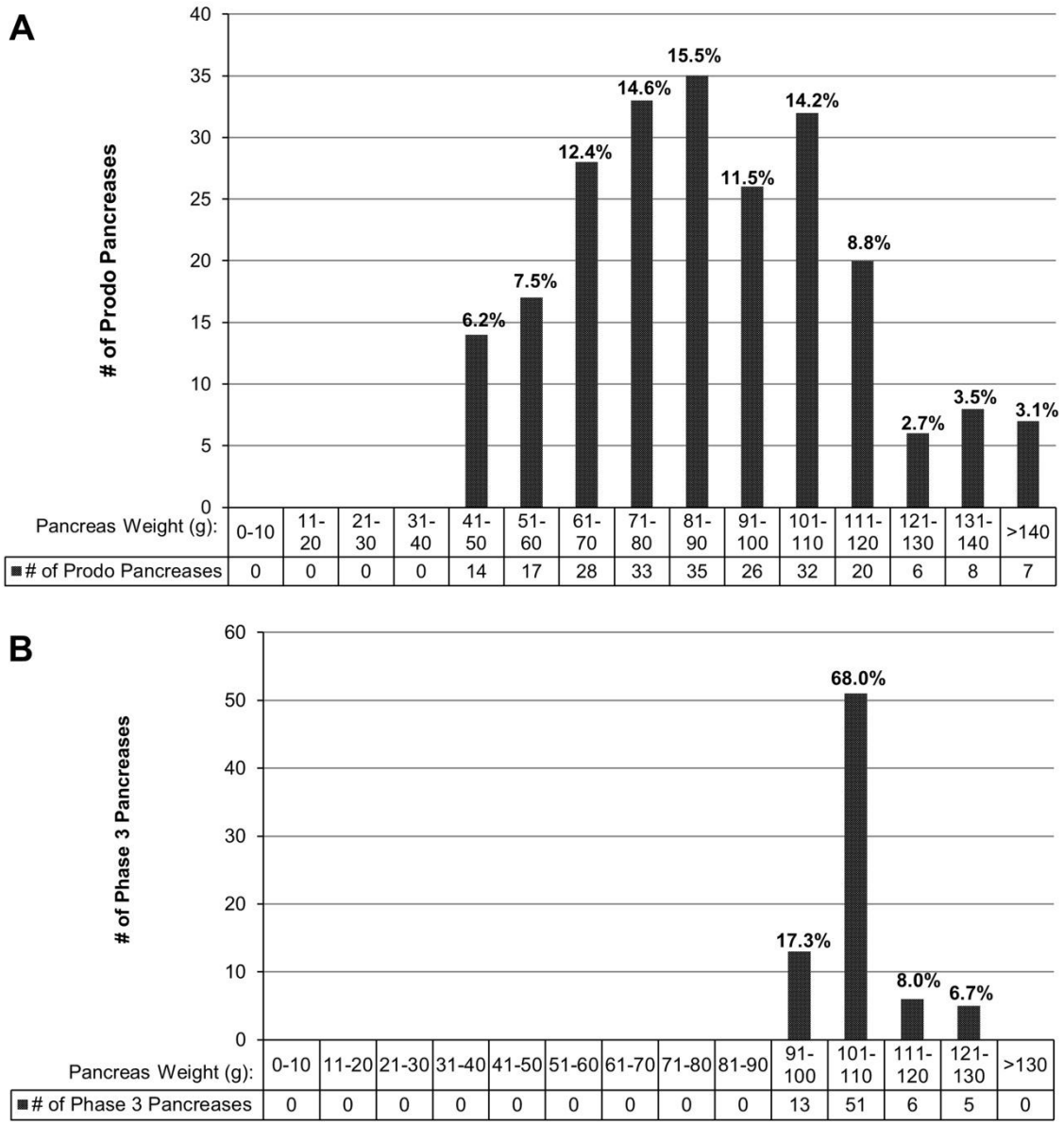


Figure 12 Effect of pancreas weight on islet processing. A: Prodo processed human pancreas weight distribution (n=226); B: Phase 3 study human pancreas weight distribution (n=75) [3, 4].

More specifically, the results shown in Table 7 confirm larger pancreases produce more islets, even though the islet yield expressed on the per gram basis are the same. Emphasizing again, the Phase 3 study averaged 6,813 IEQ/Gm Pre-Purification, 5,471 IEQ/Gm Post-Purification, and 3,599 IEQ/Gm Post-Culture that is compared to Prodo Labs 6,943 IEQ/Gm Pre-Purification, 5,484 IEQ/Gm Post-Purification, and 4,351 IEQ/Gm Post-Culture that essentially the same results. However, the results of the total IEQ processed per pancreas showed the Phase 3 study averaged 708,479 IEQ/Pancreas Pre-Purification, 582,370 IEQ/Pancreas Post-Purification, and 490,174 IEQ/Pancreas Post-Culture, while this Prodo Study only averaged 460,367 IEQ/ Pancreas Pre-Purification, 354,860 IEQ/Pancreas Post-Purification, and 279,083 IEQ/Pancreas Post-Culture. Thus, the clinical centers focusing on only processing the largest pancreases will provide more islets for

transplant from a single donor to a single recipient, even with the same islet yield expressed on a per gram basis from smaller pancreases. While this was a great strategy for the Phase 3 clinical trial of only processing the largest human pancreases for islets to implant successfully at a 50% process success level, this strategy may fall short for a BLA with the expected increase in the numbers of T1D patients needing islet transplants that will be required to be uniformly curative, will drive up the numbers of islet donors needed.

Also included in Table 7 is the last entry entitled "Historic Results" from 1988 that provides the comparison of these recent studies to the first publication of processing human islets in 12 pancreas donors with the D/FC approach. This current Prodo report shows a 23.9% IEQ per process loss of islets and a 21.2% loss of IEQ/Gm due to purification. The Historic Results show a 40.3% IEQ per process loss of islets and a 41.7% IEQ/Gm loss of islets due to purification but no Switch Times were recorded. Interestingly, the purity of this current study was 90.0% pure with the Historic Results showing 79.0% pure islets, while the current, averaged clinical islet trials (Phase 3) reports only a 59.1% purity that is being accepted in order to increase the amount of islet tissue per transplant for diabetic recipients.

The primary challenge for isolating primary islets from human pancreases from organ donors is the fact that islets, being scattered throughout the pancreas, have to be both mechanically and enzymatically released from the acinar and ductal tissues with appropriate timing, temperature, use of enzyme types, mechanical agitation, and the proper balance of purifying the freed islets. Our hypothesis and practice is that the more rapid this process can be completed with efficient removal of the isolated islets from the active digestate within the D/FC, the higher the islet yields, quality, and purity will be realized resulting in better islet implant results, research investigations, and longer quality islet culture times. As shown in Table 7, this described process does accomplish that objective of significantly reducing the digestion time compared to the other clinical centers processing islets for clinical trials. At the time of the Switch, the temperature is lowered to 10^{o}C initially reducing both the collagenase relative activity and the thermolysin activity that are then also diluted out over the combination time. Another primary difference may be the use a continuous density gradient that permits the recovery of human islets with purities on average of 90.0% rather than the purity for clinical trials of 59.4%. When using these methods, it may be possible for human islets intended for clinical trials to be processed with islet purities approaching 90% rather than the current 60%, assuming high islet yields per donor can also be accomplished. Furthermore, reducing digestion switch times to the 6-7 minute level allows one to deliver higher quality islets by reducing islet damage during this Digestion/Filtration process. In this way, most all pre-digested pancreas preparations enter the D/FC only under collagenase pre-digestion with the goal being to make each human pancreas preparation as near to the same as possible prior to the D/FC 37^{o}C digestion that permits the thermolysin to kick in and rapidly digest the acinar tissue off the islets. Then, the rapidly cooled islets can be quickly recovered while diluting out the enzymes to preserve islet integrity.

4.1.2 DCD Donors

Non-DM donors versus DCD donors islet yields are not significantly different in IEQ/Gm islet yields expressed as Pre-Purification and Post-Purification results, but Non-DM donors have a small but significantly higher islet yield than DCD donors after Post-Culture analysis.

To qualify as a DCD donor to be included in this study required the following restrictions based on our previous experience: a) less than a 15 minute downtime from the original event at the time of hospitalization without CPR and b) <31 minutes to develop cardiac arrest during the DCD process. An expansion of the donor pool of human organs for clinical transplantation has been made possible by the establishment of a controlled recovery of organs from non-heart beating donors declared DCD. This report also demonstrates that if one restricts specific conditions in DCD donors, that human pancreases can be successfully processed with the results of islet isolation nearly at the same level and quality as for non-DCD donors. If one examines the individual DCD procedure records from these cases, one consistently finds very low oxygenation levels for a significant period of time prior to the actual cardiac arrest. Thus, the restriction of the DCD time to <31 minutes until cardiac arrest reduces this period of pancreas hypoxia prior to initiating islet processing in order to protect the pancreas from increasing autolysis that occurs with longer donor hypoxic and ischemic times. These results also suggest that islets processed from DCD donors under these specific restrictions should increase the donor pool for processing islets for research, as well as for clinical islet transplantation.

4.1.3 T2D Donors

a) For the Successful Non-DM and T2D donors, the processing details were not statistically different regarding the Cold Ischemia Time, the Switch Time, and the Digested Pancreas Weight, but the percent of the pancreas processed was significantly lower for the T2D donor than the Non-DM donor.

b) For the Successful Non-DM Processes, the significant differences over the Unsuccessful Non-DM processes were a shorter Cold Ischemia Time, less of the pancreas processed, and a shorter Switch Time.

c) For the Successful T2D Processes, the significant differences over the Unsuccessful T2D Processes were a much larger pancreas weight, a much higher percent of the pancreas digested, and a much shorter Switch Time.

d) Islet Yields reported as IEQ/Gm from T2D donors were significantly reduced at all the Processing steps: Pre-Purification, Post-Purification and Post-Culture, compared to Non-DM donors.

e) Islet Indices from T2D donors were not significantly reduced at any of the Processing steps.

f) GSIR results for T2D islets compared to Non-DM islets were not significantly reduced at the 1st Glucose Stimulated Insulin Step, but were significantly reduced at both the 2nd and the 3rd Glucose Stimulated Insulin Steps as well as the Total Stimulated Insulin released. The fold increase of insulin release in response to GSIR testing showed the same responses for the Non-DM and the T2D islets at 12mM glucose, but larger insulin responses at the higher glucose stimulation steps for the Non-DM islets were consistently observed over those able to be achieved by the T2D islets.

g) The Insulin Content of T2D islets compared to the Non-DM islets were also significantly reduced to the 75.2% level. These comparisons were all made 5-8 days after processing at the time of the GSIR testing and were not obtained immediately after islet isolation.

h) The effect of T2D donor Hemoglobin A1c levels (<6.5%, 6.51-10.0%, >10.1%) on islet processing results showed changes related to these A1c levels. But, none of these results were significantly different by Linear Regression Analysis, predominantly due to the low numbers of processes in both the lowest A1c level group and the highest A1c level group.

This study also presents results of processing human islets from organ donors that have a previous diagnosis of T2D. By eliminating T2D donors under insulin treatment and those with high hemoglobin A1c levels (>10%) from islet processing, improved islet processing results were obtained. There was some difficulty in statistically comparing these two processes in that there were 226 successful Non-DM processes and 29 successful T2D processes in this study. However, we were able to demonstrate significantly lower islet yields at the different process times, reduced glucose stimulated insulin release at the higher glucose concentrations, and reduced islet insulin content in the T2D donor islets compared to the Non-DM donor islets. We purposely reduced potential differences between these two groups by eliminating processing islets from T2D donors that were on insulin therapy or had Hemoglobin A1c levels >10% prior to islet processing. We had previously encountered very marginal yields of viable and functional islets from more advanced cases of T2D. Another analysis we thought would show significant differences between Non-DM donors and T2D donors, was based on T2D donors HgbA1c levels. But, the numbers of processes separated into three HgbA1c levels did not permit statistically meaningful results with the smaller numbers in each subgroup. Those T2D donors with HgbA1c values of <6.5% had a tendency for higher islet yields with higher glucose stimulated insulin responses and higher insulin content than those with HgbA1c values >10%. Those T2D donors with HgbA1c values between 6.5% and 10.0% had intermediate islet yields and glucose stimulated insulin release levels, but somewhat less than Non-T2D donors. So additional T2D donors have to be added to those already processed to assure these preliminary impressions can hold up to statistical analysis.

4.2 Critical Donor Variables for Non-DM Donors

4.2.1 Age

a) There *is no evidence of donor age effect on Islet Yields for the Pre-Purification Step. However, due to the loss of islets with purification, especially from the younger donors from embedded islets, there now are significant differences in Islet Yields in both the Post-Purification and Post-Culture groups with increasing age. The embedded islets are predominantly in the 18-19 year old group and the 20-29 year old group that cannot be further purified by using the current density gradients.*

b) Islet Indices were not significantly changed by age for the Pre-Purification, Post-Purification, and the Post-Culture groups.

c) GSIR Total Stimulated Insulin results were not significantly changed by age as examined by Linear Regression analyses.

d) Insulin Content was not significantly changed by age differences.

The yields of islets from donors from age 30 years to 69 years were essentially the same in terms of islet yields and GSIR when divided into arbitrary 10-year age groups. However, in the younger donors from age 18 to age 29 years, the same quantity of islets were released from the pancreas prior to purification. Yet, there were significantly less islets available after purification and recovered after culture from these younger donors. The specific reason for this decrease of purified islets from young donors is directly related to the pancreatic duct used for enzymatic distension of the pancreas prior to D/FC digestion. In younger aged donors, the pancreatic duct appears to be more susceptible to disruption within the pancreas during the ductal injection of collagenase and thermolysin enzymes. The duct structure apparently fails prior to accomplishing

adequate islet separation at the islet/acinar cell interface in younger human donors. This results in what are called embedded islets that are partially or completely surrounded by non-islet acinar tissue that increases the density of these islet/acinar cell aggregates. Using continuous density gradient purification takes these embedded islets down to lower layers with much larger concentrations of acinar cell aggregates significantly reducing islet purity. Since the primary objective of researchers for human islets is receiving very pure islets, the number of pure islets available from younger donors using the current methods is certainly less per donor than the older donors that have lower levels of embedded islets. The clinical investigators transplanting human islets are focused on high quality and quantity per donor and are thus willing to implant less purified islets into their diabetic recipients. In Tables 1 & 3, the average islet purity at the 21 islet transplant centers was 59.2% that compares to the current research islets presented in this report at 90.0%. Is it possible for the clinical islet transplant centers to improve on their islet purities without sacrificing significant donor islet yields? While there have been documented complications relating to the non-islet impurities in some T1D recipients, the percentage is small even with this low islet purity. However, with improved islet processing and purification, islet processors should enable higher islet purities that should also reduce post-implant loss of islet mass for clinical transplantation.

4.2.2 Body Mass Index

a) Islet Yields were not significantly reduced for any of the three processing steps in terms of overall Linear Regression analysis for donor BMI values. However, there are some of the BMI groups that have individual significant differences in islet yields with the lowest BMI group (<19.9) having significantly lower islet yields than the highest BMI group (35.0-35.9). Also, the 20.0-24.9 group has significantly lower islet yields than the 35-35.9 group as well as the 30-34.9 group has significantly lower islet yields than the 35-35.9 group.

b) Islet Indices were significantly higher at the higher BMI levels at the Pre-Purification and the Post-Purification steps, but were not significantly different at the Post-Culture step.

c) GSIR results were not significantly different at any level.

d) Insulin Content was not significantly different with any of the BMI groups.

Evaluation of the effects of donor BMI at first analysis shows little differences from BMI levels from 19.9 to 40 in terms of islet yields and GSIR results. There is a gradual increase in islet indices in donors with BMI levels >30 as expected by observations of larger islets in these donors during processing. Since donors with BMI levels > 40 were excluded from processing due to our previous experience in their lower islet yields, we were not able to document any difference in insulin content in BMI donors >40 in this study.

4.2.3 Cold Ischemia Time (CIT)

a) Islet Yields from all three processing steps: Pre-Purification, Post-Purification, and Post-Culture, were significantly reduced by longer CIT's.

b) While Islet Indices were not significantly reduced by increasing CIT's for the Pre-Purification and the Post-Purification groups, the Post-Culture group demonstrated significant reduction in Islet indices with increasing CIT's.

c) GSIR results were not significantly reduced by longer CIT's.

d) Islet Insulin Content was significantly reduced by longer CIT's.

This report demonstrates that one of the two most critical variables affecting human islet yields is the CIT. All three processing steps resulted in significantly lower islet yields as IEQ/Gm with increasing times of pancreas cold storage. The Islet Index also showed a significant trend for decreasing values with longer cold ischemia times. But there was no observable effect on GSIR results for any of the times, including >16 hours suggesting that islets that were processed and cultured appropriately were able to function normally after 5-8 days of culture. After the completion of this study and due to these findings, we have limited our cold ischemia times prior to pancreas processing to be 12 hours or less and have eliminated accepting pancreases flushed with SPS-1 in those pancreases stored for 10 hours or more prior to processing. This decrease in the effects of longer Cold Ischemia Time induced islet damage observed in this study over other published studies may be the result of the significantly more rapid process of digesting human islets due to shorter digestion times at 35^{o}C-37^{o}C.

4.2.4 Process Switch Time

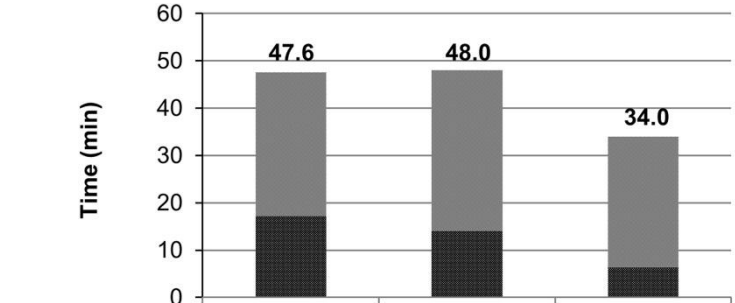
a) Islet Yields were significantly reduced by increasing the Process Switch Time at all of the times recorded (the 3-8 min, the 8.1-10 min, and the >10 min groups) from Pre-Purification to Post-Purification and on to Post-Culture levels. More specifically, while the islet yields from the 3-8 minutes were significantly higher than 8-10 minutes and the islet yields from 3-8 minutes were significantly higher than the >10 minutes, the islet yields from the 8-10 minutes and those from the >10 minutes were not statistically significantly different. In terms of processing experience, 164/226 (72.6%) of processes were switched by 8 minutes, 41/226 (18.1%) were switched by 10 minutes, and 21/226 (9.3%) were switched by >10 minutes.

b) Islet Indices in the Post-Culture group were found in the 3-8 minute time to be significantly higher than the >10 minute group suggesting increased damage to the processed islets with the longest Switch times.

c) GSIR results expressed as the Total Stimulated Insulin release were significantly lower for the Post-Culture group as the Switch time increased.

d) Insulin Content were not significantly reduced at the >10 minute time.

This report demonstrates that one of the two most critical variables involved in human pancreas processing into human islets in this study was the process Switch Time when the recirculating mode through the D/FC is switched to the collection mode that stops recycling the enzymes, brings colder basic culture media into the chamber, and empties the D/FC content into collection conicals at 10^{o}C over time. All processes with Switch Times from 3-8 minutes had the significantly highest islet yields, GSIR results, and islet insulin content. When the process Switch Time was between 8 to 10 minutes there was a small but significant drop in islet yields and GSIR responses, but not in insulin content. When the Process Switch Time was increased to >10 minutes, there was a significant drop in islet yields, GSIR insulin release, and insulin content in these islets. The Switch Times utilized in this study were only one third to one half of the Switch Times reportedly used in processing of human islets for clinical islet transplantation as shown in Figure 13. The results from this study suggest efforts to reduce process Switch Times from recirculating to collection modes in other pancreas processing systems could benefit the quality and functionality of the islets produced.



	Clinical Islet Transplant Centers 2003-2016	Phase 3 CIT 2016	Prodo Islet Processing 2011-2016
Collection Time	30.4	34	27.7
Switch Time	17.2	14	6.3

Figure 13 Switch time, collection time, and total digestion time of human pancreas processing.

4.2.5 OPO's and Organ Preservation Solutions

a) Overall Islet Yields were not significantly affected by comparing the individual, different OPO's, but, when the individual OPO's were grouped into the Region 5 Mid-West OPO group and a Region 4 West-Coast OPO group, the West Coast OPO's had significantly higher islet yields than the Mid-West OPO's with the West Coast OPO's located much closer to this islet processing center.

b) The use of UW solution resulted in significantly higher islet yields than HTK at all three processing steps.

c) Islet Yields using different preservation solutions compared at different preservation times showed no differences with UW solution nor with HTK solution between the 10.1 – 15 hour and the >15 hour storage times.

d) However, there was a very significant drop in islet yields using SPS-1 from the 10.1-15 hour and the >15 hour groups.

e) Use of scatter plot presentation of these results, showed a marked protection of islet yields using UW solution rather than HTK or SPS-1 solutions with the least protection demonstrated by the use of SPS-1.

While there were differences in the results from different OPO's regarding islet yields, these results were dependent upon the number of donors processed and the location of the OPO's. Two of the OPO's were in Texas rather than California so the effects of longer cold ischemia times and their preference for using HTK or SPS-1 pancreas preservation solutions were likely additive in their reduced yields. There was evidence at all three processing steps that preservation with UW solution rather than HTK or SPS-1 was optimal for islet yields. SPS-1 had the poorest pancreas preservation at the longest cold ischemia times >15 hours by scatter plot analysis.

Summary of all of the processing donor variables and their significant effects on islet processing are provided in Table 8.

Table 8 Summary of processing variables effects on islet outcomes.

Process Results	Donor Age Increases	BMI <40 Increases	CIT Increases	Switch Time Increases	Increased Distance from Islet Isolation Site
Islet Yield					
Pre-Pure	No Change	No Change	Decrease‡	Decrease‡	Decrease‡
Post-Pure	Increase‡	No Change	Decrease‡	Decrease‡	Decrease‡
Post-Culture	Increase‡*	No Change	Decrease‡	Decrease‡	Decrease‡
Islet Index					
Pre-Pure	No Change	Increase‡	No Change	No Change	-
Post-Pure	No Change	Increase‡	No Change	Decrease‡	-
Post-Culture	No Change	No Change	Decrease‡	Decrease	-
GSIR	No Change	No Change	No Change	Decrease	-
Total Insulin Content	No Change	No Change	Decrease‡	No Change	-

***** The use of PIM(R)® culture medium for islets increases their size Post-Culture

‡ Statistically significant change

4.2.6 Process Variables Not Affecting Islet Yields

The Cause of Death of pancreas donors did not show any significant changes in the process success of purified, functional human islets. In addition to these factors, we did not notice any differences with minor pancreas changes such as mild inter-lobular edema, superficial hemorrhage, or superficial fatty infiltration.

5. Conclusions

The following conclusions are offered from these studies:

1. High quality and highly functional human islets can consistently be produced from Human Pancreas for Research and no longer need not be considered to be reduced in quantity or in quality compared to isolated islets processed from pancreases produced for clinical islet transplantation, assuming strict adherence to optimal pancreas processing, islet recovery, islet culture, and islet transport steps are accomplished.

2. The identity and control of pancreas and process variables permits the consistent and predictable outcomes in the production of high quality Human Islets for Research through the establishment of manufacturing procedures and standards.

3. The expression of pancreas processing islet yields as IEQ/Gm of pancreas is statistically superior to relying on IEQ per Pancreas process results to compare center to center processing outcomes due to large variations in the weights of human pancreases being processed into islets.

4. While there are major differences in the cause of death in non-diabetic, DCD donors versus non-diabetic, heart-beating donors, the proper restrictions of DCD donor conditions can permit

their consideration for processing into Human Islets for Research. These selected DCD donors have now been shown in this study to provide the same islet quality and quantities as non-DCD donors, with a small but significant reduction in Post-Culture islet quantities. The use of two critical limitations of a) <15 minutes of down time without CPR at the time of hospitalization and b) <31 minutes from extubation to cardiac arrest during the DCD was followed in this study and can expand the potential pool of Human Pancreas for Research as well as Human Islets for Research. It may be possible that these same limitations could expand the donated pancreas pool available for Clinical Islet Transplantation as well, since DCD donor pancreases have recently been published for both Clinical Pancreas Transplantation and Clinical Islet Transplantation, but are not universal.

5. The demonstration that significant numbers of high quality, functional human islets can be successfully processed from selected T2D donors increases the possibilities of providing more of these rarely processed diabetic islets for critically needed research efforts. The demonstrated limited ability of T2D islets to respond to higher glucose concentrations with lowered insulin content in this report suggests studies of T2D islets need more detailed investigations into the critical mechanisms involved. In addition, new studies utilizing T2D islets immediately after processing as well as after longer times of culture may provide more insights into whether these effects related to T2D are permanent or can be improved *in vitro* that could lead to new approaches for treating T2D in patients.

6. The two most significant process variables were using shorter Switch Times from 3 to 10 minutes to start collecting as well as shorter pancreas Cold Ischemia Times of <12 hours with UW preservation that resulted in significantly higher yields and quality of islets in this study. The Switch changes the Recycling Mode of enzyme flow through the D/FC to start the Collection Mode passing the chamber content out to collection tubes. This finding suggests that re-evaluation of human pancreas processing procedures currently using much longer digestion times may benefit by decreasing their process digestion times. This opportunity requires appropriate procedural changes in order to increase the quality and the quantity of isolated islets that could be available for transplantation. The restriction of using pancreas with shorter cold ischemia times to increase transplantable human islet yields can require that clinical islet processing centers consider reducing acceptance of pancreases from distant OPO's.

7. Additional significant processing variables included a positive trend on increasing age for islet yields after islet purification due to the embedded islets that reduce available islets from younger donors, and a positive trend on increasing BMI on Islet Indices before and after purification. While the optimal collagenase type was not evaluated in this study, an optimal islet tissue culture medium "spacing" was identified. Research efforts need to be focused on how to eliminate islet embeddedness currently reducing islet yields from young brain dead donors.

8. The optimal preservation solution for preserving human pancreases for processing into Human Islets for Research was shown to be the UW solution as compared to HTK or SPS-1 solutions, especially with longer cold ischemic duration.

6. Considerations

1. Re-evaluation of large scale processing of adult human islets for acute transplantation into T1D recipients required for the BLA should consider: a) the assumption that any large-scale islet processing for this treatment may routinely require two donors for each recipient for long-term

graft function, b) the routine use of appropriate antibiotics during the processing and culture of islets for a few days can reduce the relatively high incidence of microbial contaminated islet products currently being implanted into diabetic recipients under full immunosuppression, c) the identification of superior pancreas donors for islet processing may reduce the number of pancreases processed to achieve optimal results, and d) a transition from exploratory clinical islet transplantation techniques and outcomes to defined and proven effective methods with effective protocols is needed to develop clinical islet allograft transplantation as an improved therapy for selected patients with complications from T1D. All of these factors are important for a successful BLA for future clinical islet transplants.

2. Currently, in clinical islet auto-transplantation for chronic pancreatitis, the poorly purified islets are placed into the portal vein within a few hours in most centers with little or no islet purification or culture. Adapting the procedures from this study could permit the culture of more purified human islets by delaying the implant procedure through the placement of an injection catheter in a mesenteric vein at the time of pancreatectomy or relying on radiologic guided portal vein implants. This would permit the implant procedure to be performed several days later under local anaesthesia after effective islet purification, optimal culture with appropriate antibiotics, as well as a reduced microbial risk of contamination to the recipient of the cultured islet implant have been completed. This would result in more compact and highly purified islets that should have a higher chance of survival and longer function post-implant into the liver by removing the very large quantities of damaged non-islet tissue during the processing. It should certainly increase the chance of achieving an optimal success for these islet autograft recipients with anticipated longer duration of their islet graft function.

List of Abbreviations

BD – Becton Dickinson Company, BSC – Biological Safety Cabinet, BLA – Biological License Approval, BMI – Body Mass Index, C – Centigrade, CA – California, CITR – Clinical Islet Transplant Report, CITS – Clinical Islet Transplant Sites, CMRL 1066 – tissue culture medium, COBE 2991 – Cobe Blood Processor, CIT – Cold Ischemia Time, CPR – CardioPulmonary Resuscitation, DCD – Donation after Cardiac Death, D/FC – Digestion/Filtration Chamber, DM – Diabetes Mellitus, DMSO – Dimethyl sulfoxide, DNA – Deoxyribonucleic acid, DTZ – Diphenylthiocarbazone, FDA - Federal Drug Administration, Gm – Gram, GMP – Good Manufacturing Practices, GSIR – Glucose Stimulated Insulin Release, GSI – Glucose Stimulation Index, HBSS – Hanks Balanced Salt Solution, HgbA1c – Hemoglobin A1c, HEPA – High Efficiency Particulate Air, HIR – Human Islets for Research, HIT – Human Islets for Transplantation, HPR – Human Pancreas for Research, HPT – Human Pancreas for Transplantation, HSA – Human Serum Albumen, HTK - Histidine-Tryptophan-Ketoglutarate organ preservation solution, IBMX - 3-isobutyl-1-methylxanthine, IEQ – Islet Equivalent, IIAM – International Institute for the Advancement of Medicine, IIDP – Integrated Islet Distribution Program, IP – Intra-peritoneal, IRB – Institutional Review Board, ISO - International Organization for Standardization, IBMX - 3-isobutyl-1-methylxanthine, JDRF – Juvenile Diabetes Research Foundation, M – Molar, mM – Millimolar, ml – Milliliter, ng – Nanogram, NAIDS - North American Islet Donor Score, NIH – National Institutes of Health, NHP – Non-Human Primates, Non-DM – Non-Diabetes Mellitus, OPO – Organ Procurement Organization, OPS - Organ Preservation Solution, PEG – Poly(ethylene glycol), Prodo – Prodo Laboratories, SLRI – Scharp-Lacy Research

Institute, SOP – Standard Operating Procedure, SPS-1 – Static Preservation Solution for Organs, SubQ – Subcutaneous, T1D – Type 1 Diabetes, T2D – Type 2 Diabetes, UNOS – United Network of Organ Sharing, USA – United States of America, USPTO – United States Patent and Trademark Office, UW Solution – University of Wisconsin Organ Preservation Solution.

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Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Figure S1: Current digestion/filtration circuit for human pancreas processing.
2. Figure S2: Digestion/Filtration samples from a T2D human pancreas process.
3. Figure S3: Purification samples from the same T2D human pancreas process.
4. Figure S4a: Prodo custom islet image analyser;
   Figure S4b: Output from Islet Scanner.

Author Contributions

David W. Scharp, MD – Senior scientist in islet isolation, culture, and transplantation involved in all portions of the human pancreas processing from preparing the pancreas, its distention and digestion, as well as islet purification, culture, and transportation. He also is the primary author of this report.

Jayagawri Arulmoli – Senior Islet Scientist working in all aspects of human islet processing and research and manager of the other contributors. She was involved in preparing the media and reagents used in the islet isolation, islet culture, and shipping islets. She performed the long term islet culture study in this paper. She performed, taught, and directed the GSIR assay, Picogreen assay, ELISA assay, and compiled the ELSA data for this publication. She also oversaw the compilation of data, tables, and graphs for this publication.

Kelly Morgan – Research Associate was part of the team which prepared the media and reagents, and performed islet isolation, culturing, and shipping of the islets and performed the GSIR assay and ELISA assay. She was involved in compilation of data, statistical analysis of the data preparing tables and graphs and final formatting of the paper.

Hannah Sunshine – Research Associate was part of the team which prepared the media and reagents, and performed islet isolation, culturing, and shipping of the islets and performed the GSIR assay and ELISA assay. She was involved in compilation of data, statistical analysis of the data preparing tables and graphs and final formatting of the paper.

Ergeng Hao, MD, MSC – Senior Scientist was involved in performing surgery on the pancreas to isolate islets, and was a part of the team which performed islet isolation. He was also involved in preparing the media and reagents used in the islet isolation, culturing of islets and shipping the islets. He performed the Picogreen assay to quantify the DNA content of the islets that were used in the GSIR assay. He participated in the preparation and editing the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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