

RESEARCH ARTICLE

High-mannose type *N*-glycans with core fucosylation and complex-type *N*-glycans with terminal neuraminic acid residues are unique to porcine islets

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Abstract

Objectives

Islet transplantation is an emerging treatment option for type 1 diabetes but its application is limited by the shortage of human pancreas donors. Characterization of the *N*- and *O*-glycan surface antigens that vary between human and genetically engineered porcine islet donors could shed light on targets of antibody mediated rejection.

Methods

N- and *O*-glycans were isolated from human and adult porcine islets and analyzed using matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and electrospray ionization mass spectrometry (ESI-MS/MS).

Results

A total of 57 porcine and 34 human *N*-glycans and 21 porcine and 14 human *O*-glycans were detected from cultured islets. Twenty-eight of which were detected only from porcine islets, which include novel xenoantigens such as high-mannose type *N*-glycans with core fucosylation and complex-type *N*-glycans with terminal neuraminic acid residues. Porcine islets have terminal *N*-glycolylneuraminic acid (NeuGc) residue in bi-antennary *N*-glycans and sialyl-Tn *O*-glycans. No galactose- α -1,3-galactose (α -Gal) or Sd^a epitope were detected on any of the islets.

Conclusions

These results provide important insights into the potential antigenic differences of *N*- and *O*-glycan profiles between human and porcine islets. Glycan differences may identify novel gene targets for genetic engineering to generate superior porcine islet donors.

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: BJH has an equity interest in and serves as an executive officer of Diabetes-Free, Inc, an organization that may commercially benefit from the results of this research. This interest has been reviewed and managed by the University of Minnesota in accordance with its Conflict of Interest policies, and does not alter our adherence to PLOS ONE policies on sharing data and materials.

Introduction

Phase 3 trials of transplantation of human pancreatic islets have highlighted the potential of cell replacement therapies in type 1 diabetes [1, 2]. Though highly effective, the applicability of islet transplantation is limited by the shortage of human donor pancreases. Porcine donors are a promising alternative source of islets in light of their similar physiology and size compared with humans, their high reproductive capacity, and the potential for genetic manipulation [3, 4]. Preclinical studies and initial clinical trials of islet xenotransplantation have demonstrated both the safety of porcine islet cell products and the need for developing new and improved strategies for preventing their rejection [5–16].

Carbohydrates (glycans) are one of the major classes of biomolecules found on cell surfaces and play a critical role in biological processes such as organ development and immunity [17]. They are synthesized in the endoplasmic reticulum, modified in the Golgi apparatus, and transferred to the plasma membrane [18]. As glycan expression varies depending on species, strain, individual, organs, and cell types, detailed qualitative and quantitative structural information on the target organ/animal is required. The discovery of the galactose- α -1,3-galactose (α -Gal) epitope that is present in pigs but is absent in humans and nonhuman primates (NHPs) allowed the prevention of hyperacute rejections in pig-to-NHP cardiac and renal xenotransplantation [19, 20]. Recent *in vitro* studies have demonstrated the importance of deleting N-glycolylneuraminic acid (NeuGc) and Sd^a antigen on porcine donor cells for preventing rejection of planned solid organ xenotransplants in humans [21–25].

The tissue-specific expression of glycan antigens in primarily avascular islet cell xenografts remains incompletely understood. The α -Gal antigen is expressed only on very few adult porcine islet endocrine cells and does not cause their hyperacute rejection after xenotransplantation in NHPs [7, 26, 27]. To identify potential new targets for genetic engineering of porcine donors tailored for use in islet xenotransplantation, the current study was conducted to compare N- and O-glycan profiles between human and porcine islets using mass spectrometric analysis. We found unique differences in high-mannose and complex-type N-glycan profiles between human and porcine islets, as well as the presence of NeuGc structures in N- and O-glycans of porcine islets.

Materials and methods

Pig and human islet preparation

Seven adult female pigs (*Sus scrofa*; six Mangalita and one Landrace x Yorkshire), with a median age of 3 years (range: 3–4) and a median weight of 206 kg (170–247), were evaluated as donors of islet xenografts. Aliquots of two adult human islet preparations were purchased from PRODO Laboratories Inc. (Aliso Viejo, CA) and one adult human islet preparation deemed unsuitable for clinical transplantation was provided with appropriate consent by our local islet transplant program.

Porcine islets were isolated as previously described [7]. Briefly, donor pig was anesthetized with tiletamine-zolazepam (Telazol[®]; Zoetis, Parsippany-Troy Hills, NJ) before exsanguination, and retrieved pancreas tissue was dissociated with collagenase and neutral protease. Liberated islets were purified from non-islet tissue by continuous density gradient centrifugation on a COBE 2991 cell separator (Terumo BCT, Lakewood, CO), and cultured free-floating in ME 199 media supplemented with 10% heat-inactivated porcine serum (HyClone, Logan, UT), L-glutamine and heparin (10 U/mL) at 37°C in humidified air without CO₂. From each donor islet preparation, 5,000 islet equivalents of islets were collected and shipped to the Complex Carbohydrate Research Center at the University of Georgia (Athens, GA) in culture

media for further analysis. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee and conducted in compliance with the Animal Welfare Act and adhere to principles stated in the Guide for Care and Use of Laboratory Animals (Protocol Number: 1907-37282A). For the purpose of using human islets, this project was reviewed by the Institutional Review Board of the University of Minnesota and was determined that it does not meet the regulatory definition of human subjects research, as defined by DHHS and FDA and therefore exempt.

Sample preparation

Islet samples were centrifuged at 400 rcf for 3 min and the pellet was washed five times with 1x PBS, followed by lipid extraction by the Folch method (using chloroform, methanol, and water) [28]. After lipid extraction, the sample was subjected to cold acetone:water precipitation producing a protein-rich powder.

Release of N-glycans

An aliquot of the protein-rich powder was digested with trypsin in Tris-HCl buffer overnight. After protease digestion, the sample was passed through a C18 Sep-Pak cartridge, washed with a 5% acetic acid, and the glycopeptides were eluted with a blend of isopropanol in 5% acetic acid.

The glycopeptide eluate was treated with PNGase F to release the N-glycans and the digest was passed through a C18 Sep-Pak cartridge to separate the N-glycans from O-glycopeptide fraction. The N-glycans fraction was eluted first with 5% acetic acid followed by elution of O-glycopeptides into another container with a blend of isopropanol and 5% acetic acid. After lyophilization, the N-glycans fraction was permethylated for mass spectrometry [29].

Release of O-glycans

The O-glycopeptide fraction from each sample was subjected through reductive beta-elimination procedure using sodium borohydride in sodium hydroxide solution to cleave the O-linked glycans from the peptides. Subsequently, released O-glycans were cleaned up using acid foam (H⁺) ion exchange resin and permethylated for mass spectrometry [29].

Per-O-methylation of N-linked glycans

The N- and O-glycans were permethylated for structural characterization by mass spectrometry [30]. Briefly, the dried eluates were dissolved with dimethylsulfoxide (DMSO) and methylated with NaOH-DMSO base and methyl iodide. The reaction was quenched with water and per-O-methylated carbohydrates were extracted with methylene chloride and dried under N₂.

Profiling by Matrix-Assisted Laser-Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

The permethylated glycans were dissolved with methanol and crystallized with α -dihydroxybenzoic acid (20 mg/mL in 50% methanol: water) matrix. Analysis of glycans present in the samples was performed in the positive ion mode by MALDI-TOF-MS using AB SCIEX TOF/TOF 5800 mass spectrometer (Applied Biosystem/ MDS Analytical Technologies).

Profiling by Electrospray Ionization Mass Spectrometry (ESI-MS/MS) and Higher-Energy Collisional Dissociation Tandem Mass Spectrometry (HCD-MS/MS)

We conducted ESI-MS/MS to confirm the structure of permethylated glycans. In silico fragmentation of structures predicted based on common mammalian biosynthetic pathway were generated through GlycoWorkbench software. The presence of these fragments was examined on the ESI-MS/MS spectrum of each glycan. If isomers are present or alternate structures are possible, we changed the structural assignments accordingly in order to match the corresponding ESI-MS/MS spectrum.

Aliquot from each permethylated sample glycans was analyzed by ESI-MS/MS (Thermo Orbitrap Fusion Tribrid mass spectrometer) to collect both full mass and MS/MS fragmentation data. Permethylated glycans from the samples were infused into the mass spectrometer through a nano-electrospray ionization (NSI) probe. The MS1 and MS2 spectra (Higher-energy Collisional Dissociation, HCD) of the glycans were acquired at high resolution by a simple precursor scan and total ion monitoring program respectively.

Data processing of MALDI-TOF-MS, ESI-MS and ESI-MS/MS data

Data Explorer 4.5 (MALDI-TOF-MS data) and XCalibur 4.2 (ESI-MS and ESI-MS/MS data) software were used in order to extract the raw MS data. The quantitation of glycoforms were conducted from MALDI-TOF-MS spectra by calculating the relative intensity of glycan peaks. The glycoworkbench analysis were conducted for the structural assignment of glycans without any further processing of data and by manually entering the values into the software.

Results

Characterization of N-glycans from pig and human islets

The representative MALDI-TOF-MS and ESI-MS full scan spectra of the N-glycans are shown in Fig 1, and the proposed glycan structures and their percentages are summarized in Fig 2. A total of 57 and 34 N-glycans were detected from the porcine and human islets, respectively, 21 of which were only observed from pig islets (Fig 2). High mannose type structures, which included a few fucosylated species, consisted of the majority of the N-glycans detected from the glycoprotein. Man5GlcNAc2 (m/z 1580) and Man6GlcNAc2 (m/z 1784) showed the largest signals of all possible N-glycans of porcine islets (Fig 2). Man3GlcNAc2 (m/z 1172) and Man3-GlcNAc2Fuc1 (m/z 1346), as well as Man5GlcNAc2 and Man6GlcNAc2, showed large signals in human islets. There were 2 high mannose type N-glycans with core fucosylation (m/z 1550 and 1754) annotated in porcine islets, both of which were only found from porcine islets (Fig 3) [31, 32].

Approximately 4% of the total N-glycans were with terminal neuraminic acid residue, including the two signals (m/z 2997 and 3027) that appear to be bi-antennary oligosaccharides contained NeuGc (Fig 4) [33]. Sialylated N-glycans in tri- and tetra-antennary structures and hybrid-type structure were only found from porcine islets (Figs 4 and 5) [33]. Moreover, tetra-antennary N-glycans with LacNAc elongations (with or without N-acetylneuraminic acid [NeuAc] terminal) are only found from porcine islets (Fig 5). Three N-glycans with Lewis type structures (m/z 2418, 2592, and 3216) were detected from human islets (Fig 2).

Characterization of O-glycans from pig and human islets

The representative MALDI-TOF-MS and ESI-MS full scan spectra of the O-glycans are shown in Fig 6, and the proposed glycan structures and their percentages are summarized in Fig 7.

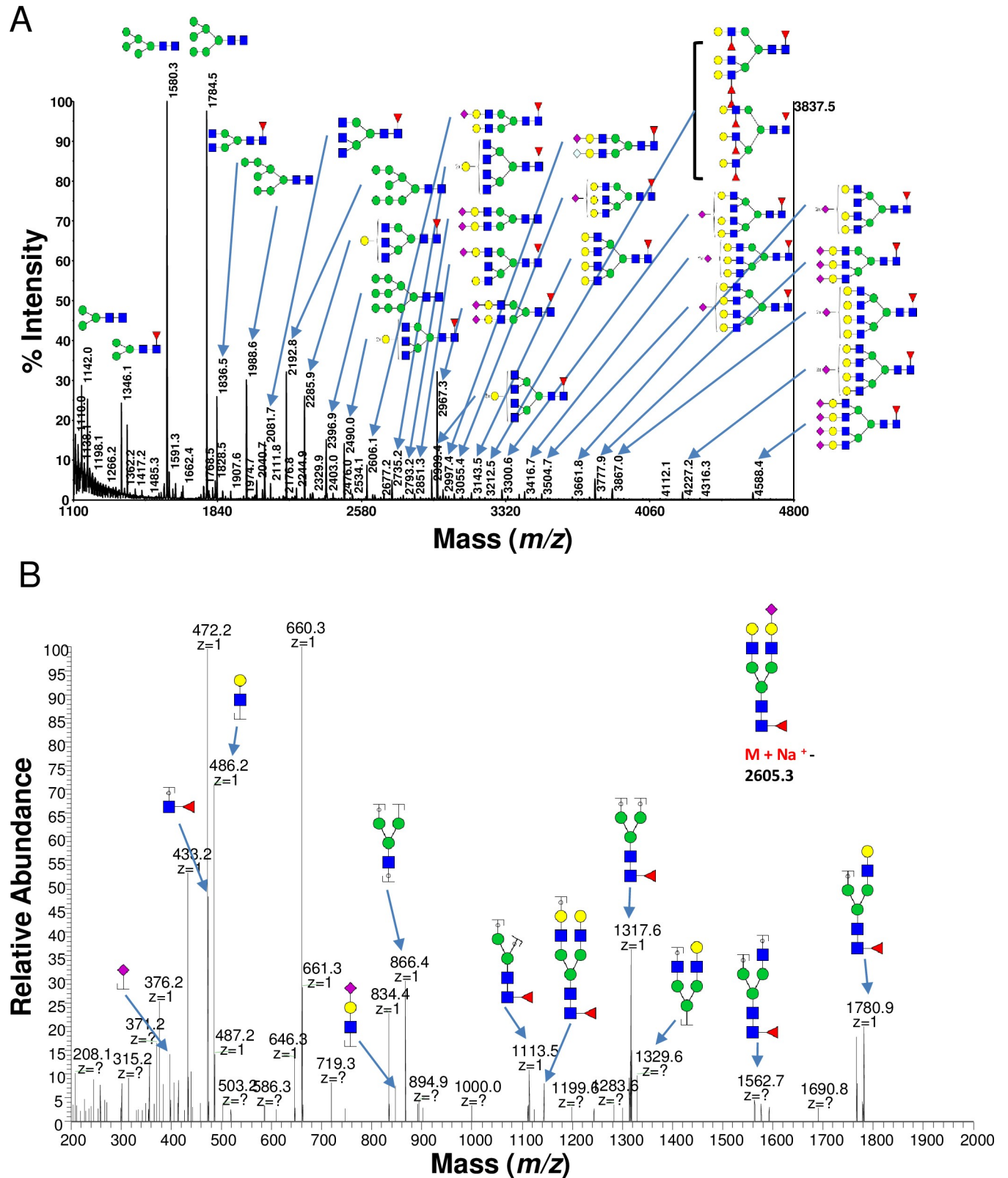


Fig 1. The representative MALDI-TOF-MS and ESI-MS/MS spectrum of N-glycans. MALDI-TOF-MS spectrum of permethylated N-glycans released by PNGaseF (A) and representative HCD MS2 fragmentation spectra at m/z 884 (3+) $[M + Na]^+ \rightarrow 2605.3$ (B).

<https://doi.org/10.1371/journal.pone.0241249.g001>

| Mass (m/z) | Text description of structures | Cartoon representation of structures | Porcine islets (%) ² | | | | | | | Human islets (%) ² | | | Uniqueness |
|------------|---|--------------------------------------|---------------------------------|-------|-------|-------|-------|-------|-------|-------------------------------|----------|----------|------------|
| | | | P1103 | P1104 | P1111 | P1134 | P1136 | P1137 | P1138 | H1081 | HP 13049 | HP 15059 | |
| 1172 | Man ₅ GlcNAc ₂ | | 13.9 | 2.5 | 5.2 | 2.7 | 4.0 | 4.1 | 3.9 | 8.8 | 11.4 | 14.5 | Shared |
| 1346 | Man ₅ GlcNAc ₂ Fuc ₁ | | 11.3 | 6.1 | 4.3 | 1.9 | 3.8 | 6.3 | 2.7 | 10.9 | 24.3 | 15.8 | Shared |
| 1376 | Man ₅ GlcNAc ₂ | | 8.0 | 2.1 | 3.3 | 1.3 | 2.3 | 2.6 | 2.1 | 2.5 | 2.5 | 2.4 | Shared |
| 1417 | GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.6 | 0.6 | 0.7 | 1.1 | 0.4 | 0.5 | 0.6 | 0 | Shared |
| 1550 | Man ₅ GlcNAc ₂ Fuc ₁ | | 0 | 0 | 0.4 | 0 | 0 | 0.9 | 0.3 | 0 | 0 | 0 | Pig Only |
| 1580 | Man ₅ GlcNAc ₂ | | 32.7 | 40.5 | 19.1 | 20.3 | 25.7 | 31.2 | 28.3 | 18.5 | 16.2 | 40.8 | Shared |
| 1591 | GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0.6 | 1.2 | 0.5 | 0.7 | 1.3 | 0.6 | 1.3 | 0.9 | 0 | Shared |
| 1621 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.3 | 0 | 0 | 0 | 0.2 | 0.8 | 0.7 | 0.6 | Shared |
| 1662 | GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.6 | 2.0 | 0.5 | 0 | 0.2 | 0 | 0.6 | 0 | Shared |
| 1754 | Man ₅ GlcNAc ₂ Fuc ₁ | | 0 | 0 | 0.3 | 0 | 0 | 0 | 0.3 | 0 | 0 | 0 | Pig Only |
| 1784 | Man ₅ GlcNAc ₂ | | 24.6 | 30.4 | 19.5 | 25.8 | 29.0 | 35.4 | 36.9 | 21.1 | 12.8 | 9.2 | Shared |
| 1795 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 | 0.8 | 0.8 | 0.4 | Shared |
| 1826 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.3 | 0 | 0 | 0 | 0 | 0 | 0.3 | 0 | Shared |
| 1836 | GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 1.9 | 4.9 | 24.1 | 1.3 | 1.5 | 1.0 | 2.5 | 1.8 | 0.4 | Shared |
| 1866 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.4 | 0.8 | 0 | 0 | 0 | 0 | 0.6 | 0 | Shared |
| 1907 | GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.4 | 0 | 0 | 0 | 0.2 | 0 | 0.4 | 0 | Shared |
| 1988 | Man ₅ GlcNAc ₂ | | 5.9 | 6.2 | 6.0 | 6.5 | 7.2 | 7.2 | 9.2 | 10.1 | 5.1 | 2.7 | Shared |
| 2030 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0 | Shared |
| 2041 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0.6 | 0.9 | 2.9 | 0 | 0 | 0.2 | 0 | 0.7 | 0 | Shared |
| 2071 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.5 | 0 | 0 | 0 | 0 | 0 | 1.1 | 0.7 | Shared |
| 2082 | GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0.6 | 2.6 | 0.6 | 0.7 | 0.6 | 0.5 | 1.8 | 1.3 | 0 | Shared |
| 2112 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.8 | 0 | 0 | 0 | 0 | 0 | 0.6 | 0.4 | Shared |
| 2157 | NeuAc ₁ Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Pig Only |
| 2193 | Man ₅ GlcNAc ₂ | | 3.7 | 5.7 | 6.3 | 5.7 | 6.4 | 6.3 | 8.9 | 7.6 | 5.9 | 3.5 | Shared |
| 2228 | NeuAc ₁ Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Pig Only |
| 2245 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0.3 | 0.6 | 0.3 | 0 | 0 | 0.2 | 0.9 | 1.1 | 0.5 | Shared |
| 2286 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0.8 | 4.7 | 0.3 | 0.5 | 0 | 0.4 | 0 | 0.3 | 0 | Shared |
| 2316 | Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0 | Shared |
| 2327 | GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0 | 0.3 | 0 | 0 | 0 | 0 | 2.8 | 0.6 | 0 | Shared |
| 2391 | NeuAc ₁ Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ | | 0 | 0 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Pig Only |
| 2397 | Man ₅ GlcNAc ₂ | | 0 | 1.5 | 2.6 | 1.9 | 1.9 | 1.7 | 2.9 | 6.1 | 4.1 | 2.9 | Shared |
| 2402 | NeuAc ₁ Gal ₁ GlcNAc ₁ Man ₃ GlcNAc ₂ Fuc ₁ | | 0 | 0 | 0.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Pig Only |
| 2418 | Gal ₁ GlcNAc ₁ Fuc ₁ Man ₃ GlcNAc ₂ Fuc ₁ or Gal ₁ GlcNAc ₁ Fuc ₂ Man ₃ GlcNAc ₂ | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.6 | 1.1 | 3.1 | Human Only |

Fig 2. Summary of N-linked glycans detected from adult porcine and human islets by MALDI-TOF-MS and ESI-MS. The structures were confirmed by ESI-MS/MS. ¹All masses (mass+Na) are permethylated and single-charged values measured by MALDI-TOF-MS. The structure assignment were based on common biosynthetic pathway and ESI-MS/MS fragmentation by HCD. ²% N-glycans were calculated from the area units of detected N-glycans.

<https://doi.org/10.1371/journal.pone.0241249.g002>

The data from two human islet samples were not obtained due to detergent contamination on the sample which interfered with lower mass O-glycan peaks. A total of 21 porcine and 14 human O-glycans were detected from islet samples, respectively. Core 1 (m/z 534, also known as T antigen) and sialylated core 1 (m/z 896) showed the largest signals of all possible O-glycans of porcine islets, followed by disialylated core 1 (m/z 1257). Most of the O-glycans detected from human islets contained the core 1 structure. Three signals that possibly bear the terminal NeuGc residue were observed (m/z 722, 926, and 1317) from porcine islets, including sialyl-Tn antigen (m/z 722). Lewis type structures such as (Gal)-(FucGlcNAc) and (FucGal)-(FucGlcNAc) were present on the terminal of O-glycans of higher mass from sample H1081. To determine the presence of the Sd^a antigen, all sialic acid bearing ions from MS/MS spectra were evaluated, however, the presence of the Sd^a antigen was not detected.

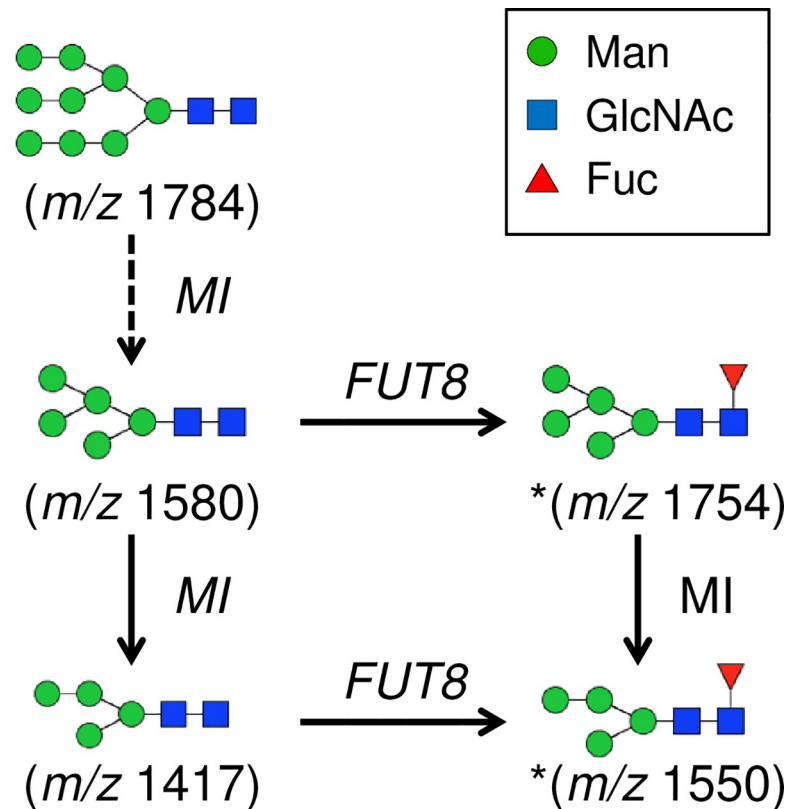


Fig 3. Possible biosynthetic pathways of high-mannose type N-glycans with the fucosylated core. FUT8, α 1,6-fucosyltransferase; MI, α -mannosidase I.

<https://doi.org/10.1371/journal.pone.0241249.g003>

Discussion

The demonstration of prolonged diabetes reversal after porcine islet xenotransplantation in NHPs suggests that porcine islets could be developed into a widely available cell source for cell replacement therapy in diabetes. To work toward this end, safer and more effective strategies for preventing islet xenograft rejection will be necessary. Understanding the differences in carbohydrate antigens expressed on porcine and human islets could minimize the immunogenicity of islets from porcine donors custom-engineered for use in islet xenotransplantation.

Our MALDI-TOF-MS and ESI-MS/MS analysis of glycans identified differences between porcine and human islets. Our results confirm that porcine islets have NeuGc in N- and O-glycan forms, albeit no α -Gal or Sd^a epitope was detected from any of the islets (Figs 2 and 7). The study also proposed novel glycan structures that exist only in porcine islets; the high-mannose type N-glycans with core fucosylation and complex-type N-glycans with terminal neuraminic acid residues, which can be potential gene targets for genetic engineering to generate superior porcine islet donors (Figs 3–5).

Our findings build upon two series of studies that performed qualitative and quantitative analyses of carbohydrate antigens of porcine islets using mass spectrometry (Table 1) [34–38]. Kim *et al.* detected 80 N-glycans, which includes NeuGc epitopes and a negligible amount of α -Gal epitope, but they did not include in their analysis a direct comparison between porcine and human islet glycan profiles [34, 35]. Miyagawa *et al.* compared porcine and human islets and reported that 12 of 28 and 9 of 24 N-glycans found from adult and neonatal wildtype pigs were detected exclusively on porcine islets, respectively [36, 38]. Within a larger number of 7

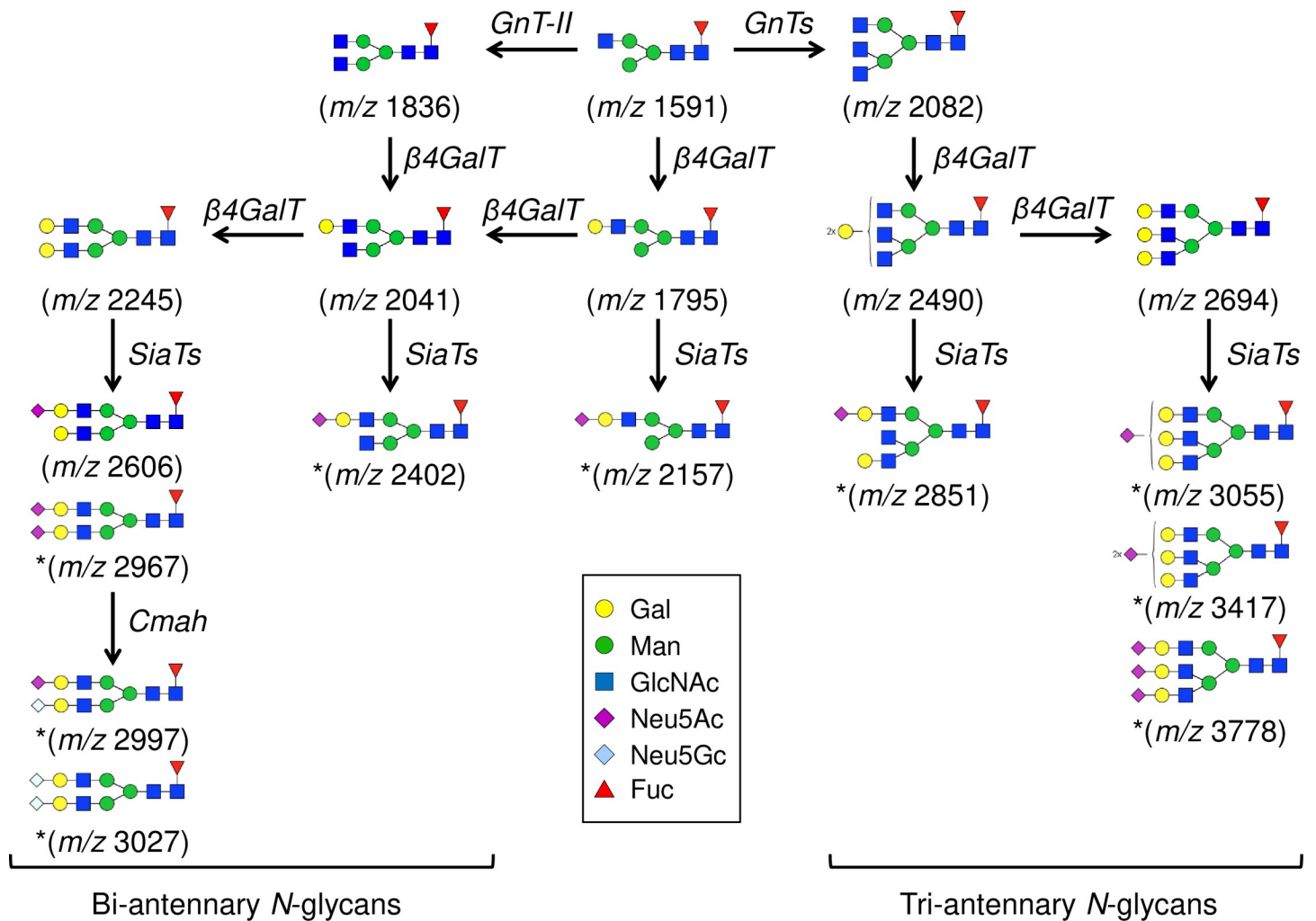


Fig 4. Biosynthetic pathways of bi- and tri-antennary N-glycans. $\beta 4GalT$, beta-1,4-galactosyltransferase; Cmah, cytidine monophospho-N-acetylneuraminic acid hydroxylase; GnT, N-Acetylglucosaminyltransferases; SiaTs, Sialyltransferases.

<https://doi.org/10.1371/journal.pone.0241249.g004>

porcine donors, we demonstrated 57 N-glycans and 21 O-glycans including the NeuGc epitope and other potential non- α -Gal antigens, although further studies are needed to confirm these findings as comparison to only 3 and 1 human samples were performed with respect to N- and O-glycan analysis, respectively. All these studies including ours confirm a negligible amount of α -Gal epitopes and a considerable amount of NeuGc epitopes expressed on adult porcine islets. The number of identified glycans differs between the porcine donors used in our study and donors used in previous studies, which may be attributed to the culture conditions or islet isolation methods. Although direct comparison between the studies may be difficult because of these reasons, the two tri-antennary N-glycans (m/z 2157 and 2402) were only found in adult porcine islets in ours and Miyagawa's studies [36].

An abundance of high-mannose type N-glycans is assumed to be a typical feature of islets [36]. In the current study, among the high-mannose type N-glycans, a rare type of the high-mannose N-glycan that has a fucosylated core (m/z 1550 and 1754) were measured from porcine islets. These glycans are previously detected from porcine cathepsin D or lamia bean lectin [39, 40]. Burlak *et al.* has recently described that anti-fucose antibodies in human serum are

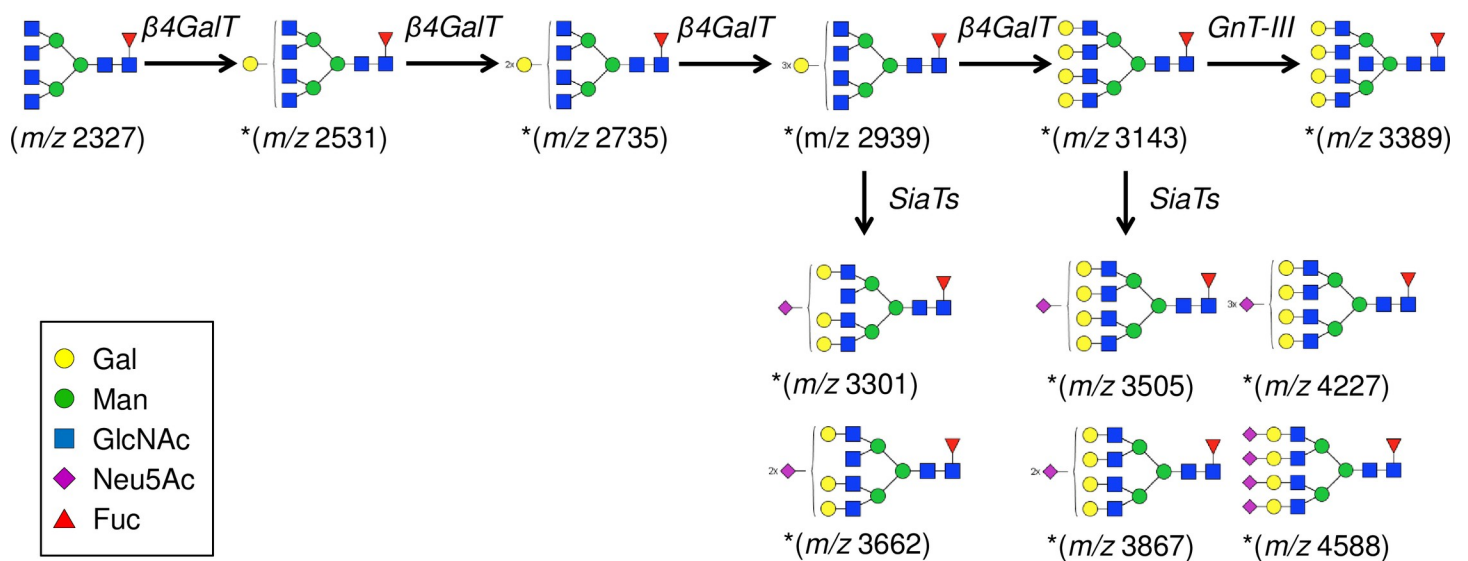


Fig 5. Biosynthetic pathways of tetra-antennary N-glycans. $\beta 4\text{GalT}$, beta-1,4-galactosyltransferase; SiaTs, Sialyltransferases.

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involved in the antibody-mediated rejection of xenogeneic porcine tissue from donors lacking α -Gal and NeuGc epitopes [23]. It may be reasonable to include high-mannose N-glycans with the fucosylated core in future studies to more thoroughly understand xenograft rejection.

In the current study, up to 16% of total N-glycans on porcine islets had terminal neuraminic acid residues. Interestingly, all detected sialylated N-glycans in tri- and tetra-antennary forms or the hybrid form, which were also detected by Kim *et al.* [34], were only measured from porcine islets but not from human islets. Human islet sialylated N-glycans were only found in the bi-antennary form. These findings are in line with the previous study by Komoda *et al.* that demonstrated that the origin of xenoantigenicity of porcine islets is mainly N-glycans including sialic acid antigens (NeuAc and NeuGc) [41].

In addition to the antigens that we discussed above, it has been proposed that Thomsen-Friedenreich antigen (T antigen), T and sialyl-Tn antigens, P antigen, and I or i antigens are potential non- α -Gal carbohydrate antigens against which humans have naturally occurring antibodies [42]. Among these antigens, we successfully observed sialyl-Tn antigens (m/z 692 and 721) exclusively in porcine islets (Fig 7), which also can be a target of rejection. To the best of our knowledge, this is the very first study that analyzed O-glycan structures of pig and human islets. However, as mentioned above, the condition of the islet isolation and culture might affect the result of the analysis and further studies are warranted to confirm these findings.

In this study, islets from 3-year old adult pigs were examined for N- and O-glycan analyses building on studies of glycan expression previously reported by age [43] Future studies should examine the glycan expression of neonatal porcine islets to describe alternative age donors for clinal transplantation.

In conclusion, evidence presented in this study indicates that core-fucosylated high-mannose N-glycans and complex-type N-glycans with terminal neuraminic acid residue are unique structures found on porcine islets. Also, the structural analysis suggested that NeuGc structures are found as a part of mature bi-antennary N-glycans or sialylated Tn antigens in O-glycans. This study underscores the opportunities associated with improved understanding of specific N- and O-glycans expressed on porcine islets. Future studies will focus on the antigen-specific reactions against these novel glycan structures, which can be a potential target of xenoreactive antibodies.

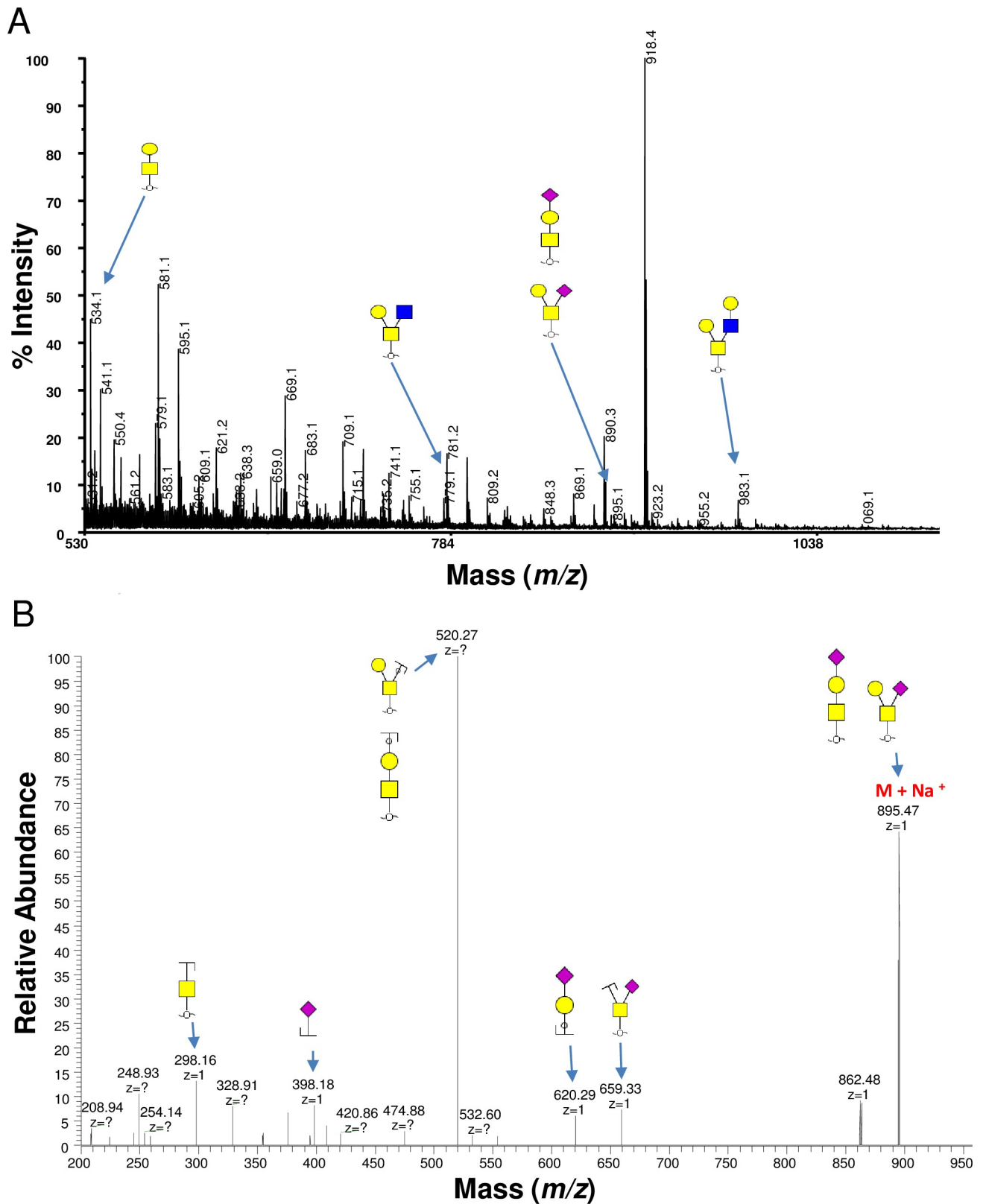


Fig 6. The representative MALDI-TOF-MS and ESI-MS/MS spectrum of O-glycans. MALDI-TOF-MS spectrum of permethylated O-glycans released by β -elimination (A) and representative HCD MS2 fragmentation spectra at m/z 895 $[M + Na]^+$ (B).

<https://doi.org/10.1371/journal.pone.0241249.g006>

| Mass (<i>m/z</i>) ¹ | Text description of structures | Cartoon representation of structures | Porcine islets (%) ² | | | | | | | Human islets (%) ² | | | Uniqueness |
|-------------------------------------|--|--|---------------------------------|-------|-------|-------|-------|-------|-------|-------------------------------|---------|---------|------------|
| | | | P1103 | P1104 | P1111 | P1134 | P1136 | P1137 | P1138 | H1081 | HP13049 | HP15059 | |
| 534 | Gal ₁ GalNAc ₁ |  | 70 | 75.1 | 51.8 | 41.9 | 45.4 | 83.6 | 47.7 | 16.5 | NA | NA | Shared |
| 575 | GlcNAc ₁ GalNAc ₁ |  | 0 | 0 | 1.0 | 0 | 0 | 0 | 0 | 9.1 | NA | NA | Shared |
| 691 | NeuAc ₁ GalNAc ₁ |  | 0 | 0 | 2.2 | 0 | 0 | 0 | 0 | 0 | NA | NA | Pig Only |
| 721 | NeuGc ₁ GalNAc ₁ |  | 0 | 0 | 1.5 | 0 | 0 | 0 | 0 | 0 | NA | NA | Pig Only |
| 779 | GlcNAc ₁ Gal ₁ GalNAc ₁ |  | 14.5 | 7.2 | 6.9 | 0 | 0 | 0 | 0 | 4.1 | NA | NA | Shared |
| 820 | GlcNAc ₂ GalNAc ₁ |  | 0 | 0 | 1.5 | 0 | 0 | 0 | 0 | 0 | NA | NA | Pig Only |
| 895 | NeuAc ₁ Gal ₁ GalNAc ₁ |  | 5.5 | 9.6 | 12.0 | 42.5 | 46.3 | 14.2 | 46.5 | 17.8 | NA | NA | Shared |
| 925 | NeuGc ₁ Gal ₁ GalNAc ₁ |  | 0 | 2.0 | 2.6 | 0 | 0 | 0 | 0 | 0 | NA | NA | Pig Only |
| 936 | NeuAc ₁ GlcNAc ₁ GalNAc ₁ |  | 0 | 0 | 0.7 | 0 | 0 | 0 | 0 | 0 | NA | NA | Pig Only |
| 984 | Gal ₁ GlcNAc ₁ Gal ₁ GalNAc ₁ |  | 10.1 | 5.0 | 12.5 | 0 | 0 | 0 | 0 | 6.5 | NA | NA | Shared |
| 1025 | Gal ₁ GlcNAc ₂ GalNAc ₁ |  | 0 | 0 | 0.7 | 0 | 0 | 0 | 0 | 0 | NA | NA | Pig Only |
| 1158 | Gal ₁ Fuc ₁ GlcNAc ₁ Gal ₁ GalNAc ₁ | or  or  or  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7.4 | NA | NA | Human Only |
| 1257 | NeuAc ₂ Gal ₁ GalNAc ₁ |  | 0 | 1.1 | 5.5 | 15.6 | 8.3 | 2.2 | 5.8 | 19.5 | NA | NA | Shared |
| 1317 | NeuGc ₂ Gal ₁ GalNAc ₁ |  | 0 | 0 | 0.6 | 0 | 0 | 0 | 0 | 0 | NA | NA | Pig Only |
| 1332 | Gal ₁ Fuc ₂ GlcNAc ₁ Gal ₁ GalNAc ₁ | or  or  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.6 | NA | NA | Human Only |
| 1345 | Gal ₁ GlcNAc ₁ NeuAc ₁ Gal ₁ GalNAc ₁ |  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.1 | NA | NA | Human Only |
| 1519 | NeuAc ₁ Gal ₁ GlcNAc ₁ Fuc ₁ Gal ₁ GalNAc ₁ | or  or  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.9 | NA | NA | Human Only |
| 1693 | Gal ₁ Fuc ₂ GlcNAc ₁ NeuAc ₁ Gal ₁ GalNAc ₁ |  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.6 | NA | NA | Human Only |
| 1706 | NeuAc ₂ Gal ₁ GlcNAc ₁ Gal ₁ GalNAc ₁ |  | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 | 1.8 | NA | NA | Shared |
| 1968 | Gal ₂ Fuc ₁ GlcNAc ₂ NeuAc ₁ Gal ₁ GalNAc ₁ |  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.6 | NA | NA | Human Only |
| 2142 | Gal ₂ Fuc ₂ GlcNAc ₂ NeuAc ₁ Gal ₁ GalNAc ₁ | or  or  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.5 | NA | NA | Human Only |

■ GlcNAc (N-acetylglucosamine) ● Gal (galactose) ▲ Fuc (fucose) ◆ NeuAc (N-acetylneuraminic acid) ◇ NeuGc (N-glycolylneuraminic acid)

Fig 7. Summary of O-linked glycans detected from adult porcine and human islets by MALDI-TOF-MS and ESI-MS/MS. ¹All masses (mass +Na) are permethylated and single-charged values measured by MALDI-TOF-MS. The structure assignment were based on common biosynthetic pathway and ESI-MS/MS fragmentation by HCD. ²% O-glycans were calculated from the area units of detected O-glycans.

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Table 1. Characteristics of mass spectrometry studies for pig islets.

| Study | Islet source | Age | # of analysis | Methods | Target | # of detected glycans | α -Gal | NeuGc | Proposed potential non- α -Gal antigens |
|---------------|---|----------|----------------|-------------------------------|------------------------|-----------------------|----------------|--------|---|
| Kim [34] | SPF CMS miniature pig | N/A | 1 | MALDI-TOF-MS, ESI-MS/MS, HPLC | N-Glycans | 80 | 1 ¹ | 13 | N/A |
| Kim [35] | | N/A | 1 | MALDI-TOF-MS, ESI-MS/MS | GSL | 47 | 0 | 4 | N/A |
| Miyagawa [36] | WT pigs (Large white/Landrace x Duroc) | 6 months | 1 ² | MALDI-TOF-MS, HPLC | N-Glycans | 28 | 0 | 0 | Sulfated N-glycans |
| Eguchi [37] | | 6 months | 1 ² | LC-MS | N-Glycans | 16 | 0 | 0 | - |
| Miyagawa [38] | | 1–3 days | 1 ² | MALDI-TOF-MS, HPLC | N-Glycans | 24 | 0 | 0 | β -GlcNAc Man α 1-3Man α 1-6Man |
| Current study | WT pigs (Mangalista and Landrace x Yorkshire) | 3 years | 7 | MALDI-TOF-MS, ESI-MS/MS | N-Glycans O-Glycans | 57 21 | 0 0 | 2 3 | Hi-mannose N-glycans with core fucosylation Sialylated complex N-glycans |

Numbers in brackets show reference. CMS, Chicago Medical School; ESI-MS/MS, Electrospray Ionization Mass Spectrometry; GSL, Glycosphingolipids; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; MALDI-TOF-MS, Matrix-Assisted Laser-Desorption Time-of-Flight Mass Spectrometry; N/A, not available; SPF, specific pathogen-free; WT, wild-type.

¹detected in ESI-q-TOF MS analysis but the quality was negligible. Zero α -Gal epitope was observed in HPLC analysis.

²Islets from several pigs may be combined into one analysis.

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References

1. Hering BJ, Clarke WR, Bridges ND, Eggerman TL, Alejandro R, Bellin MD, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. *Diabetes Care*. 2016; 39:1230–40. <https://doi.org/10.2337/dc15-1988> PMID: 27208344
2. Lablanche S, Vantghem MC, Kessler L, Wojtusciszyn A, Borot S, Thivolet C, et al. Islet transplantation versus insulin therapy in patients with type 1 diabetes with severe hypoglycaemia or poorly controlled glycaemia after kidney transplantation (TRIMECO): a multicentre, randomised controlled trial. *Lancet Diabetes Endocrinol*. 2018; 6:527–37. [https://doi.org/10.1016/S2213-8587\(18\)30078-0](https://doi.org/10.1016/S2213-8587(18)30078-0) PMID: 29776895

3. Cowan PJ, Tector AJ. The resurgence of xenotransplantation. *Am J Transplant*. 2017; 17:2531–6. <https://doi.org/10.1111/ajt.14311> PMID: 28397351
4. Ekser B, Li P, Cooper DKC. Xenotransplantation: past, present, and future. *Curr Opin Organ Transplant*. 2017; 22:513–21. <https://doi.org/10.1097/MOT.0000000000000463> PMID: 28901971
5. Groth CG, Korsgren O, Tibell A, Tollema J, Möller E, Bolinder J, et al. Transplantation of porcine fetal pancreas to diabetic patients. *Lancet*. 1994; 344(8934):1402–4. [https://doi.org/10.1016/s0140-6736\(94\)90570-3](https://doi.org/10.1016/s0140-6736(94)90570-3) PMID: 7968077
6. Wang W, Mo Z, Ye B, Hu P, Liu S, Yi S. A clinical trial of xenotransplantation of neonatal pig islets for diabetic patients. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*. 2011; 36:1134–40. <https://doi.org/10.3969/j.issn.1672-7347.2011.12.002> PMID: 22246351
7. Hering BJ, Wijkstrom M, Graham ML, Hårdstedt M, Aasheim TC, Jie T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med*. 2006; 12:301–3. <https://doi.org/10.1038/nm1369> PMID: 16491083
8. Cardona K, Korbitt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med*. 2006; 12:304–6. <https://doi.org/10.1038/nm1375> PMID: 16501570
9. van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. *Am J Transplant*. 2009; 9:2716–26. <https://doi.org/10.1111/j.1600-6143.2009.02850.x> PMID: 19845582
10. Thompson P, Badell IR, Lowe M, Cano J, Song M, Leopardi F, et al. Islet xenotransplantation using galactose-deficient neonatal donors improves engraftment and function. *Am J Transplant*. 2011; 11:2593–602. <https://doi.org/10.1111/j.1600-6143.2011.03720.x> PMID: 21883917
11. Bottino R, Wijkstrom M, van der Windt DJ, Hara H, Ezzelarab M, Murase N, et al. Pig-to-monkey islet xenotransplantation using multi-transgenic pigs. *Am J Transplant*. 2014; 14:2275–87. <https://doi.org/10.1111/ajt.12868> PMID: 25220221
12. Hawthorne WJ, Salvaris EJ, Phillips P, Hawkes J, Liuwantara D, Burns H, et al. Control of IBMIR in neonatal porcine islet xenotransplantation in baboons. *Am J Transplant*. 2014; 14:1300–9. <https://doi.org/10.1111/ajt.12722> PMID: 24842781
13. Thompson P, Badell IR, Lowe M, Turner A, Cano J, Avila J, et al. Alternative immunomodulatory strategies for xenotransplantation: CD40/154 pathway-sparing regimens promote xenograft survival. *Am J Transplant*. 2012; 12:1765–75. <https://doi.org/10.1111/j.1600-6143.2012.04031.x> PMID: 22458586
14. Thompson P, Cardona K, Russell M, Badell IR, Shaffer V, Korbitt G, et al. CD40-specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. *Am J Transplant*. 2011; 11:947–57. <https://doi.org/10.1111/j.1600-6143.2011.03509.x> PMID: 21521467
15. Valdes-Gonzalez R, Rodriguez-Ventura AL, White DJ, Bracho-Blanchet E, Castillo A, Ramirez-Gonzalez B, et al. Long-term follow-up of patients with type 1 diabetes transplanted with neonatal pig islets. *Clin Exp Immunol*. 2010; 162:537–42. <https://doi.org/10.1111/j.1365-2249.2010.04273.x> PMID: 20964645
16. Shin JS, Kim JM, Min BH, Yoon IH, Kim HJ, Kim JS, et al. Pre-clinical results in pig-to-non-human primate islet xenotransplantation using anti-CD40 antibody (2C10R4)-based immunosuppression. *Xenotransplantation*. 2018; 25:10.1111/xen.12356. <https://doi.org/10.1111/xen.12356> PMID: 29057561
17. Sterner E, Flanagan N, Gildersleeve JC. Perspectives on Anti-Glycan Antibodies Gleaned from Development of a Community Resource Database. *ACS Chem Biol*. 2016; 11:1773–83. <https://doi.org/10.1021/acschembio.6b00244> PMID: 27220698
18. Stanley P, Taniguchi N, Aebi M. 2017. N-Glycans. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al. *Essentials of Glycobiology* [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015–2017.
19. Kuwaki K, Tseng YL, Dor FJ, Shimizu A, Houser SL, Sanderson TM, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med*. 2005; 11:29–31. <https://doi.org/10.1038/nm1171> PMID: 15619628
20. Yamada K, Yazawa K, Shimizu A, Iwanaga T, Hisashi Y, Nuhn M, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 2005; 11:32–4. <https://doi.org/10.1038/nm1172> PMID: 15619627
21. Byrne G, Ahmad-Villiers S, Du Z, McGregor C. B4GALNT2 and xenotransplantation: A newly appreciated xenogeneic antigen. *Xenotransplantation*. 2018; 25(5):e12394. <https://doi.org/10.1111/xen.12394> PMID: 29604134

22. Martens GR, Reyes LM, Li P, Butler JR, Ladowski JM, Estrada JL, et al. Humoral reactivity of renal transplant-waitlisted patients to cells from GGTA1/CMAH/B4GalNT2, and SLA Class I knockout pigs. *Transplantation*. 2017; 101:e86–e92. <https://doi.org/10.1097/TP.0000000000001646> PMID: 28114170
23. Burlak C, Taylor RT, Wang ZY, Tector AJ. Human anti- α -fucose antibodies are xenoreactive toward GGTA1/CMAH knockout pigs. *Xenotransplantation*. 2020. Forthcoming <https://doi.org/10.1111/xen.12629> PMID: 32697003
24. Kim YG, Kim SY, Hur YM, Joo HS, Chung J, Lee DS, et al. The identification and characterization of xenoantigenic nonhuman carbohydrate sequences in membrane proteins from porcine kidney. *Proteomics*. 2006; 6:1133–42. <https://doi.org/10.1002/pmic.200500275> PMID: 16470663
25. Burlak C, Paris LL, Lutz AJ, Sidner RA, Estrada J, Li P, et al. Reduced binding of human antibodies to cells from GGTA1/CMAH KO pigs. *Am J Transplant*. 2014; 14:1895–900. <https://doi.org/10.1111/ajt.12744> PMID: 24909344
26. McKenzie IF, Koulmanda M, Mandel TE, Sandrin MS. Pig islet xenografts are susceptible to "anti-pig" but not Gal $\alpha(1,3)$ Gal antibody plus complement in Gal o/o mice. *J Immunol*. 1998; 161(10):5116–9. PMID: 9820477
27. Kirchof N, Shibata S, Wijkstrom M, Kulick DM, Salerno CT, Clemmings SM, et al. Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. *Xenotransplantation*. 2004; 11:396–407. <https://doi.org/10.1111/j.1399-3089.2004.00157.x> PMID: 15303976
28. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957; 226(1):497–50. PMID: 13428781
29. Shajahan A, Heiss C, Ishihara M, Azadi P. Glycomic and glycoproteomic analysis of glycoproteins—a tutorial. *Anal Bioanal Chem*. 2017; 409:4483–4505. <https://doi.org/10.1007/s00216-017-0406-7> PMID: 28585084
30. Anumula KR, Taylor PB. A comprehensive procedure for preparation of partially methylated alditol acetates from glycoprotein carbohydrates. *Anal Biochem*. 1992; 203(1):101–8. [https://doi.org/10.1016/0003-2697\(92\)90048-c](https://doi.org/10.1016/0003-2697(92)90048-c) PMID: 1524204
31. Lin AI, Philipsberg GA, Haltiwanger RS. Core fucosylation of high-mannose-type oligosaccharides in GlcNAc transferase I-deficient (Lec1) CHO cells. *Glycobiology*. 1994; 4:895–901. <https://doi.org/10.1093/glycob/4.6.895> PMID: 7734851
32. Akama TO, Nakagawa H, Wong NK, Sutton-Smith M, Dell A, Morris HR, et al. Essential and mutually compensatory roles of α -mannosidase II and α -mannosidase IIx in N-glycan processing in vivo in mice. *Proc Natl Acad Sci U S A*. 2006; 103:8983–8. <https://doi.org/10.1073/pnas.0603248103> PMID: 16754854
33. Nagae M, Yamaguchi Y, Taniguchi N, Kizuka Y. 3D Structure and Function of Glycosyltransferases Involved in N-glycan Maturation. *Int J Mol Sci*. 2020; 21:437. <https://doi.org/10.3390/ijms21020437> PMID: 31936666
34. Kim YG, Gil GC, Jang KS, Lee S, Kim HI, Kim JS, et al. Qualitative and quantitative comparison of N-glycans between pig endothelial and islet cells by high-performance liquid chromatography and mass spectrometry-based strategy. *J Mass Spectrom*. 2009; 44:1087–104. <https://doi.org/10.1002/jms.1587> PMID: 19373860
35. Kim YG, Harvey DJ, Yang YH, Park CG, Kim BG. Mass spectrometric analysis of the glycosphingolipid-derived glycans from miniature pig endothelial cells and islets: identification of NeuGc epitope in pig islets. *J Mass Spectrom*. 2009; 44:1489–99. <https://doi.org/10.1002/jms.1638> PMID: 19760646
36. Miyagawa S, Maeda A, Kawamura T, Ueno T, Usui N, Kondo S, et al. A comparison of the main structures of N-glycans of porcine islets with those from humans. *Glycobiology*. 2014; 24:125–38. <https://doi.org/10.1093/glycob/cwt088> PMID: 24100142
37. Eguchi H, Kawamura T, Kashiya N, Matsuura R, Sakai R, Nakahata K, et al. Supplemental Analysis for N-linked Sugars in Adult Pig Islets. *Transplant Proc*. 2016; 48:1302–3. <https://doi.org/10.1016/j.transproceed.2015.10.071> PMID: 27320609
38. Miyagawa S, Maeda A, Eguchi H, Kawamura T, Kashiya N, Ueno T, et al. A structural analysis of N-glycans of neonatal porcine islet-like cell clusters (NPCC). *Transpl Immunol*. 2014; 31:48–53. <https://doi.org/10.1016/j.trim.2014.05.003> PMID: 24867127
39. Takahashi T, Schmidt PG, Tang J. Oligosaccharide units of lysosomal cathepsin D from porcine spleen. Amino acid sequence and carbohydrate structure of the glycopeptides. *J Biol Chem*. 1983; 258:2819–30. PMID: 6826541
40. Misaki A, Goldstein IJ. Glycosyl moiety of the lima bean lectin. *J Biol Chem*. 1977; 252:6995–9. PMID: 903350

41. Komoda H, Miyagawa S, Kubo T, Kitano E, Kitamura H, Omori T, et al. A study of the xenoantigenicity of adult pig islets cells. *Xenotransplantation*. 2004; 11:237–46. <https://doi.org/10.1111/j.1399-3089.2004.00121.x> PMID: 15099203
42. Ezzelarab M, Ayares D, Cooper DK. Carbohydrates in xenotransplantation. *Immunol Cell Biol*. 2005; 83:396–404. <https://doi.org/10.1111/j.1440-1711.2005.01344.x> PMID: 16033535
43. Rayat GR, Rajotte RV, Hering BJ, Binette TM, Korbitt GS. In vitro and in vivo expression of Galalpha-(1,3)Gal on porcine islet cells is age dependent. *J Endocrinol*. 2003; 177:127–35. <https://doi.org/10.1677/joe.0.1770127> PMID: 12697044