¹H MAS NMR of Donor Pancreatic Tissue is a Useful Predictor of Islet Viability Prior to Islet Isolation.

Running Title: ¹H MAS NMR of pancreatic tissue

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Abstract

A significant limitation and cost to any clinical islet program is the related to processing human pancreas and not recovering significant numbers of viable islets for clinical transplantation. The development of an assay system that could be utilized and provide an index of cell and tissue viability before islet isolation would provide a major impact on the scientific aspects of organ preservation and a huge cost saving to any clinical islet transplantation program.

Metabolomic analysis by ^1H MAS NMR was used to assess samples of donor pancreatic tissue taken prior to islet isolation. A significant correlation was observed between the ratio of the combined integrals of the sugar (3.5-4.5 ppm) and choline (3.0-3.5 ppm) regions to the integrals of the CH_3 (0.9 ppm) and CH_2 (1.3 ppm) peaks of the ^1H MAS NMR spectra of pancreatic tissue samples taken prior to islet isolation and the glucose responsiveness, a measure of islet viability, of the isolated islets ($P < 0.05$). The effect of the two-layer (University of Wisconsin solution/perfluorochemical [UW/PFC]) cold-storage method, previously shown to restore ischemically damaged pancreases by increasing oxygenation, was also studied using ^1H MAS NMR spectroscopy. PFC recovery of the donor pancreas also correlated with an increase in the combined integrals of the sugar and choline regions to the CH_3 and CH_2 peaks of the ^1H MAS NMR spectra ($P < 0.05$). In addition, significant differences in the integrals of the sugar region and CH_2 peaks were observed between the pre- and post-PFC samples ($P < 0.05$). These results support the notion that specific metabolites observed in ^1H MAS NMR can be used as a means to assess reversible/irreversible tissue damage and offers a means to assess donor pancreatic tissue prior to islet isolation for transplantation.

Keywords: solid state NMR, magic angle spinning, diabetes, pancreas, islet isolation, transplantation

Abbreviations: NMR, Nuclear Magnetic Resonance; MAS, Magic Angle Spinning; CIT, cold ischemia time; SI, stimulation index; PFC, perfluorochemical; UW solution, University of Wisconsin solution

Introduction

The World Health Organization reports that the scale of the problem that diabetes poses to world health is still widely under recognized. At least 177 million people worldwide suffer from diabetes, a figure which will likely more than double by 2030. Type 1 diabetes accounts for 5 – 10% of all diabetics with incidences increasing at roughly 2 – 5% per year worldwide (1). The success of islet transplantation from cadaveric organ donors and the recent development of the “Edmonton Protocol” has become a promising and viable option for some in the treatment of type-1 diabetes to restore euglycemia and achieve insulin independence (2). In this case, islets of Langerhans, which make up only approximately 1% of the organ, are isolated from the surrounding exocrine tissue of cadaveric pancreases. However, many donor variables have been shown to influence islet yield and viability and inevitably patient outcome following islet transplantation. These factors, which include cold-ischemia time (CIT), donor age, and body mass index, contribute to the variability in donor organs that may result in lower islet yields requiring recipients to have repeated islet transplants from as many as 3 donor pancreases to become completely insulin independent (3). Moreover, islet isolation is a difficult, time-consuming and expensive process. Therefore, it would be advantageous to screen donor organs prior to islet isolation and transplantation.

Metabolomics, the study of small molecule metabolites in biofluid or tissue samples, is an emerging science and provides information about an individual’s unique cellular and biochemical profile. In this regard, high-resolution NMR spectroscopy is a method with which to resolve and quantify hundreds of molecular signatures within a sample. Recently it has become possible to obtain high resolution ^1H NMR spectra of intact tissue using solid state magic angle spinning (MAS) NMR. Spinning of solid samples at an angle of 54.7° (where $(3 \cos^2\theta - 1)/2 = 0$) with respect to the external magnetic field results in averaging of the chemical shift anisotropies and dipolar couplings thus sharpening lines to produce spectra that may be comparable in resolution and spectral quality with those produced by conventional solution state NMR.

^1H MAS NMR has been applied in cancer research in various tissues, including breast (4), kidney (5;6), brain (7), adipose tissue (8), and lymph nodes (9). This technique has proven useful for studying lipids and other endogenous metabolites within cells and tissues to distinguish normal from damaged or abnormal cells and tissues. In addition, ^1H MAS NMR offers several advantages over conventional NMR techniques. Sample preparation does not require extraction and data can be acquired in less than 30 minutes with low cell volume or tissue sample size. Intracellular lipids which are not water soluble can be detected by ^1H MAS NMR and have not only been associated with malignancy but also apoptosis. In cultures of Jurkat T-cells, treated with Doxorubicin to induce apoptosis, there was a correlation between the CH_2/CH_3 lipid signal intensity ratio of the ^1H MAS NMR spectra and the percentage of apoptotic cells as detected by nuclear morphologic analysis, surface expression of phosphatidylserine, and DNA fragmentation (10). Moreover, increases in the CH_2 signal intensity was observed in a variety of cells when apoptosis was induced, but not in necrotic death, suggesting that this may reflect a common biological mechanism after the onset of apoptosis (10;11). It has also been

shown in β TC3 cells, an insulin-secreting mouse insulinoma cell line, that oxygen deprivation and cell viability is associated with changes in the absolute levels and/or the ratios of specific metabolites observed in the ^1H MAS NMR spectra of specific metabolites, suggesting that these may be sensitive markers of oxygenation status as well as reversible/irreversible damage (12).

This paper represents the first account of using tissue metabolomics to predict cellular viability prior to islet isolation and transplantation. In this paper, we show a correlation between viability of isolated islets and the integrations of the “sugar” and “choline” regions and methylene (CH_2) and methyl (CH_3) peaks of ^1H MAS NMR spectra obtained prior to islet isolation. We also show a correlation between these spectral regions and oxygen recovery of the pancreas with perfluorochemical (PFC) prior to islet isolation.

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Experimental

Human pancreases were obtained from brain-dead cadavers with informed consent from relatives. Tissue procurement and experimental protocols were approved by the Human Ethics Committee of the University of Alberta. Pancreases were removed following *in situ* vascular perfusion using cold University of Wisconsin (UW) solution as part of a multi-organ procurement. Pancreases were placed in cold UW solution on ice and immediately transported to Clinical Islet Isolation Laboratory at the University of Alberta for tissue processing.

Purification and islet quantification

The method of human islet isolation has been previously described (2). Briefly, to isolate islets, the ducts were perfused in a controlled fashion with a cold enzyme (Liberase human islet, Roche Biochemicals Inc.). The islets were then separated by gentle mechanical dissociation and purified with the use of continuous gradients of Ficoll-diatrizoic acid (Seromed-Biochrom) in an apheresis system (Cobe Laboratories Inc.). Islet recovery following purification was assessed in duplicate by counts of dithizone-stained aliquots of the final suspension of tissue and expressed in terms of islet equivalents (IE), the standard unit for reporting variations in the volume of islets, with the use of a standard islet diameter of 150 μm (13).

Characterization of islet cell preparations.

For assessment of *in vitro* viability, the insulin secretory response of the islets to a glucose challenge was determined following isolation and 18-24 h of tissue culture at 37°C in humidified air (5% CO₂, 95% air) in CMRL-1066 medium, 10% fetal calf serum, 25 mM HEPES buffer using a static incubation assay as previously described (2;14). Briefly, a known number of duplicate aliquots of islets were incubated in a low concentration of glucose (50 mg per dl [2.8 mM]) and a high concentration of glucose (360 mg per deciliter [20 mM]) for two hours, and the amount of insulin generated in response to the high-glucose challenge was divided by the amount generated by the low-glucose challenge to yield the mean insulin-release stimulation index (SI).

MAS NMR

All spectra were run on a Varian Unity 300 NMR spectrometer equipped with a chemagnetics solid-state MAS probe. Samples were prepared by freeze grinding approximately 100 – 120 mg of a pancreatic biopsy and packing into 5 mm chemagnetics rotors. The samples were spun at 4000 kHz at room temperature (~21°C). A “rotor-synchronized” CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence was used along with solvent presaturation as a T₂ filter to minimize water, protein and fat resonances as described by Cheng et al. (9). Spectra were acquired with 1024 transients into 40k data-points utilizing a spectral width of 10000 Hz and a relaxation delay of 2 seconds. Chemical shifts were referenced to the methyl peak at 0.9 ppm. The relative areas under the methylene (CH₂), methyl (CH₃), “sugar” and “choline” (PC) resonances at 1.3, 0.9,

3.5 – 4.5, and 3.0 – 3.5 ppm respectively were determined by integrating the spectrum, using relatively flat areas between the peaks as baseline reference.

Statistical Method

All results are expressed as mean $\pm$ SEM. Statistical analysis was performed using GraphPad Software (San Diego, CA). Individual post-PFC groups were analyzed using two-tailed, unpaired t-tests. Comparisons between pre- and post-PFC groups were analyzed using two-tailed, paired t-tests. Linear regression analysis was performed to determine whether the slope was significantly non-zero. Differences were considered significant when $P < 0.05$.

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Results

MAS NMR and stimulation index as a measure of islet viability

Pancreatic tissue samples were collected at the commencement of islet isolation, snap frozen in liquid N₂ and stored at -80°C until further analysis. Donor related variables and islet isolation results were recorded. Donor age ranged from 16-66 years (43.3±6.7 years), body mass index ranged from 18-32 (23.0±1.5), total CIT ranged from 7.5-19.5 h (13.9±1.3 h), CIT (UW) ranged from 3-15.5 h (9.6±1.2), CIT (PFC) ranged from 3-7 h (4.3±0.5 h) (Table 1).

Figure 1 shows the NMR spectra of nine pancreatic biopsies (post-PFC, see below) from different donors. A list of metabolic signal assignments and regions have been previously characterized and reported with chemical shifts and multiplicities (15-17). As reported, the region between δ 3.0 and δ 4.5 is particularly complex containing resonances from several small metabolites including *myo*-inositol, sorbitol, alanine, glutamine, glutamate, glycine, lysine, taurine, choline, betaine, as well as from α and β glucose, and glycogen and with broader signals arising from lipid and protein components. The “choline” region also is somewhat complex, containing choline (3.21 ppm), phosphocholine (3.23 ppm), glycerophosphatidylcholine (3.24 ppm), phosphatidylcholine (3.27 ppm), and *myo*-inositol (3.27, 3.29, 3.31 ppm) (15). Triglycerides contribute to the resonances (multiplets at 4.1 and 4.3 ppm) in this region originating from the glycerol backbone and also contribute to the resonance centered at 0.9 ppm, originating from terminal methyl protons on fatty acyl chains (15). For our purposes, the regions of interest are defined as the “sugar” region (3.5 – 4.5 ppm), “choline” (PC) region (3.0 – 3.5 ppm), CH₂ peak (1.3 ppm), and CH₃ peak (0.9 ppm).

Stimulation indices from the isolated islets, a measure of islet viability, were compared with the spectral integrations of the “sugar” and “choline” regions and the CH₂ and CH₃ peaks (see Figure 1) and are shown in Table 1. Three of the nine samples were not considered for further analysis as the stimulation indexes for two of these samples were not determined (P7 and P8). Furthermore, two of the spectra (P8 and P9) did not exhibit well-defined peaks in the NMR spectra. Interestingly, the islet isolation for P8 was aborted mid-way through the procedure and considered a complete failure, and for P9, although the islet isolation was completed, was also not considered successful, with a yield of only approximately 65,000 islet equivalents. When the stimulation indices of the isolated islets were compared with the NMR spectra for P1 through P6, it became apparent that the stimulation indices were significantly and positively correlated to the size of the “sugar” and “choline” regions and inversely related to the size of the CH₂ and CH₃ peaks. When the stimulation index was plotted vs. the integrals of the (“sugar” + “PC”)/(CH₂ + CH₃) regions, the slope was significantly different from zero (P=0.0247) (Figure 2A).

The stimulation index for islets purified from donors P1, P2 and P3 was 2.4±0.26 (mean ± SEM) and was 1.327±0.1214 for P4, P5, and P6, which are significantly different as compared by an unpaired t-test and (P=0.0194) (Figure 2B). These two groups of samples

also showed a significant difference in the ratio of the (“sugar” + “PC”) to ($\text{CH}_2 + \text{CH}_3$) integrals for tissue samples taken prior to islet isolation. The ratio of the integrals of the (“sugar” + “PC”)/($\text{CH}_2 + \text{CH}_3$) regions for P1, P2 and P3, which showed an SI above 2, was 12.84 ± 2.293 (mean $\pm$ SEM) while the mean for the group of three samples with a SI below 2, was significantly less (3.160 ± 1.445 , $P=0.0234$) (Figure 2C).

It has been our unpublished observation that the islet yield increases following a brief period of cold storage following organ procurement. This observation is also supported in a unique experiment in which a pancreatic sample was collected at the time of multi-organ procurement and immediately snap frozen. Following procurement, the pancreas was then brought back to the Islet Isolation Laboratory where it remained in cold storage for an additional 3 hours in UW solution before the commencement of the islet isolation process. After the 3 hour period, another tissue sample was collected and the islet isolation procedure was initiated. Figure 3 shows the spectra of the tissue sample collected at the time of procurement and a sample collected following 3 hours of cold storage. It is evident that there is an increase in the “sugar” and “PC” regions and a decrease in the CH_3 and CH_2 peaks following cold storage. In fact, the ratio of the (“sugar” + “PC”) regions to the ($\text{CH}_3 + \text{CH}_2$) peaks of the spectra increased 10-fold from 0.994 to 10.11, and the islets following isolation, showed a stimulation index of 3.05. It is interesting that a short period of cold storage, known to increase islet yield, is consistent with the changes of the ^1H MAS NMR metabolic profile associated with improved viability. This observation underlines the use of this technique in predicting islet isolation outcome.

MAS NMR and PFC

It has been shown that the two-layer (University of Wisconsin solution/perfluorochemical [UW/PFC]) cold-storage method for whole pancreas preservation, which delivers sufficient oxygen to the pancreas during preservation, restores the ischemically damaged pancreas (18-20). It was further demonstrated that this method of preservation reduced cold ischemic injury by sustaining higher levels of adenosine triphosphate (ATP), maintaining cellular integrity and controlling ischemic swelling (21); and that this method of preservation improved islet recovery from human pancreases with prolonged cold storage in UW (22). We therefore examined whether there was an effect of the two-layer cold storage method on the ^1H MAS NMR spectra of pancreatic donor tissue samples. In these experiments, donor organs were stored and transported to the Islet Isolation Laboratory in UW solution. Upon arrival to the Islet Isolation Laboratory pancreatic tissue biopsies were collected and snap frozen prior to transfer to the two-layer storage medium containing PFC. Following 3-7 h of recovery in PFC another tissue sample was collected and snap frozen for NMR analysis before the commencement of the islet isolation process.

Figure 4 shows the comparison of ^1H MAS NMR spectra of eight donor pancreatic samples obtained pre- and post-PFC recovery (see also Figure 1). Pre-PFC data for P6 was not analyzed as the sample was lost during tissue preparation. The integration of the

areas of interest in the ^1H MAS NMR spectra show a correlation between the NMR spectral regions and recovery of the pancreas in PFC, except for P8 and P9 where there appeared little change between pre- and post-PFC recovery. As previously stated, the islet isolations for P8 and P9 were unsuccessful and the peaks in the NMR spectra were not well resolved. For P1 to P7, a similar result to the stimulation index data was obtained, where the SI was correlated to the ratio of the integrals of the (“sugar” + “PC”) regions to the ($\text{CH}_2 + \text{CH}_3$) peaks; recovery of the pancreas in PFC is also associated with an increase in the combined area of the “sugar” and “PC” regions and a decrease in the combined area of the CH_3 and CH_2 peaks (Figure 5). Table 1 shows the pre-PFC integrals and post-PFC integrals. Moreover, the differences between integrals of the spectra of the pre- and post-PFC samples appears to be much more striking than that observed between samples of high and low SI when donor organs were collected and stored on UW solution alone.

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Discussion

Examination of the solid state ^1H MAS NMR metabolic profile of pancreatic tissue in advance of islet isolation gives a qualitative measure of the viability of the tissue and is directly correlated with the stimulation index of the isolated islets. An increase in the ratio of the combined “sugar” and “choline” integrated regions to the combined integrated CH_3 and CH_2 peaks in the tissue sample was associated with islet viability, post isolation, as defined by glucose responsiveness. We also show that reoxygenation of the pancreas in PFC (3-7 hours), which is reported to restore ischemic damage incurred during preservation, storage and transportation, in UW solution is associated with changes in the ^1H MAS NMR metabolic profile in a manner similar to that associated with islet viability. PFC recovery was associated with a significant increase in the integrated “sugar” region and a significant decrease in the CH_2 region of the ^1H MAS NMR spectra.

It has been observed that there is a correlation between the orderly processes of apoptosis and the CH_2 peak observed in ^1H MAS NMR (23). In cells undergoing apoptosis, the caspase enzyme cascade is activated, leading to the rapid redistribution of phosphatidylserine (PS) from the inner leaflet of the plasma membrane lipid bilayer to the outer leaflet and is followed by membrane bleb formation and DNA degradation. Blankenberg et al demonstrated that the CH_2 resonance intensity as observed by ^1H MAS NMR was directly proportional to the surface expression of phosphatidylserine, an early marker of apoptosis, in lymphoblasts in vitro (9). Insight into the change in the ^1H MAS NMR lipid profile have also recently been forthcoming. It has been shown that an increase in the CH_2/CH_3 ratio observed in the early stage of induced apoptosis in Jurkat cells, results from an accumulation of mobile triacylglycerols mostly in the form of cytosolic lipid droplets (24;25). In their model, the onset of apoptosis and alterations in the CH_2/CH_3 ratio is associated with an increase in mobile neutral lipid triacylglycerol concentration, and based on confocal microscopy images of cells stained with Nile Red, they conclude that neutral lipids detected by ^1H MAS NMR could originate from lipid droplets that are located within the cell cytoplasm (24). Further, Griffin et al. show a distinct temporal progression in terms of a metabolic profile of the glioma tissue and cells after programmed cell death and that polyunsaturated fatty acid resonances arising from a nonmembrane source of lipids is consistent with the source being cytosolic lipid droplets (26).

Several pieces of evidence suggest that the ^1H MAS NMR profile may also reflect the state of oxygenation of the tissue. Papas et al has shown that oxygen deprivation was associated with an increase in the CH_2 resonance in βTC3 cells, an insulin-secreting mouse insulinoma cell line (12). And more recently, we have shown the recovery of viable islets was significantly increased in the UW/PFC group compared to the UW group (22).

On the basis that PFC dissolves approximately 10 times as much oxygen as does water at 4°C , Kuroda et al developed the two layer method of cold storage preservation (18). Moreover, Kuroda et al has shown tissue ATP concentration can be elevated and maintained during cold storage if the dog pancreas is in direct contact with the PFC layer,

reflecting higher oxygenation and viability of the pancreas (20;27). They also demonstrated that ATP has an essential role in supplying energy for processes that maintain cellular integrity by sufficiently oxygenating the pancreas and by simultaneously blocking ATP production with DNP, an uncoupler of mitochondrial oxidative phosphorylation (28). Interestingly, Papucci et al demonstrated that Ubiquinone Q₁₀ which is an electron transporter in the mitochondrial respiratory chain and is a free radical scavenger, attenuated the decrease of ATP and prevented induced apoptotic death in keratinocytes (29).

In addition, it has been shown that ATP levels are directly affected by cold storage. It is known that hypothermia induces cell swelling. The ratio of high Na⁺ and low in K⁺ in the extracellular fluid is maintained by the Na⁺ pump (Na-K ATPase), which uses much of the energy (ATP) derived from oxidative phosphorylation. Anaerobic-hypothermic preservation suppresses the activity of the Na⁺ pump and decreases the membrane potential of the plasma membrane. Ultimately, the cell swells resulting from Na⁺ and Cl⁻ entering the cell down a concentration gradient and the accumulation of water. Secondly, ATP rapidly degrades during hypothermic storage. The rapid regeneration of Na pump activity, as well as other energy-requiring steps in the metabolism that require ATP become necessary for cellular function with restoration of physiological temperatures (reviewed in (21)).

The previous observation of higher yield of viable islets with the two-layer preservation method together with the ¹H MAS NMR profiles observed here suggests that at least some of ischemic damage in the pancreas following cold storage is reversible. Following 3-7 hours of recovery of the pancreas in PFC, the CH₂ resonances decreased with a coincident increase in the “sugar” region in the ¹H MAS NMR spectra. This suggests that the increase in CH₂ and decrease in the “sugar” region represent early stages of cellular injury and/or apoptosis. It is interesting to speculate that this reversibility of damage may have clinical significance. It will be of interest to see whether graft function of islets isolated from donor pancreases recovered with the two-layer method of cold storage is better than graft function of islets isolated from pancreases stored in UW solution alone. There is some evidence to suggest that this might be true. Kuroda et al demonstrated in dogs that graft function was better after transplantation following preservation with the two-layer method. (30). Regardless of whether this is the case in human islet transplantation, the ¹H MAS NMR metabolic profiles may prove to be a profound predictor of patient outcome following islet transplantation.

Conclusions: We have used the ¹H MAS NMR profile of donor tissue as a predictor of isolated islet viability. ¹H MAS NMR profiles of donor pancreatic tissue correlate with the glucose responsiveness of islets following isolation and are consistent with the observation that the two layer method of cold-storage improves viability of the pancreas. ¹H MAS NMR offers many advantages, requiring a small sample size (<100 mg tissue) and minimal sample preparation, and is a fast (under 30 minutes) and sensitive measure of viability.

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Figure Legends:

Figure 1: 300 MHz ^1H MAS NMR spectra of 9 donor pancreas biopsies. The regions of interest are the “sugar” region (3.5-4.5 ppm) in yellow, the “choline” region, (3.0-3.5 ppm) in green, the methylene (CH_2) peak (1.3 ppm) in blue, and the methyl (CH_3) peak (0.9 ppm) in orange. The large “peak” near 4.7 - 4.8 ppm is water.

Figure 2: (A) Correlation of the stimulation index (SI) of isolated islets versus the ratio of the integrals (“sugar” + “PC”) / (CH_2 + CH_3) of the ^1H MAS NMR spectra of pancreatic tissue prior islet isolation. (B) The stimulation index (SI) of isolated islets. SI > 2 (P1, P2, P3), black; SI < 2 (P4, P5, P6), hatched (mean $\pm$ SEM). (C) The ratio (“sugar” + “PC”) / (CH_2 + CH_3) of the ^1H MAS NMR integrals of the pancreatic samples with SI > 2 (black) vs. SI < 2 (hatched) (mean $\pm$ SEM).

Figure 3: 300 MHz ^1H MAS NMR spectra of donor pancreas samples taken during procurement (A) and after 3 hours of cold storage in UW solution (B). The integrals of the areas of interest and the ratio of the integrals (“sugar” + “PC”) / (CH_2 + CH_3) of the procurement and post-cold storage samples are shown.

Figure 4: 300 MHz ^1H MAS NMR spectra of 8 donor pancreas biopsies before recovery in PFC (A), or following recovery in PFC for 3-7 hours (B).

Figure 5: 300 MHz ^1H MAS NMR integrals from donor pancreatic samples before and after recovery of the pancreas in PFC. The integrals of the areas of interest and the ratio of the integrals (“sugar” + “PC”) / (CH_2 + CH_3) of the pre-PFC (black) and post-PFC (hatched) samples are shown. (mean $\pm$ SEM; n=8)

TABLE 1.

	Donor Variables		Cold Ischemia Time (hours)			¹ H MAS NMR Integrals					SI
	Age (years)	BMI (kg/m ²)	UW	PFC	Total	Sugar	PC	CH ₂	CH ₃	(Sugar+PC) (CH ₂ +CH ₃)	
P1	48	18	10	4	14	0.165 0.861	0.008 0.077	0.705 0.037	0.121 0.025	0.210 15.13	2.95
P2	62	21	6.5	3	9.5	0.761 0.882	0.068 0.056	0.123 0.040	0.048 0.022	4.85 15.13	2.23
P3	21	27	12.5	7	19.5	0.590 0.836	0.0086 0.055	0.312 0.073	0.089 0.035	1.49 8.25	2.1
P4	62	26	9	6	15	0.249 0.477	0.0057 0.047	0.669 0.381	0.077 0.096	0.341 1.10	1.53
P5	55	32	8.5	3	11.5	0.171 0.750	0.016 0.106	0.679 0.101	0.135 0.043	0.230 5.94	1.34
P6	66	20	11	5.5	16.5	N/D 0.667	N/D 0.042	N/D 0.214	N/D 0.077	N/D 2.44	1.10
P7	16	22	15.5	3	18.5	0.712 0.778	0.139 0.120	0.098 0.052	0.051 0.050	5.71 8.80	N/D
P8	19	21	3	4.5	7.5	0.806 0.752	0.097 0.140	0.054 0.077	0.043 0.031	9.31 8.26	N/D
P9	41	20	10.5	3	13.5	N/D	N/D	N/D	N/D	N/D	1.08

Figure 1

Figure 2

Figure 3.

WITHDRAWN
see manuscript DOI for details

Figure 4

see manuscript DOI for details

	Sugar	PC	CH ₂	CH ₃	$\frac{(\text{Sugar}+\text{PC})}{(\text{CH}_2+\text{CH}_3)}$
Pre-PFC	0.441±0.113	0.041±0.0219	0.431±0.117	0.087±0.015	2.139±1.018
Post-PFC	0.750±0.053*	0.072±0.012	0.128±0.048*	0.050±0.010	8.113±2.099*

* p < 0.05

Figure 5