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Serum metabolite signature predicts the acute onset of diabetes in spontaneously diabetic congenic BB rats.

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Metabolite signature predicts BB rat diabetes

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Abstract

The clinical presentation of type 1 diabetes is preceded by a prodrome of beta cell autoimmunity. We probed the short period of subtle metabolic abnormalities, which precede the acute onset of diabetes in the spontaneously diabetic BB rat by analyzing the serum metabolite profile detected with combined gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). We found that the metabolite pattern prior to diabetes included 17 metabolites, which differed between individual diabetes prone (DP) BB rats and their age and sex matched diabetes resistant (DR) littermates. As the metabolite signature at the 40 days of age baseline failed to distinguish DP from DR, there was a brief ten-day period after which the diabetes prediction pattern was observed, that includes fatty acids (e.g. oleamide), phospholipids (e.g phosphocholines) and amino acids (e.g. isoleucine). It is concluded that distinct changes in the serum metabolite pattern predict type 1 diabetes and precede the appearance of insulitis in spontaneously diabetic BB DP rats. This observation should prove useful to dissect mechanisms of type 1 diabetes.

Key words: type 1 diabetes; metabolomics; OPLS-DA; dynamic modeling

Type 1 diabetes (T1D) is one of the most common chronic diseases among children and young adults. The number of patients diagnosed every year steadily increases while age of onset decreases (Gillespie et al., 2004, Gale, 2002). T1D in humans is a well-defined "two step" disease affecting children at genetic risk primarily conferred by HLA-DQ on chromosome 6 (Onengut-Gumuscu and Concannon, 2006, Rich et al., 2009). The first step is the appearance of islet autoimmunity marked by autoantibodies against one or several of the autoantigens, GAD65, insulin, IA-2 or ZnT8 (for a review see (Pihoker et al., 2005, Wenzlau et al., 2007). Recently serum metabolite signatures were reported in children at genetic risk for T1D demonstrating reduced serum levels of succinic acid and phosphatidylcholine at birth, increased levels of lysophosphatidylcholine months before seroconversion to islet autoantibodies but that all changes were normalized after the seroconversion (Oresic et al., 2008). In some of these children, serum samples were available as close as three months prior to the presentation of their diabetes. Studies in spontaneously diabetic animals would therefore be useful to further analyze the mechanisms by which serum metabolites change during the days prior to the onset of diabetes.

The diabetes prone (DP) congenic DP DR.lyp BioBreeding (BB) rats (Fuller et al., 2006, Fuller et al., 2009) develop diabetes in a manner comparable to human T1D. Spontaneous diabetes occurs in both DP males and females at the time of puberty, which is also associated with the peak incidence of T1D in humans. As in humans, the DP BB rats have classic T1D symptoms including weight loss, polydipsia, polyuria and ketoacidosis as well as insulitis at the time of clinical onset (reviewed in Mordes et al. (Mordes et al., 2004). The MHC RT1Bu/u, the paralogue of human HLA-DQ confers the primary genetic susceptibility (Jacob et al., 1992). The spontaneous BB rat

diabetes is linked to a frame-shift mutation in the gene for the anti-apoptotic protein, Gimap5, a null allele resulting in lymphopenia (lyp) from birth (MacMurray et al., 2002, Hornum et al., 2002). Diabetes develops at 50-80 days of age in all congenic DR.lyp/lyp BB rats while both DR.lyp/+ (DR1) and DR.+/+ (DR2) littermates remain diabetes resistant (DR) (Fuller et al., 2006, Fuller et al., 2009). The first step in BB rat diabetes development is signs that the beta cells fail in function. Initially, islet autoantibodies were observed but in outbred BB rats only (Baekkeskov et al., 1984). In early congenic DR.lyp rats, pre-diabetic DP BB rats displayed reduced weight gain up to nine days before diabetes (Markholst et al., 1993). We hypothesized therefore that an autoimmune attack on the pancreatic β cells might be marked by subtle metabolic abnormalities. In support of this hypothesis, we recently showed that pre-diabetic DP rats failed to maintain core body temperature (Akesson et al., 2007) and that diabetes onset was heralded by a progressive shift from carbohydrate metabolism to lipid oxidation (Akesson et al., 2008). It can be suggested that these pre-diabetic metabolic changes, which preceded beta cell destruction and insulitis (Bieg et al., 2000), might be reflected in an altered metabolite profile in the peripheral blood.

In order to test the hypothesis that metabolic changes in serum predict diabetes we followed with repeat serum metabolomics analyses by LC/MS (Bruce et al., 2008) and GC/MS (A et al., 2005) combined with statistical dynamic modeling (Trygg et al., 2007, Trygg and Lundstedt, 2007, Stenlund et al., 2009), congenic DP BB rats along with their DR1 and DR2 littermates from 40 days of age until the rapid onset of diabetes. In contrast to Oresic et al. (2008), our study is specifically addressing to what extent the acute onset of diabetes in the BB rat is preceded by changes in the serum metabolite profile that would distinguish the pre-diabetic DP from the DR BB rats.

2 Materials and Methods

2.1 Reference compounds, stable isotope-labelled internal standards and other reagents.

The reference compounds were purchased from Sigma (St. Louis. MO, USA), Merck (Darmstadt, Germany), Aldrich (Steinhein, Germany) and Serva (Heidelberg, Germany). The compounds and reagents were all of analytical grade except where stated otherwise. The stable-isotope-labeled internal standard compounds (IS), [$^{13}C_5$]-proline, [$^{2}H_4$]-succinic acid, [$^{13}C_5$, ^{15}N]-glutamic acid, [1,2,3-$^{13}C_3$]-myristic acid, [$^{2}H_7$]-cholesterol, [$^{13}C_{12}$]-sucrose, [$^{13}C_4$]-palmitic acid and [$^{2}H_4$]-butane-diamine 2HCl from Campro (Veenendaal, the Netherlands) and [$^{2}H_6$]-salicylic acid from Icon (Summit, NJ, USA). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) plus 1% trimethylchlorosilane (TMCS) and pyridine (silylation grade) were purchased from Pierce Chemical Company, USA.

2.2 Congenic BB rats.

The parental DR.*lyp* (BBDR.BBDP.*lyp/lyp***)** line used in the present study was derived from animals with two independent recombination events developed from our previously described introgression of lymphopenia by cyclic cross-intercross breeding of BBDP with BBDR (Bieg et al., 2000). The first recombination event was flanked by simple sequence–length polymorphism (SSLP) marker D4Rhw10 and the second flanked by the SSLP marker D4Rhw11. These DR rats thus have a 2-Mb fragment of DP on chromosome 4 (Fuller et al., 2006, Fuller et al., 2009). The remainder of the genome represents BBDR as verified by genome-wide scanning (Fuller et al., 2006). The BBDR rats used to secure introgression of the recombination have been kept in

sister/brother breeding since 1983.

2.3 Housing

All animals were kept and bred in specific pathogen free (SPF) environment (http://depts.washington.edu/compmed/rodenthealth/index.html). The rats were given free access to food and water, on a 12-h light, 12-h dark cycle. All experiments were approved by the IACUC at University of Washington, Seattle, WA.

2.4 Genotyping

Five mm tail snips were obtained from the rats between 25 and 30 days of age and DNA was isolated using a phenol/chloroform protocol as previously described (Fuller et al., 2006). PCR reactions were performed in 10 μl master mix containing 10X reaction buffer, 50 ng isolated DNA (2 ng μl^{-1}), 2 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 0.05 μmol L^{-1} M IRDye 700 labelled primer, 1 μmol L^{-1} unlabeled reverse primer, 0.5 U Taq Polymerase and 0,04 mg ml^{-1} BSA. All samples were then subjected to one hold of 95°C for 5 min prior to 30 cycles of amplification (95°C for 20 s, 60°C for 20 s and 72°C for 30 s), which was followed by one final hold of 72°C for 3 min. Samples were kept at 4°C until use. PCR products were diluted to 25% with STOP solution and analysed using a NEN Global IR^2 DNA Analyser System (Model 4200S-2) using 6.5% gel matrix.

2.5 Phenotyping

Two drops of tail vein blood was diluted in Gey's solution and subjected to FACS analysis as described previously (Fuller et al., 2009). Cells were re-suspended in 100 μl FITC-labelled R73, diluted 1:400 in 4% BSA-PBS. The cells were incubated in the dark for 10 min and then washed by centrifugation in 100 μl BSA-PBS. The

supernatant was removed and the cells were re-suspended in 200 µl PBS and transferred to FACS-tubes to be analysed the same day. The frequency of TCR-positive T cells among mononuclear cells was determined on an EPICS Elite Flow Cytometer (Beckman Coulter, Fullerton, CA).

2.6 Diabetes diagnosis.

Starting at 40 days of age, all rats were weighed daily (Sartorious, Edgewood, NY) and blood glucose (Acsencia Elite XL, Bayer, Leverkusen, Germany) was tested if the rat did not gain weight as compared to the previous day. Diabetes was diagnosed when blood glucose exceeded 11.1 mmol L^{-1} (200 mg dL^{-1}) for two consecutive measurements the same day (AM and PM blood glucose) or in the morning the following day.

2.7 Study design.

Two series of experiments were carried out. In the first study, a tail vein blood sample (100-500 µl blood) was obtained mid-day from 16 DP, 11 DR1, 7 DR2 rats at 40 days of age. The 46 rats were from seven different litters with 7-13 rats in each litter, the average being nine rats. In the second series, a total of 21 rats were followed from 40 days of age until the onset of diabetes. In each litter of seven, 1-2 rats of each DR.lyp genotype were selected to be followed for a tail vein blood sample every five days. The DP rats (n=4) were followed until the onset of hyperglycemia and diagnosis of diabetes. The DR1 (n=4) and DR2 (n=4) rats were followed in parallel to each DP littermate.

2.8 Extraction of metabolites from rat serum

Extraction of metabolites from serum samples was essentially performed as described in A et al. (A et al., 2005). A total of 630 µl of MeOH:H_2O (9:1; v/v) including internal standards was added to 70 µl of serum. The solution was vortex mixed for 10 sec, kept on ice for 10 min, and then vigorously extracted at a frequency of 30 Hz for 3 min using a MM301 vibration Mill (Retsch GmbH & Co. KG, Haan, Germany). After 120 min on ice, the samples were centrifuged at 19 600 *g* for 10 min at 4°C and 200 µl of the supernatant was transferred to a GC vial, while 200 µl was transferred to a LC/MS and a GC/MS vial and evaporated to dryness.

2.9 GC/MS analysis.

Prior GC/MS analysis the samples were derivatised by shaking for 10 min at 5°C with 30 µL of methoxyamine hydrochloride (15 mg mL^{-1}) in pyridine and then incubating them for 16 h at room temperature. The samples were next trimethylsilylated by adding 30 µL of MSTFA with 1% TMCS and incubating them for 1 h at room temperature. After silylation, 30 µL of heptane (containing 0.5 µg methyl stearate as internal standard) was added.

The derivatised sample (1 µl) was injected split less by an Agilent 7683 Series Autosampler (Agilent, Atlanta; GA, USA) into an Agilent 6980 GC equipped with a 10 m×0.18 mm ID, fused silica capillary column chemically bonded with 0.18 µm DB5-MS stationary phase (J&W Scientific, Folsom, CA, USA). The injector temperature was set at 270°C. Helium was used as carrier gas at a constant flow rate of 1 ml/min through the column. For every analysis, the purge time was set to 60s at a purge flow rate of 20 ml min^{-1} and an equilibration time of 1 min. The column

temperature was initially kept at 70°C for 2 min, then increased from 70 to 320 °C at 40 °C min^{-1}, where it was held for 2 min. The column effluent was introduced into the ion source of a Pegasus III TOFMS (Leco Corp., St Joseph, MI, USA). The transfer line temperature was set at 250°C and ion source temperature at 200°C. Ions were generated by a 70 eV electron beam at a current of 2.0 mA. Masses were acquired from m/z 50 to 800 at a rate of 30 spectra s^{-1}, and the acceleration voltage was turned on after a solvent delay of 150 s.

2.10 LC/MS analysis

Chromatography was performed on a Waters Acquity™ system, equipped with column oven, coupled to a Waters LCT premier time-of-flight (Tof) mass spectrometer. An aliquot of the extracted sample was injected onto a 2.1 x 100 mm, 1.7 µm C_8 UPLC™ column. The gradient elution buffers were **A** (H_2O, 0.1% formic acid) and **B** (acetonitrile, 0.1% formic acid), and the flow-rate was 500 µl min^{-1}. The column was eluted with a linear gradient consisted of 1-20% B over 0-4 min, 20-40% of B 4-6 min, 40-95% B 6-9 min, the composition was held at 95% B for 4.5 min, and returned to 1% B at 14.50 min, the composition was kept at 1% B for a further 4.5 min before the next injection.

The mobile phase was introduced into an electrospray ion source. The source temperature was 120ºC with a cone gas flow of 10 L hr^{-1}, a desolvation temperature of 320ºC and a nebulization gas flow of 600 L hr^{-1}. The capillary voltage was set at 3 kV for positive ion mode, with a cone voltage of 0 V, a data acquisition rate of 0.1 s, an interscan delay of 0.1 s, with dynamic range enhancement (DRE) mode activated. Leucine enkephalin was employed as the lockmass compound for accurate mass measurements, infused straight into the MS at a concentration of 400 pg μL^{-1} (in

50:50 ACN:water) at a flow rate of 20 μL min^{-1}. The normal lockmass in the DRE mode was the positive ion 2^{nd} C^{13} peak of leucine enkephalin at 558.2829, and the extended lockmass peak was the normal positive ion peak observed at 556.2771. All mass spectral data were acquired in the centroid mode, 50 - 1000 m/z, with a data threshold value set to 3. "PseudoMSMS" analysis was performed by increasing the aperture 1 voltage from 5 to 45V.

2.11 Data processing of MS-data

All non-processed MS-files from the metabolic analysis were exported from the ChromaTOF (GC/MS; (Leco Corp., St Joseph, MI, USA) or MassLynx™ (LC/MS; Waters, Manchester, UK) software in NetCDF format to MATLAB™ software 7.0 (Mathworks, Natick, MA, USA), in which all data pre-treatment procedures, such as base-line correction, chromatogram alignment, time windows setting and Hierarchical Multivariate Curve Resolution (H-MCR) were performed using custom scripts according to Jonsson et al. (Jonsson et al., 2005, Jonsson et al., 2006). All manual integrations were performed using ChromaTOF 2.32 software, QuanLynx™ or custom scripts. In total were approx. 300 and 120 putative metabolites detected by GC/MS and LC/MS, respectively.

2.12 Mass spectra analysis

Metabolites from the GC/MS analysis were identified by comparing retention indices and mass spectra with data in retention index and mass spectra libraries (Schauer et al., 2005). Metabolites from the LC/MS analysis were identified by comparing retention time and mass spectra with data in the *in-house* retention time and mass

spectra library. Metabolites not available in the *in-house* library were identified or classified by determining the elemental composition from measured exact mass, followed by database searches (ChemSpider, http://www.chemspider.com; METLIN, http://metlin.scripps.edu; Lipid MAPS, http://www.lipidmaps.org/) and spectra interpretation of "PseudoMSMS analysis".

2.13 Statistical analysis

Principal component analysis (PCA) (Jackson, 1991) is frequently used to get an overview of a data table **X,** detect clusters and identify anomalies and outliers in the data. In metabolomics, the data table **X** [NxK] , where N defines the number rows or metabolite profiles, and K the number of quantified variables or metabolites is compressed into a few new "latent" variables, called scores **T**. The loading matrix **P** describes the influence of each of the original variables in the construction of the scores **T**:

$$X = TP^T + EX = TP^T + EX = TP^T + E \quad (1)$$

Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA)

OPLS-DA (Bylesjö et al., 2007, Trygg and Wold, 2002) was used for Prediction of Class (PoC) parameter prediction. In OPLS-DA, all metabolic profiles predicted by the model ($\hat{y}$) are assigned class-specific values of 1 or 0 by using a threshold. The threshold is determined by the number of samples in each class. For class balanced models, the threshold is 0.5. Further details of the OPLS-DA algorithm have been described previously (Bylesjö et al., 2007).

In OPLS the data are modeled by the model:

$$X = t_P p_P^T + T_O P_O^T + E \quad (2a)$$

$$y = t_P q_P^T + fX = t_P p_P^T + T_O P_O^T + E \quad (2b)$$

In OPLS-DA, the predictive component loading provides a direct measure of the influence of each variable. In metabolomics studies, the correlation scaled loading values reveal the most discriminatory variables. Correlation scaled p(corr) loading values are calculated as:

$$p(corr) = \frac{t^T x_i}{(\|t\| \|x_i\|)} \quad (3)$$

X_i represents the i^{th} variable in the X matrix (quantified metabolites).

All multivariate modeling was done in Simca-P+ version 12 (Umetrics, Umeå, Sweden).

The amount of explained variance in a model was calculated as:

$$R^2(X) = \frac{\Sigma(\hat{x}-x)^2}{\Sigma x^2} \quad (4)$$

being the modeled variation of **X**. Model complexity was determined using leave-one-out cross-validation (Wold, 1978a).

Dynamic modelling (Trygg et al., 2007, Trygg and Lundstedt, 2007, Stenlund et al., 2009) is a methodology that makes it possible to evaluate and handle different types of variations such as individual differences in metabolic kinetics, circadian rhythm, fast and slow responders and is also capable of handling sparse and unevenly sampled time series.

3 Results

Our investigation was based on a longitudinal study design of DP, DR1 and DR2 BB rats (Fig. 1a). The heterozygous breeding ensures that the offspring of 25% DP, 50% DR1 and 25% DR2 are born to non-diabetic parents. All rats were followed from 40 days of age until diabetes occurred in the DP BB rats (Fig. 1b) when the littermates kept in the same cage were killed in parallel. All DP rats developed acute hyperglycemia within 24 hours and were diagnosed when morning serum sample was obtained (Fig. 1b). The growth of DP BB rats was indistinguishable from DR1 and DR2 rats until the day of diabetes onset (Fig. 1c) when the DP rats rapidly started to lose weight unless given insulin (data not shown). The age at onset has in our previous studies varied between about 45 to 70 days of age (Fig. 1d). The unique and reproducible spontaneous onset of diabetes made it possible to test the hypothesis that a serum metabolite signature would predict acute diabetes in the DP and thereby distinguish these rats from the DR BB rats.

Our longitudinal study design included a subset of twelve (12) rats where serum samples from DP, DR1 and DR2 rats were first collected (Fig. 1e, X) around 40 days of age until DP rats was diagnosed for diabetes (Fig. 1e, red circles). Serum samples were collected simultaneously for rats housed in the same cage.

3.1 Comparison of serum profiles using PCA and dynamic modeling

The serum samples subjected to both GC/MS and LC/MS metabolomics analysis revealed a large number of quantified putative metabolites. We first analyzed the LC/MS data from all DP and DR samples at the 39-40 days of age baseline using

principal component analysis (PCA) (Jackson, 1991) (Supplementary Fig. 1). The PCA scores plot (t1-t2) of these analyses in normoglycemic DP (n=16), DR1 (n=7) and DR2 (n=12) rats revealed no systematic difference in the serum metabolite profiles between DP and DR rats. We then used PCA to model the temporal metabolic changes in the DP (n=4), DR1 (n=4) and DR2 (n=4) rats over all 66 samples (Fig. 2a). Each point in the scatter plot corresponds to a single sample, colored by genotype and labeled by its individual number (1-4) in that genotype class. Points that lie in close proximity are more similar compared to points that lie far from each other. For clarity, the t1-t2 temporal score values for each individual rat are also shown in a separate scatter plot. Inter-individual variations were noted between all twelve rats since they were found to be individually clustered at different areas of the scores plot (Fig. 2b). We therefore used PCA to map the metabolic trajectory for each individual rat in accordance with the dynamic modeling approach (Trygg et al., 2007, Trygg and Lundstedt, 2007, Stenlund et al., 2009). This means that each rat is used as its own reference, allowing subtle changes, specific to each individual rat over time to be examined. We then determined the multivariate temporal metabolic change between the two sampling points immediately preceding the onset of diabetes (linked by a horizontal line between red circles in Fig. 1e). This corresponds to the relative metabolic changes, between animal ages (days) 50-55 for all DP (n=4) rats, except the DP-2 rat and their age matched controls, DR1 (n=4) and DR2 (n=4), respectively. We could thereafter test our hypothesis that subtle distinct serum metabolite changes predict diabetes in the individual DP rat using orthogonal projections to latent structures discriminant analysis (OPLS-DA).

3.2 OPLS-DA to reveal the metabolite signature that distinguishes pre-diabetic DP

from DR rats

The metabolic profiles were predicted using OPLS-DA (Fig. 3a, b). The resulting OPLS-DA Prediction of Class (PoC) parameter based on leave-one-out cross-validated predictions (Wold, 1978b) revealed a metabolite signature, based on 17 identified or classified metabolites detected by LC/MS that was sufficient to clearly distinguish pre-diabetic DP from DR rats (sensitivity=100%, specificity=75 %) (Fig. 3a). The data in Figure 3b show the OPLS-DA correlation scaled loading values of these 17 metabolites that differentiated the pre-diabetic DP rats compared to the age matched DR rats. Six metabolites were increased and eleven decreased in the DP rats prior to any onset of diabetes. Prior to diabetes onset, all four DP rats had modulated concentrations of fatty acid derivatives (e.g. oleamide), phospholipids (e.g phosphocholines) or amino acids (e.g. isoleucine) compared to their diabetes-resistant DR1 and DR2 littermates. The versatility of our metabolomics approach to predict diabetes in the DP rats was further evident from the metabolites detected by GC/MS (Fig. 3c). Heat maps are used to illustrate relative metabolic changes prior to diabetes onset for amino acids, carbohydrates, organic acids, lipids and other diverse compounds (Fig. 3c). It is noted that the differences in isoleucine, certain lipids and fatty acids were independently detected by both LC/MS and GC/MS.

4 Discussion

Type 1 diabetes is defined according to current criteria solely by an increase in fasting plasma glucose above 11.1 mmol L^{-1} (Genuth et al., 2003). This single parameter definition or threshold level indicating a deviation from normal has been used for more than 90 years (Genuth et al., 2003). Our study in the well-characterized spontaneous diabetes of the BB rat has the advantage that the presentation of diabetes

to fulfill these criteria is acute as the 11.1 mmol L^{-1} is passed and much higher glucose concentrations reached within 24 hours. As the blood samples were obtained in the 40-50 days of age time span before any DP rat had developed diabetes, our data demonstrate that the glucose single parameter abnormality was preceded by significant alterations of several serum metabolites. This is important as this observation alone suggest that there may be other insulin-sensitive metabolic pathways that are more sensitive to a reduction in plasma insulin than the insulin-dependent uptake of glucose primarily in the liver. Based on the LC/MS analysis a predictive metabolite profile composed of 17 metabolites with decreased or increased levels distinguished DP from DR rats. These abnormalities in the DP rats appear spontaneously between 40 and 50 days of age. As the 40 days of age baseline metabolite measurements failed to distinguish DP from DR rats we conclude therefore that age and lymphopenia in the DP rats is not the explanation of the ability of our metabolomics approach to predict DP. Hence, the major finding in our study was that there are asymptomatic disease processes causing an increase in some and a decrease other metabolites resulting in a clear and easily detectable metabolite pattern that is unique to the DP rats which are about to develop diabetes. Our findings of major metabolite changes in asymptomatic pre-diabetic DP rats therefore surpass the expected major alterations including hyperglycemia, which were documented at the time of clinical onset of diabetes in the DP rats (Supplementary Fig. 2).

In children who developed T1D later in life it was reported that succinic acid and phosphatidylcholine were reduced already in the cord blood (Oresic et al., 2008). During follow up, increased levels of lysophosphatidylcholine were detected months before seroconversion to islet autoantibodies (Oresic et al., 2008). The metabolic

disturbance was partially normalized after seroconversion, which did not allow these authors to detect a metabolic profile that would predict the clinical onset of diabetes (Oresic et al., 2008). It is well accepted that the number of islet autoantibodies increases the risk for diabetes but does not tell the time to clinical onset (DPT-1, 2002, Sosenko et al., 2008). In our congenic BB rats, the metabolic pattern is similar to children who progressed to islet autoimmunity (Oresic et al., 2008). However, in the BB rat the progression to clinical onset is genetically controlled and simplified as there is no complicating phase of preclinical islet autoimmunity as in humans (Pihoker et al., 2005) or peri-insulitis as in the spontaneously diabetic NOD mouse (Mordes et al., 2004). The evaluation of metabolites in our BB rat serum samples was complicated by a high degree of normal physiological variation, such as individual differences in metabolic dynamics and individual responses to disease progression. This also implies that the often used modeling approach to compare controls with disease is not optimal as individual dynamics are not taken into account. For this reason, our study design was laid out as sequential samples of each individual rat over an appropriate time course to capture individual metabolic changes over time. This design allowed us to use each rat as its own control.

The second major finding in our investigation was therefore that according to LC/MS analysis, the changes in levels of only 17 identified or classified metabolites were sufficient to predict DP from DR rats. The additional identification of serum metabolites by GC/MS confirmed the findings by LC/MS but also extended the change in the serum metabolome that further underlined the dramatic changes that may take place in the DP BB rat metabolism prior to diabetes. The recent investigation of children followed from birth until onset of diabetes did for obvious

reasons not include serum samples obtained immediately prior to the clinical onset of diabetes (Oresic et al., 2008). As it is currently not possible to predict the clinical onset of childhood type 1 diabetes, the study schedule with a visit every three months enabled sampling at least as close as 3 months prior to onset (Oresic et al., 2008). At this point in time, no other significant differences but diminished phospholipids were reported (Oresic et al., 2008). However, it cannot be excluded that additional changes in metabolite pattern may emerge as the beta cell function deteriorates prior to onset. Our observation of significant alterations of several serum metabolites, to a degree consistent with the findings at 3 months prior to onset (Oresic et al., 2008), support the notion that there are subclinical disturbances in metabolism that precede the onset of diabetes caused by a rapid and specific loss of pancreatic islet beta cells.

The change in the serum metabolite profile prior to the acute onset of diabetes support our hypothesis that subtle metabolic changes precede the beta cell killing in DP-rats. We speculate that the previously reported increased in core body temperature (Akesson et al., 2007) and the progressive shift towards lipid oxidation relative carbohydrate metabolism (Akesson et al., 2008) might coincide with the observed change in the serum metabolite profile. Indeed, the long-chain fatty acid amide, oleamide has been shown to regulate several physiological functions, including an ability to decrease body core temperature. Oleamide does not have a designated receptor, but most likely act via cannabinoid receptors (CB-1 and CB-2), GABA A receptors and several serotonin receptors. The impact of oleamide and other bioactive amides on diabetes development is largely unknown but intriguing, as several of the metabolites in our report stem from the biosynthesis or breakdown of such molecules. It is important to note in this regard that both cannabinoid- and serotonin receptors are involved in the control of insulin secretion. Further, CB1 receptors have been

proposed to contribute to insulin resistance in human skeletal muscle cells (Eckardt et al., 2009) and in mouse adipocytes (D'Eon et al., 2008). Further studies will be needed to uncover the mechanism by which a decreasing beta cell mass prior to the clinical onset of diabetes may induce a change in the output of specific phospholipids.

The increased lipid oxidation prior to the onset of hyperglycemia (Akesson et al., 2008) is expected to increase serum free fatty acids. Our metabolomics data suggested a complex pattern of altered free fatty acid levels. Furthermore, it was of interest to note that a diacylglycerol was decreased and contributed to the model that distinguished DR from DP rats. The reduction in the diacylglycerol may reflect an increase in lipid oxidation as the demand for fatty acid synthesis is increasing and more fatty acids are needed for energy metabolism since the relative availability of insulin is decreasing. What are the consequences for serum levels of metabolites when lipid oxidation is increased? The relative reduction of insulin without altering blood glucose levels may contribute to a relative insulinopenia in the liver. The hepatocyte may respond with an altered metabolism to include an increase in lipid oxidation that may alter serum levels of not only lipids but also of amino acids.

The mechanisms of the rapid beta cell killing in the DP BB rat are not fully understood. While the strong linkage to MHC RT1B u/u is suggestive of T cell mediated killing (Mordes et al., 2004) recent analysis of the gene expression profile in both the pancreatic islets and lymphnodes draining the pancreas suggest that it cannot be excluded that mast cells may also contribute (Geoffrey et al., 2006, Hessner et al., 2004). It has previously been shown that the islets of Langerhans are not infiltrated with mononuclear cells until the time of onset but that there is subtle infiltration of the

pancreas by e.g. dendritic cells prior to hyperglycemia (Bieg et al., 2000). We therefore speculate that the serum metabolite signature may reflect spontaneous processes that eventually trigger the cellular killing of the pancreatic islet beta cells. Alternatively, it cannot be excluded that a slowly progressive loss of beta cells is resulting in a relative reduction in the pancreatic output of insulin to the liver. As the liver is extracting 80% of the insulin secreted from the pancreas we speculate that the hepatocytes are the first to experience the relative reduction in insulin.

The present findings provide the possibility to predict the prediabetic state not only in the DP rat but also human subjects (Oresic et al., 2008). There is evidence to suggest that gestational events may increase the risk for T1D in genetically susceptible children (Lynch et al., 2008, Larsson et al., 2007, Larsson et al., 2008). In 56 children who progressed to T1D serum succinic acid and phosphatidyl choline were reduced already at birth (Oresic et al., 2008). Seroconversion to islet autoantibodies long before the clinical onset is well documented in several studies including BABY DIAB (Bonifacio et al., 2004), DIPP (Nejentsev et al., 1999), DAISY (Lamb et al., 2009), DiPiS (Larsson et al., 2008) and TEDDY(TEDDY-Study-Group, 2008). The latter study in particular has the statistical power and frequent sampling (every three months until 4 years of age) to detect metabolite profiles that might predict step one, step two, or both of the disease. It is therefore of interest that both humans (Oresic et al., 2008) and our DP BB rats showed changes in phosphatidyl choline associated with diabetes risk. A direct comparison between human T1D and BB rat is complicated by the fact that their etiologies are vastly different. Nonetheless, the potential similarities in metabolomic findings suggest that the BB rat may be useful to establish cross-species markers that would facilitate interpretation of data from preclinical studies in BB rats,

aiming to prevent diabetes.

5 Concluding remarks

Our study demonstrates that the combined use of metabolomics (GC/MS and LC/MS) and statistical modeling based on the individual rat's metabolic response (dynamic modeling) are effective means to detect an altered serum metabolite profile that predict diabetes onset. The study of congenic DR.lyp rats prior to diabetes in the DP DR.lyp/lyp BB rat indicate that the ensuing diabetes is associated with pronounced yet subclinical metabolic abnormalities that predict diabetes. It is striking to note that serum metabolites completely distinguished DP from DR rats before the DP rats had developed diabetes. These findings are of great value, as early prediction would allow early intervention and preservation of pancreatic islet beta cells and thus endogenous insulin production.

Acknowledgement

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Figure Legends

Figure 1. Experimental design. A)Heterozygot breeding of BB rats, resulting in 25% diabetes prone DR.lyp/lyp (DP, red), 50% diabetes resistant DR.lyp/+ (DR1, shown in blue) and 25% DR.+/+ (DR2, blue) littermates were used for metabolic profiling. All rats were sampled daily for blood glucose (B) and weight (C). D) Colony data for diabetes distribution shows that no rat was hyperglycemic before 47 days of age and all of the rats were diagnosed before 79 days of age, median age being 64 days of age. E) Starting at 40 days of age, blood samples were drawn approximately every five days until hyperglycemia onset, and subject to metabolomics analyses (samples marked by "X"). Red circles (O) represent onset samples and horizontal lines indicate pairs of samples used in the Prediction of class modeling (Fig. 3A).

Figure 2. A) Principal components analysis to map the overall temporal metabolic changes in all DP and DR rats. B) For clarity the t_1-t_2 score values for each individual rat are also shown in a separate scatter plot. Inter-individual variations are observed between individual rats as their score values are clustered at different areas of the scores plots.

Figure 3. A) OPLS-DA Prediction of Class (PoC) based on LC/MS data on relative changes in 17 serum metabolites from DP and DR rats. A PoC value above the threshold value of 0,33 predicts diabetes in the individual DP rat prior to hyperglycemia onset. B) The correlation scaled loading values of the OPLS-DA predictive component based on the LC/MS data revealed the most influential

metabolites. Out of the 17 metabolites that differentiated the pre-diabetic DP rats from the age matched DR rats, six metabolites showed increased relative levels (shown as positive P(corr) value), and eleven decreased levels in the DP rats (shown as negative P(corr) value). C) Correlation based heat map of amino acids, carbohydrates, organic acids, lipids and other diverse compounds illustrate the relative metabolic changes from GC/MS based analysis prior to hyperglycemia onset.

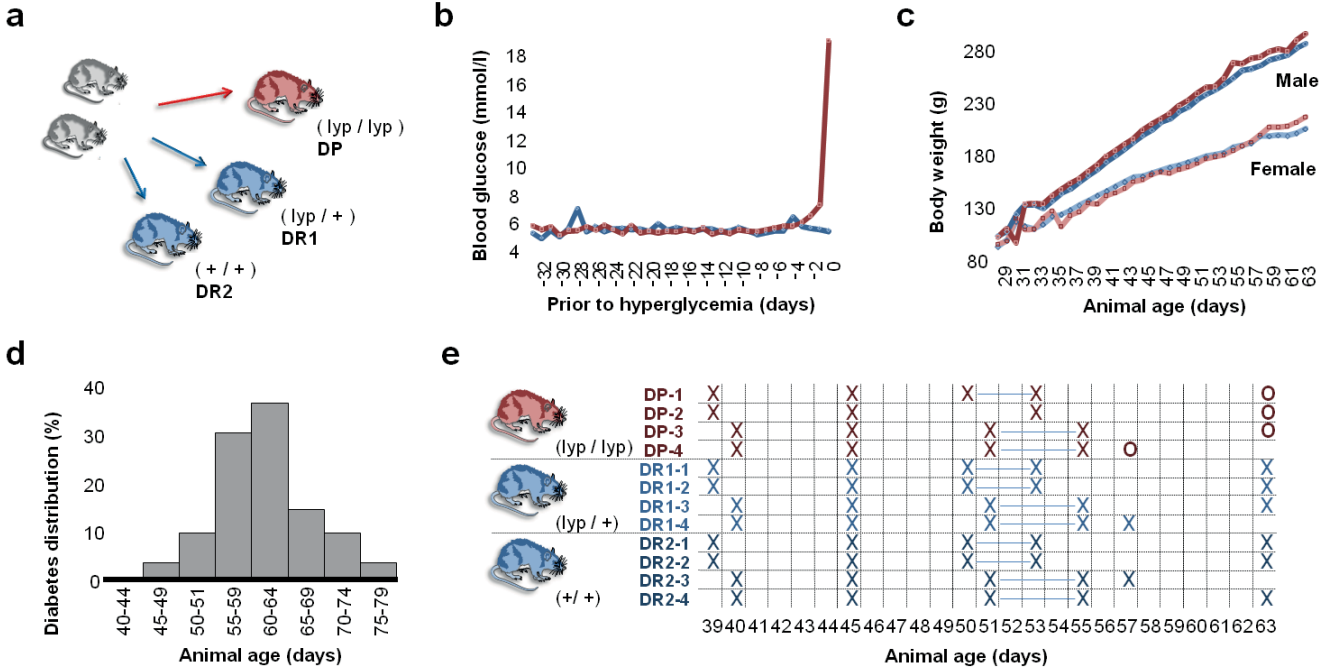

a
(lyp / lyp)
DP
(lyp / +)
DR1
(+ / +)
DR2
b
Blood glucose (mmol/l)
Prior to hyperglycemia (days)
c
Body weight (g)
Male
Female
Animal age (days)
d
Diabetes distribution (%)
40-44
45-49
50-51
55-59
60-64
65-69
70-74
75-79
Animal age (days)
e
DP-1
DP-2
DP-3
DP-4
(lyp / lyp)
DR1-1
DR1-2
DR1-3
DR1-4
(lyp / +)
DR2-1
DR2-2
DR2-3
DR2-4
(+/ +)
Animal age (days)

Figure 1

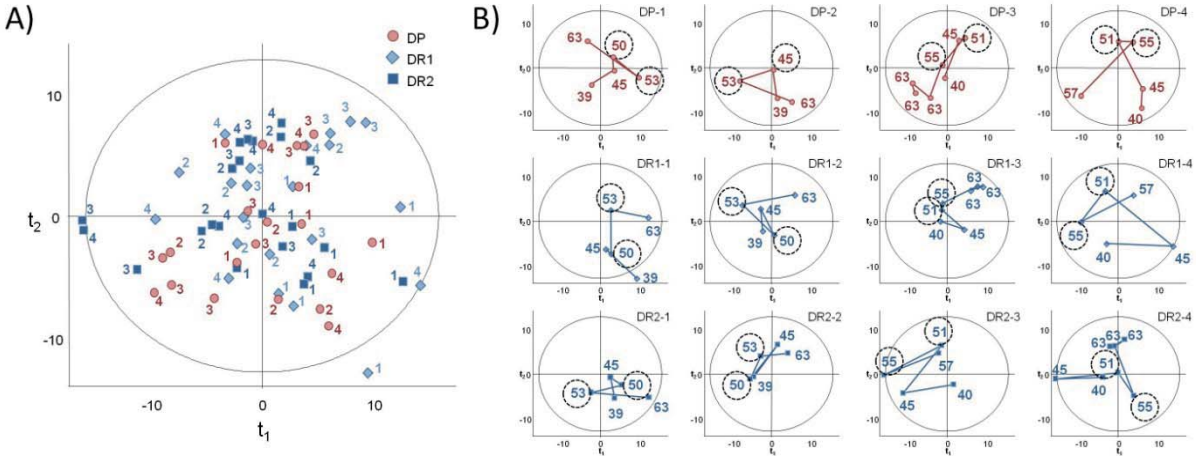

A)
DP
DR1
DR2
10
0
-10
t_2
-10
0
10
t_1
B)
DP-1
DP-2
DP-3
DP-4
DR1-1
DR1-2
DR1-3
DR1-4
DR2-1
DR2-2
DR2-3
DR2-4

Figure 2

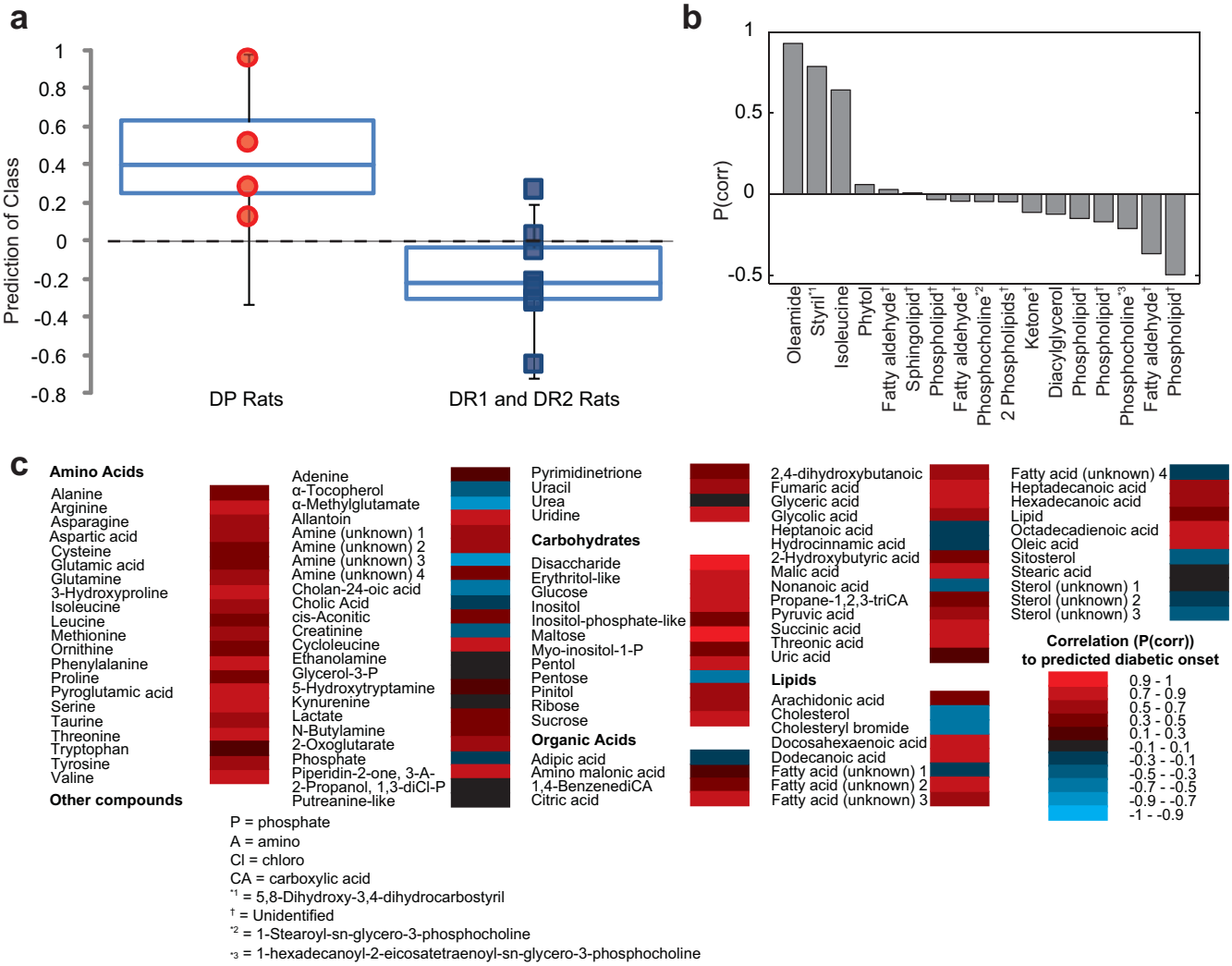

a
Prediction of Class
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
DP Rats
DR1 and DR2 Rats
b
P(corr)
1
0.5
0
-0.5
Oleamide
Styril*1
Isoleucine
Phytol
Fatty aldehyde†
Sphingolipid†
Phospholipid†
Fatty aldehyde†
Phosphocholine*2
2 Phospholipids†
Ketone†
Diacylglycerol
Phospholipid†
Phospholipid†
Phosphocholine*3
Fatty aldehyde†
Phospholipid†
c
Amino Acids
Alanine
Arginine
Asparagine
Aspartic acid
Cysteine
Glutamic acid
Glutamine
3-Hydroxyproline
Isoleucine
Leucine
Methionine
Ornithine
Phenylalanine
Proline
Pyroglutamic acid
Serine
Taurine
Threonine
Tryptophan
Tyrosine
Valine
Other compounds
Adenine
α-Tocopherol
α-Methylglutamate
Allantoin
Amine (unknown) 1
Amine (unknown) 2
Amine (unknown) 3
Amine (unknown) 4
Cholan-24-oic acid
Cholic Acid
cis-Aconitic
Creatinine
Cycloleucine
Ethanolamine
Glycerol-3-P
5-Hydroxytryptamine
Kynurenine
Lactate
N-Butylamine
2-Oxoglutarate
Phosphate
Piperidin-2-one, 3-A-
2-Propanol, 1,3-diCl-P
Putreanine-like
Pyrimidinetrione
Uracil
Urea
Uridine
Carbohydrates
Disaccharide
Erythritol-like
Glucose
Inositol
Inositol-phosphate-like
Maltose
Myo-inositol-1-P
Pentol
Pentose
Pinitol
Ribose
Sucrose
Organic Acids
Adipic acid
Amino malonic acid
1,4-BenzenediCA
Citric acid
2,4-dihydroxybutanoic
Fumaric acid
Glyceric acid
Glycolic acid
Heptanoic acid
Hydrocinnamic acid
2-Hydroxybutyric acid
Malic acid
Nonanoic acid
Propane-1,2,3-triCA
Pyruvic acid
Succinic acid
Threonic acid
Uric acid
Lipids
Arachidonic acid
Cholesterol
Cholesteryl bromide
Docosahexaenoic acid
Dodecanoic acid
Fatty acid (unknown) 1
Fatty acid (unknown) 2
Fatty acid (unknown) 3
Fatty acid (unknown) 4
Heptadecanoic acid
Hexadecanoic acid
Lipid
Octadecadienoic acid
Oleic acid
Sitosterol
Stearic acid
Sterol (unknown) 1
Sterol (unknown) 2
Sterol (unknown) 3
Correlation (P(corr))
to predicted diabetic onset
0.9 - 1
0.7 - 0.9
0.5 - 0.7
0.3 - 0.5
0.1 - 0.3
-0.1 - 0.1
-0.3 - -0.1
-0.5 - -0.3
-0.7 - -0.5
-0.9 - -0.7
-1 - -0.9
P = phosphate
A = amino
Cl = chloro
CA = carboxylic acid
*1 = 5,8-Dihydroxy-3,4-dihydrocarbostyril
† = Unidentified
*2 = 1-Stearoyl-sn-glycero-3-phosphocholine
*3 = 1-hexadecanoyl-2-eicosatetraenoyl-sn-glycero-3-phosphocholine

Figure 3

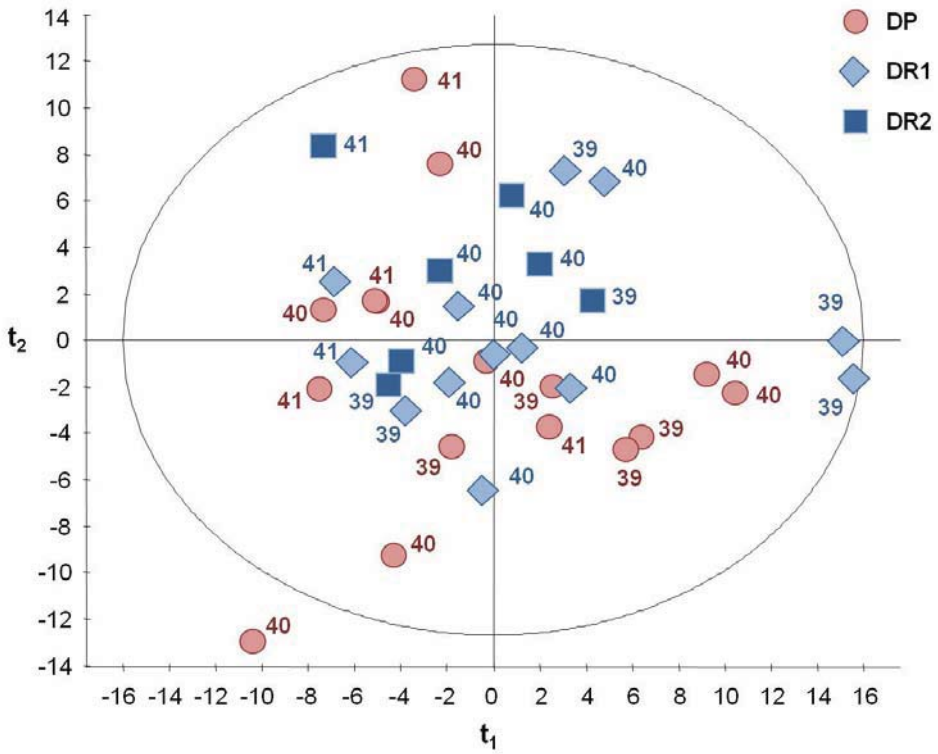
Figure 1-S.

An overview of the serum metabolite profiles from all DP and DR samples at the 39-40 days of age baseline. The PCA scores plot (t_1-t_2) of the normoglycemic DP (n=16), DR1 (n=7) and DR2 (n=12) rats reveals no systematic difference in the serum metabolite profiles at baseline day. The metabolite profiles were analyzed by LC/MS.

Figure 2-S

Cross validated OPLS-DA Prediction of Class (PoC) of onset of hyperglycemia in the DP rats compared to the DR rats.

SUPPLEMENTARY MATERIAL



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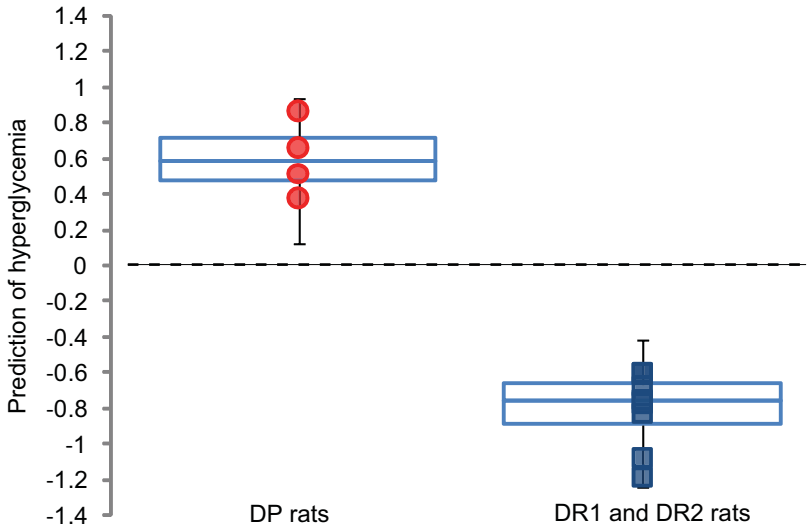

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