Diagnosing Impaired Glucose Tolerance Using Direct Infusion Mass Spectrometry of Blood Plasma

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**Abstract**

The goal of this study was to evaluate the capacity for mass spectrometry of blood plasma to diagnose impaired glucose tolerance (IGT). For this study, blood plasma samples from control subjects (n = 30) and patients with IGT (n = 20) were treated with methanol and low molecular weight fraction were then analyzed by direct infusion mass spectrometry. A total of 51 metabolite ions strongly associated with IGT were detected. The area under a receiver operating characteristic (ROC) curve (AUC) for diagnosing IGT that was based on an analysis of all these metabolites was 0.93 (accuracy 90%, specificity 90%, and sensitivity 90%). The associated reproducibility was 85%. The metabolites identified were also consistent with risk factors previously associated with the development of diabetes. Thus, direct infusion mass spectrometry of blood plasma metabolites represents a rapid, single-step, and reproducible method for the analysis of metabolites. Moreover, this method has the potential to serve as a prototype for clinical analyses that could replace the currently used glucose tolerance test with a more patient-friendly assay.

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**Introduction**

Impaired glucose tolerance (IGT) is a pre-diabetic state that is associated with insulin resistance and an increased risk of cardiovascular pathology. Moreover, IGT has been shown to precede type 2 diabetes mellitus by many years [1]. To prevent or delay the development of diabetes in pre-diabetic individuals, changes in diet and increased physical activity are recommended [2,3]. Currently, the oral glucose tolerance test (OGTT) represents the ‘gold standard’ for detecting IGT. However, this test has exhibited low reproducibility despite being considered useful for a diagnosis of IGT, as well as for diabetes and other cardiovascular risk factors [4–6]. In addition, an OGTT is time consuming (takes 2 h) and some people may experience sugar shock during it [7]. Therefore, a more rapid and reproducible test for diagnosing IGT is needed.

In this study, a metabolomics approach was evaluated for its ability to diagnose IGT. In metabolomics, a large number of small molecules (metabolites) can be detected in samples, and in the case of bodily fluid samples, this capacity provides great potential for the development of diagnostic assays [8,9]. Previously, the majority of metabolomic studies of blood plasma samples have been conducted using multi-stage protocols [10], and numerous diagnostic metabolites have been identified, including metabolites related to a pre-diabetic state. More recently, prospective nested case control studies identified five branched chain and aromatic amino acids as predictors of type 2 diabetes [11]. In another study, the metabolites, glycine, lysophosphatidylcholine, and acetylcarnitine, exhibited significantly altered levels in patients with IGT compared to individuals with normal glucose tolerance [12].

Of the available metabolomics technologies, direct infusion mass spectrometry appears to be the most suitable for clinical application. Using this technique, biological materials can be directly applied to an ionization source of a mass spectrometer without any preliminary separation, and the capacity for this approach to be used for diagnostics has been demonstrated in previous studies [13–17]. Consequently, direct infusion mass spectrometry can characterize a metabolome without additional distortion being introduced by a separation step. Moreover, this should simplify the translation of this metabolomics-based method into the clinic. Therefore, in the present study, direct infusion mass spectrometry (DIMS) of blood plasma metabolites was performed to evaluate this method as a diagnostic assay for a pre-diabetic state characterized by IGT.

**Materials and Methods**

**Patient cohorts**

Study participants were recruited at the Polyclinic Department of the Endocrinology Research Centre (Moscow, Russia). The study was approved by the ethical review committee #27-01 of the RAMS (Moscow, Russia), approval number #64 (statement # 01-
Blood sampling and sample preparation

Blood samples for metabolomic analysis were taken from the vein before the morning meal. Samples (3 ml) were placed into glass tubes containing K$_2$EDTA (BD Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged within 15 min of blood collection at 1600 g. The resulting blood plasma was subjected to one freeze/thaw cycle. To test the reproducibility of the protocol, an additional set of blood samples (n = 20) were centrifuged at 13000 x g (MiniSpin plus centrifuge; Eppendorf AG, Hamburg, Germany) for 10 min. Deproteinized supernatants were then transferred to clean plastic Eppendorf tubes, and fifty volumes of methanol containing 0.1% formic acid (Fluka) was added to each tube. The resulting solutions were subjected to mass spectrometry analysis.

Mass spectrometry analysis

Samples were analyzed with a maXis hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray ionization (ESI) source (Supporting Information S1). The mass spectrometer was set up to prioritize the detection of ions with a mass-to-charge ratio (m/z) ranging from 50 to 1000, with a mass accuracy of 1–3 parts per million (ppm). Spectra were recorded in the positive ion charge detection mode. Samples were injected into the ESI source using a glass syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) connected to a syringe injection pump (KD Scientific, Holliston, MA, USA). The flow rate of samples to the ionization source was 180 μl/h, and samples were injected in a randomized order (e.g., control samples were run between case samples). Mass spectra were obtained using DataAnalysis version 3.4 (Bruker Daltonics) to summarize one minute signals. Ion metabolite masses were determined from the mass spectrum peaks obtained using the DataAnalysis program. All peaks above noise level (signal to noise ratio >1) were selected, and the metabolite ion masses were pooled and processed using Matlab version R2010a (MathWorks, Natick, MA, USA). Alignment of mass peaks was performed as described previously [16]. This and all other calculations were performed using Matlab software.

| Table 1. Clinical characteristics of patient cohort. |
|-----------------------------------------------|
| CHARACTERISTICS | VALUES (average ± SD/range) | Subjects with IGT | AUC | t-test (p-value) |
| Number | 30 | 20 | - | - |
| Sex (male/female) | 15/15 | 10/10 | 0.51 | - |
| Age (years) | 53.3 ± 14.0/32–82 | 61.8 ± 12.2/38–85 | 0.66 | 0.03 |
| BMI (kg/m$^2$) | 35.0 ± 8.2/24.5–53.2 | 33.9 ± 8.9/23.2–57.0 | 0.46 | 0.66 |
| Fasting glucose (mmol/l) | 5.5 ± 0.4/4.8–6.4 | 5.6 ± 0.4/4.5–6.1 | 0.55 | 0.51 |
| Glucose in OGTT (mmol/l) | 6.4 ± 1.0/4.1–7.8 | 9.8 ± 1.0/8.2–11.0 | 1.00$^*$ | 0.00 |
| Insulin (μU/ml) | 16.1 ± 20.0/3.3–100.8 | 14.5 ± 7.9/4.9–31.7 | 0.59 | 0.74 |
| HbA1c (%) | 5.8 ± 0.4/5.1–6.4 | 6.1 ± 0.4/5.4–6.6 | 0.77 | 0.001 |
| LDL (mmol/l) | 3.4 ± 0.7/1.7–5.3 | 3.1 ± 1.0/1.6–4.7 | 0.41 | 0.16 |
| HDL (mmol/l) | 1.2 ± 0.4/0.7–1.9 | 1.1 ± 0.4/0.6–1.9 | 0.40 | 0.23 |
| Cholesterol (mmol/l) | 5.2 ± 0.8/3.9–6.6 | 5.1 ± 1.2/3.0–6.8 | 0.47 | 0.58 |
| Uric acid (μmol/l) | 374 ± 82/223–514 | 386 ± 83/265–581 | 0.54 | 0.61 |
| Triglycerides (mmol/l) | 1.3 ± 0.6/0.5–3.0 | 1.69 ± 0.9/0.8–3.9 | 0.62 | 0.07 |
| HOMA-IR | 4.0 ± 4.8/0.8–24.2 | 3.6 ± 2.1/1.1–8.6 | 0.59 | 0.79 |
| HOMA-β | 160 ± 202/31–1061 | 139 ± 76/55–283 | 0.58 | 0.65 |
| mbGTT | 9.9 ± 7.2/0–26 | 32.3 ± 9.9/10–46 | 0.93 | 0.0000 |

$^*$The AUC for glucose (OGTT) is equal to 1 since the OGTT test was used to establish control and IGT groups. AUC, a receiver operating characteristic (ROC) curve; OGTT, oral glucose tolerance test; mbGTT, mass spectrometry-based GTT; HOMA, homeostatic model assessment; BMI, body mass index.
For mass spectrometric peaks included in IGT pattern and having clear isotope patterns, the correspondence to the specific metabolites from the database “Human Metabolome Database” (http://www.hmdb.ca) [19] and/or Metlin (Scripps Center for Mass Spectrometry, USA; http://metlin.scripps.edu) [20] was established. Theoretical isotope patterns for each of these metabolites were generated using the Molecular Weight Calculator v.6.46 program (http://ncrr.pnl.gov).

Score calculation for the mass spectrometry-based GTT

Metabolite ions with peak intensities strongly associated with IGT (n = 51) were included in the calculation of a mass spectrometry-based glucose tolerance test (mbGTT) score. To this end, the intensity of each peak was considered as a measure for a separate two-stage test, where the final mbGTT score was represented as the number of positive results from these tests. To define the threshold values that would separate positive and negative results for all single-ion tests and the final mbGTT score, ROC curves were generated using the rocplot function of the Matlab program. This function returns all required thresholds with the corresponding accuracies, sensitivities, specificities, and area under ROC curve (AUC) values. Mass peaks having clear isotope patterns, the correspondence to the specific metabolites from the database “Human Metabolome Database” (http://www.hmdb.ca) [19] and/or Metlin (Scripps Center for Mass Spectrometry, USA; http://metlin.scripps.edu) [20] was established. Theoretical isotope patterns for each of these metabolites were generated using the Molecular Weight Calculator v.6.46 program (http://ncrr.pnl.gov).

Metabolic pattern of IGT

A metabolic pattern was established using a list of ion masses strictly associated with IGT and which were detected using ESI-DIMS. For each metabolite ion, the threshold for the mass peak intensity (derived from the metabolite concentration) was defined in order to separate positive and negative results for IGT, and these threshold values were expressed in quintiles that were defined based on the control set of mass spectra (n = 30). For example, if the intensity of a mass peak with a m/z of 133.097 is higher than that of 0.57 quintiles, i.e., 133.0970.57, then the mbGTT score should be increased by one. For metabolite ions expressing a lower intensity with IGT (these ion masses are near the limit-of-detection (LOD), and therefore, potentially did not exhibit valid distributions in the mass spectral data) were not included in calculations of mbGTT scores.

Metabolite identification

Metabolites associated with IGT that were identified from the samples analyzed based on high AUC values included fatty acids, amides of fatty acids, and five other metabolites: butanediol, phosphoglycolic acid, p-cresol sulfate, ornithine, and phosphatidycholine (Table 2). In addition, metabolites present at lower concentrations, yet having high AUC values, were found to represent quasi-molecular ions containing potassium ions.

Discussion

Development of an mbGTT

Mass spectrometry techniques that are currently available facilitate the capture of high-throughput ‘snapshots’ of the metabolome [8,14]. Moreover, for the analysis of bodily fluids, mass spectrometry has exhibited great potential for its application in diagnostic assays. In the present study, DIMS of plasma samples using an ESI source in positive ion mode (optimal for the ionization of many blood plasma substances [22]), resulted in the detection of ~4000 metabolite ions per sample. This comprehensive dataset provided valuable insight into the metabolome of blood plasma, and also demonstrated the potential for this approach to diagnose metabolic disorders related to prediabetes.

Efficacy of the mbGTT

Calculation of an AUC value is an ideal method for classifying the efficacy of a two-stage test. For example, it was previously demonstrated that AUC values ranging from 0.5–0.6 indicate a test does not work; 0.6–0.7, a poor, yet functional, test; 0.7–0.8 – a good test; 0.8–0.9, an excellent test [23]. The AUC value for the mbGTT performed in the present study was 0.93, thereby classifying this test as having excellent efficacy. However, the mbGTT did not achieve maximum accuracy based on the use of OGTG results to distinguish IGT and control groups.

An AUC value comprehensively characterizes the diagnostic power of the two-stage test, to which the mbGTT also belongs, and threshold values specified in the IGT pattern are defined using the control set of samples (i.e., the samples influence the diagnostic pattern). Correspondingly, the capacity for the mbGTT to diagnose IGT was additionally validated using the leave-one-out test. Negligible decreases that were observed in the diagnostic parameters of the leave-one-out test relate to slight fluctuations in the threshold values from one run to another during testing. More
stable threshold values could be established if a larger control set was used to define the quintiles.

Reproducibility of the mbGTT

The low reproducibility of the OGTT is a key shortcoming of this assay [4–6]. Moreover, glucose levels, as well as the level of most metabolites in blood, can widely vary. For example, the coefficient of variation (CV) for glucose levels for a 2 h OGTT is ~25% [4], thereby leading to very low reproducibility of the OGTT test for diagnosing IGT. Typically, such a CV value is not acceptable for bioanalytical assays where a CV of 15% is considered a maximum permissible value [24].

Metabolite concentrations measured by mass spectrometry also generally exhibit a very high CV (median CV of 46%) [25]. As a result, mass spectrometry-based metabolomic tests are characterized by very low reproducibility, and this prevents their implementation in clinics [26]. However, the mbGTT overcomes this shortcoming by using a score calculation method which averages metabolite fluctuations. Thus, mbGTT can accommodate the concomitant increase and decrease in levels of different subsets of metabolites. Ideally, when metabolite levels are independent from each other and their number is high, the CV of mbGTT scores should approach zero. The CV for the mbGTT score obtained in the present study was 8.4%, which is significantly better than the CV value permissible for bioanalytical assays.

Figure 1. A typical mass spectrum of human plasma metabolites. This mass spectrum was obtained following the direct injection of a deproteinated blood plasma sample into an electrospray ion source of a hybrid quadrupole time-of-flight mass spectrometer. The main metabolite groups detected are labeled. The embedded figure in the right upper corner shows the single-stage workflow that was used to obtain the corresponding metabolome profile of blood plasma by ESI-DIMS.

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Figure 2. ROC curve for the mbGTT. A total of 50 samples from IGT cases (n = 20) and control individuals (n = 30) were used to build a ROC curve. The mbGTT score was based on data for 51 metabolite ions. The point represented on the ROC curve represents the maximum mbGTT accuracy value. The area under the ROC curve (AUC) is shaded.

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### Table 2. Blood plasma metabolites with high AUC values that are associated with IGT.

| #  | Metabolite                        | Identification # in database | Molecular weight | Detected Ion | Elemental composition | Wilcoxon test (p-value) | AUC  |
|----|-----------------------------------|-----------------------------|------------------|--------------|----------------------|-------------------------|------|
|    |                                   |                             |                  |              |                      |                         |      |
|    | **Increased with IGT**            |                             |                  |              |                      |                         |      |
| 1  | 2,3-Butanediol                    | HMD03156                    | 135.0390         | 135.0392     | [M+2Na-H]^+          | 0.0027                  | 0.76 |
| 2  | Linoleamide                       | Metlin ID 43435             | 280.2631 302.2842| 280.2635 302.2854 | [M+H]^+ [M+Na]^+     | 0.0012 0.0101           | 0.77 0.71 |
| 3  | Oleamide                           | HMD802117                   | 282.2778         | 282.2711     | [M+H]^+ [M+Na]^+     | 0.0178                  | 0.71 |
| 4  | Stearamide                         | HMD834146                   | 284.2916         | 284.2948     | [M+H]^+              | 0.0052                  | 0.74 |
| 5  | Decenedioic acid                  | HMD800603                   | 223.0943         | 223.0941     | [M+Na]^+             | 0.0288                  | 0.72 |
| 6  | α- (or β-) keto-octanoic acid     | HMD813211 (or HMD819721)    | 181.0827         | 181.0835     | [M+Na]^+             | 0.0335                  | 0.71 |
| 7  | Octenoic acid                     | HMD800392                   | 165.0859         | 165.0886     | [M+Na]^+             | 0.0183                  | 0.73 |
| 8  | Malic acid                        | HMD800156                   | 178.9882         | 178.9927     | [M+2Na-H]^+          | 0.0204                  | 0.72 |
| 9  | Glucuronic acid                   | HMD800127                   | 239.0164         | 239.0138     | [M+2Na-H]^+          | 0.0303                  | 0.71 |
| 10 | Phosphoglycolic acid              | HMD800816                   | 200.9532 156.9831| 200.9535 156.9879 | [M+2Na-H]^+ [M+H]^+ | 0.016                   | 0.74 |
| 11 | p-Cresol sulfate                  | HMD811635                   | 211.0033         | 211.0035     | [M+Na]^+             | 0.0050                  | 0.76 |
| 12 | Ornithine                         | HMD800214                   | 133.0962         | 133.0972     | [M+H]^+              | 0.0227                  | 0.72 |
| 13 | Phosphatidylcholine               | HMD808097                   | 366.7848         | 366.7805     | [M+2H]^2+            | 0.058                   | 0.71 |
|    | **Decreased with IGT**            |                             |                  |              |                      |                         |      |
| 14 | K$_2$Cl$^-$                       | -                           | 112.8943         | 112.8957     | -                    | 0.0027                  | 0.79 |
| 15 | K$_2$NaCl$_2$                    | -                           | 170.8530         | 170.8543     | -                    | 0.0335                  | 0.79 |

Metlin ID refers to an identification number in the METLIN metabolite database, and ‘HMD’ refers to an identification number in the Human Metabolome Database. AUC, a receiver operating characteristic (ROC) curve; IGT, impaired glucose tolerance.

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Mass spectrometry pattern of IGT

An IGT pattern was identified from metabolite ion masses strictly associated with the disease state. For each metabolite ion, a threshold value was established for the mass peak intensity in order to distinguish positive and negative results for the IGT state. The mass values presented in the pattern are expressed in absolute units (i.e., m/z, which is generally equal to daltons). Mass peak intensities, derived from each metabolite’s concentration in blood, are expressed in units which depend on the type, model, and settings of the mass spectrometer, as well as detector consumption, purity of used solutions, the operating state of the ion source and ion transferring system, and the exact pH value of the samples. Therefore, these units are not reproducible from one mass spectrometer to another. To overcome this problem and to make the IGT pattern acceptable for diagnostics, defined threshold values were expressed in quintiles.

Generally, if one variable is higher than another, this will be detected by an instrument. If the variables have a range according to their values, this order should be preserved independently from the instrument used (i.e., the order of variables cannot be changed). This statement is the basis for establishing a metabolic pattern for IGT that can be adapted for different mass spectrometers. Quintiles are often used to set cut-off points for a given dataset. For example, a 0.3 quintile defines a threshold that separates 30% of the lower variables from the others, and this set of variables, as well as other sets separated by quintiles, are non-alterable and independent from the measuring instrument used. Therefore, the IGT pattern with threshold values expressed in quintiles represents an acceptable method for accommodating the use of different mass spectrometers.

Metabolite contributions to the mbGTT

The identification of the metabolites which contributed to the mbGTT results was an additional step that was performed to validate the proposed approach for diagnosing IGT. Blood plasma signatures for prediabetes have been well-characterized, including recent data from metabolomics studies [11,12]. In particular, amidic of the fatty acids, also known as endocannabinoids, play an important role. For example, activation of the endocannabinoid system has been shown to increase food intake, promote weight gain [27,28], and can also contribute to the worsening of a cardiovascular profile (i.e., body weight, body mass index (BMI), waist circumference, insulin and adiponectin levels) [29]. The increased endocannabinoid levels detected in IGT patients in the present study is consistent with these data. Increased fatty acid levels were also detected in subjects with IGT, and this is consistent with previously published data as well. It is known that prediabetic metabolic syndrome has been characterized by increased levels of lipids [30], including fatty acids [31].

Decenedioic acid is a dicarboxylic acid. For diabetic patients, increased urinary excretion of dicarboxylic acids occurs, and is considered to be a marker of an oxidative attack on fatty acids [32]. Increased levels of decenedioic acid were detected in the present study, and may represent evidence that this oxidative attack can occur in a prediabetic state. However, further studies will be needed to confirm this.

Phosphatidylcholine with the elemental composition C_{26}H_{45}NO_{15}P was present at higher levels in the IGT patients analyzed in this study. Phosphatidylcholine may also be related to metabolic syndromes associated with IGT that are characterized by lipid disorders.

\(\alpha\)-ketoacids is a branch-chain keto acid and an intermediate metabolite of the branch chain amino acid, leucine. Branched-chain amino acids have recently been discovered to be biomarkers of diabetes risk [11]. In the present study, an association between \(\alpha\)-ketoacids levels and IGT was observed, \(\beta\)-ketoacids are another fatty acid that is formed from the precursor molecule, malonyl-CoA, and may be another metabolite related to identified keto acid. Higher levels of \(\beta\)-ketoacids have been detected in subjects with obesity and diabetes [33,34]. Therefore, it is possible that at least one, or both, of these metabolites contributed to the mbGTT performed.

Another metabolite identified in the present study based on its high AUC value was p-cresol sulfate, which is a microbial metabolite that likely derives from secondary metabolism of p-cresol. Diabetic patients have previously shown to have higher concentrations of both free and total p-cresol concentrations in their blood [35].

Ornithine, another metabolite associated with IGT, is an amino acid produced by the urea cycle with the release of urea from arginine. It was previously established that plasma ornithine concentrations are higher in diabetic subjects [36], and this is a marker of arginine activity. In general, the latter tends to be lower in diabetic patients [36].

Phosphoglycolic acid is a substrate for triose-phosphate isomerase, and an increase in its levels has not previously been associated with IGT.

Butanediol is produced by a variety of microorganisms during a process known as butanediol fermentation [37]. This process involves the anaerobic fermentation of glucose and butanediol is one of the end products. It is possible that this metabolite is reflected in the IGT pattern since gut microbiota have been shown to play a role in the development of type 2 diabetes [38].

Of the metabolites of IGT that exhibited lower levels, changes in potassium levels were a key finding. Previously it was shown that potassium loss occurs with diabetic ketoacidosis. Specifically, there is an obligate loss of positively charged potassium ions from kidney tubules due to increased levels of negatively charged ketones present during IGT. The results of the present study are consistent with these findings.

Although the levels of other metabolites were also observed to decrease, the identification of these metabolites was not successful. ESI is a technique that can include the addition of H\(^+\), K\(^+\), and Na\(^+\) to substances being subjected to mass spectrometry. In the present study, H\(^+\), Na\(^+\) levels were found to be constant in all samples. In contrast, K\(^+\) (Table 2), and as consequence all potassium-containing quasi-ions, exhibited high AUC values for the IGT samples. Therefore, additional studies using a technique other than ESI-DIMS are needed to identify the other metabolites associated with IGT that undergo a decrease in blood plasma levels.

It should be noted, that metabolic pattern of IGT, intended for diagnostics by direct infusion mass spectrometry, and metabolite identification results (Table 2) should not exactly match each other. There is not capacity to identify all metabolites in IGT pattern. Especially it concerns low-abundant metabolites which may have high diagnostic power but do not show clear isotopic pattern in mass spectrum or metabolite interference disturbs isotopic pattern in mass spectrum. Moreover, metabolite interference may lead to situation when not first metabolite’s isotope (i.e. the reference molecular weight of metabolite in database) is included in IGT pattern.

Conclusions

In conclusion, the metabolites identified by ESI-DIMS of blood plasma samples from IGT patients and healthy controls confirm...
that the mbGTT is affected by the metabolite signature of blood plasma, and can indicate the development of diabetes in its early stages. Additional validation of this mbGTT are needed using a larger population. However, it is anticipated that the mbGTT may replace the OGTT for establishing a diagnosis of IGT based on the improved reproducibility of this test that is also more rapid to conduct and more patient friendly.

Future studies are also needed to validate the potential for capillary blood and its dried droplets to be used for this test. The small volume of sample that is required for direct infusion mass spectrometry suggests that capillary blood could be used to perform the mbGTT. Currently, dried droplets of blood are generally accepted for metabolic blood analyses. Therefore, if there are difficulties in performing the mbGTT in clinical laboratories, the use of dried capillary blood could be an alternative means by which samples could be transported to centralized laboratories that would perform the mbGTT.

Supporting Information

Table S1 Clinical characteristics of patients. (DOC)

Supporting Information S1 Mass spectra of blood plasma metabolites obtained by direct infusion mass spectrometry (DIMS) of blood plasma samples from control subjects (n=30) and patients with impaired glucose tolerance (n=20). (ZIP)

Author Contributions

Conceived and designed the experiments: PGL AIA IID. Performed the experiments: DLM EEB EAS. Analyzed the data: PGL. Contributed reagents/materials/analysis tools: AIA IID MVS. Contributed to the writing of the manuscript: PGL.

References

1. Tabač AG, Herder C, Rathmann W, Brunner Ej, Knvi maki M (2012) Prediabetes: Spectrum state for diabetes development. Lancet 379: 2279–2290. doi:10.1016/S0140-6736(12)60289-9.

2. Tuomilhoj, L, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, et al. (2001) Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 344: 1393–1398. Available: http://www.ncbi.nlm.nih.gov/pubmed/11546019.

3. Raina Elley C, Kenealy T (2008) Lifestyle interventions reduced the long-term risk of diabetes in adults with impaired glucose tolerance. Evid Based Med 13: 173. Available: http://www.ncbi.nlm.nih.gov/pubmed/19043011.

4. McDonald GW, Fisher GF, Burnham C (1965) Reproducibility of the Oral Glucose Tolerance Test. Diabetes 14: 473–480. Available: http://www.ncbi.nlm.nih.gov/pubmed/14334038.

5. Balion CM, Raina PN, Gerstein HC, Santaguida PL, Morrison KM, et al. (2007) Reproducibility of impaired glucose tolerance (IGT) and impaired fasting glucose (IFG): classification: a systematic review. Clin Chem Lab Med CCLM FESCOC 43: 1180–1105.

6. Ko GT, Chan JC, Woo J, Lau E, Yeung VT, et al. (1998) The reproducibility and usefulness of the oral glucose tolerance test in screening for diabetes and other cardiovascular risk factors. Ann Clin Biochem 35 (Pt 1): 62–67.

7. Julius Sagel, Colwell VA (1973) Shock During Oral Glucose Tolerance Testing. JAMA 226: 667–670.

8. Grossa GN, Zhang S, Gu H, Asiago V, Shazaiah N, et al. (2008) Metabolomics-based methods for early disease diagnostics. Expert Rev Med Diagn 8: 617–633.

9. Ellis DI, Dunn WB, Griffin JL, Allwood JW, Goodacre R (2007) Metabolic fingerprinting as a diagnostic tool. Pharmacy Communications 3: 1243–1286.

10. Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics. Mass Spectrom Rev 26: 51–78.

11. Strathmann FG, Hoofnagle AN (2011) Current and future applications of mass spectrometry to the clinical laboratory. Am J Clin Pathol 136: 609–616. doi:10.1309/AJCPWOTAOBNGCK.

12. Metz CE (1978) Basic principles of ROC analysis. Semin Nucl Med 8: 283–298.

13. U.S. Department of Health and Human Services, FDA, CIDER C (2001) Guidance for Industry: Bioanalytical Method Validation Guidance for Industry. Bioanalytical Method Validation. 246.

14. Krebs B, Wikoff WR, Patti GJ, Woo H-K, Kalisiak E, et al. (2009) Variability analysis of human plasma and cerebral spinal fluid reveals statistical significance of changes in mass spectrometry-based metabolomics data. Anal Chem 81: 8538–8544.

15. Martens HA, Dardenne P (1998) Validation and verification of regression in small data sets. Chemometrics and Intelligent Laboratory Systems. Vol. 44. pp. 99–121. doi:10.1016/S0169-7439(98)00167-1.

16. Lin L, Bennett BD, Rabinowitz JD (2008) Analytical strategies for LC-MS-based targeted metabolomics. J Chromatogr B Anal Technol Biomed Life Sci 871: 236–242.

17. Engeli S, Bolonne J, Feldpausch M, Gorzelniak K, Janke J, et al. (2005) Activation of the peripheral endocannabinoid system in human obesity. Diabetes 54: 2091–2097.

18. WHO (1999) Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. Report of a WHO Consultation. doi:10.1002/SCIC:1996-915619980715:7:239:AID-DIAA667:3.0.CO;2-S.
37. Geckil H, Barak Z, Chipman DM, Erenler SO, Webster DA, et al. (2004) Enhanced production of acetoin and butanediol in recombinant Enterobacter aerogenes carrying Vitreoscilla hemoglobin gene. Bioprocess Biosyst Eng 26: 325–330. doi:10.1007/s00449-004-0373-1.

38. Qin J, Li Y, Cai Z, Li S, Zhu J, et al. (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490: 55–60. doi:10.1038/nature11450.