EBioMedicine 2 (2015) 2016–2023

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Article

# A High Level of Intestinal Alkaline Phosphatase Is Protective Against Type 2 Diabetes Mellitus Irrespective of Obesity☆

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ARTICLE INFO

*Article history:*
Received 11 July 2015
Received in revised form 15 November 2015
Accepted 16 November 2015
Available online 1 December 2015

*Keywords:*
Fatty liver
Glucose intolerance
Hypertension
Incipient diabetes
Insulin resistance
The metabolic syndrome
Obesity

ABSTRACT

Mice deficient in intestinal alkaline phosphatase (IAP) develop type 2 diabetes mellitus (T2DM). We hypothesized that a high level of IAP might be protective against T2DM in humans. We determined IAP levels in the stools of 202 diabetic patients and 445 healthy non-diabetic control people. We found that compared to controls, T2DM patients have approx. 50% less IAP (mean +/− SEM: 67.4 +/− 3.2 vs 35.3 +/− 2.5 U/g stool, respectively; $p < 0.000001$) indicating a protective role of IAP against T2DM. Multiple logistic regression analyses showed an independent association between the IAP level and diabetes status. With each 25 U/g decrease in stool IAP, there is a 35% increased risk of diabetes. The study revealed that obese people with high IAP (approx. 65 U/g stool) do not develop T2DM. Approx. 65% of the healthy population have < 65.0 U/g stool IAP, and predictably, these people might have 'the incipient metabolic syndrome', including 'incipient diabetes', and might develop T2DM and other metabolic disorders in the near future. In conclusion, high IAP levels appear to be protective against diabetes irrespective of obesity, and a 'temporal IAP profile' might be a valuable tool for predicting 'the incipient metabolic syndrome', including 'incipient diabetes'.



## 1. Introduction

Type 2 diabetes mellitus (T2DM) is characterized by hyperglycemia, and it is a major global health problem that affects nearly 5.3% population of the world with devastating consequences in the context of healthcare cost, morbidity and mortality. T2DM causes long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (American Diabetes Association, 2014; Abdullah et al., 2014; Kahn et al., 2014). It is a major global health problem that affected 387 million people worldwide in 2014 and cost 612 billion US dollars (Abdullah et al., 2014). In the United States, estimated 24.4 million people (9.2% population) had diabetes in 2013, costing 306 billion dollars (Abdullah et al., 2014).

Etiologically, various factors have been postulated to be involved in the development of T2DM, such as autoimmunity, the metabolic syndrome, diets, obesity, infection, ethnicity, genetic polymorphism and predisposition, drugs, stress, sedentary lifestyle, pregnancy, etc. (Velloso et al., 2013; Kahn et al., 2014; Stumvoll et al., 2005; Masters et al., 2011). Recently, a low-grade systemic inflammation, induced by persistently increased levels of endotoxin lipopolysaccharides (LPS) in blood (metabolic endotoxemia), has been implicated as an etiological factor for T2DM (Cani et al., 2007). In our previous work, we have shown that mice deficient in the brush-border enzyme intestinal alkaline phosphatase (IAP) (*Akp3* knockout, $Akp3^{-/-}$) develop T2DM (Kaliannan et al., 2013). We have also shown that IAP detoxifies LPS and reduces metabolic endotoxemia, and oral supplementation with IAP not only prevents but also cures high-fat diet-induced T2DM in wild-type mice (Kaliannan et al., 2013). Because IAP deficiency results in T2DM in mice, we hypothesized that a high level of IAP might play a protective role against T2DM in humans. Therefore, using a case-control design we recruited T2DM and non-T2DM subjects from a community in the suburb of Dhaka, Bangladesh, and determined the levels of IAP in the stools of these participants. Here, we report that people with diabetes have less amounts of IAP in their stool compared to their healthy counterparts. Also, we found that obese people with high IAP do not develop T2DM. Further, results of this study suggest that 'temporal IAP profiling' might be a valuable tool for identifying 'incipient' T2DM and other metabolic disorders.

*Abbreviations:* AP, alkaline phosphatase; BMI, body mass index; FPG, fasting plasma glucose; IAP, intestinal alkaline phosphatase; IAP-KO, IAP knockout; L-Arg, L-homoarginine; L-Phe, L-phenylalanine; LPS, lipopolysaccharides; TNAP, tissue non-specific alkaline phosphatase.

☆ Funding: Harvard Biotech BD Ltd., Dhaka, Bangladesh.

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http://dx.doi.org/10.1016/j.ebiom.2015.11.027

## 2. Materials and Methods

### 2.1. Study Design and Participants

A case-control study was used to assess the difference in the concentrations of IAP in stools of diabetic and control healthy populations. Participants, aged 30–70 yr., were recruited from a suburban community of Dhaka, Bangladesh by advertisement through local dignitaries, hospitals, clinics and physicians' offices. Based on preliminary data (unpublished) the sample size of each group was calculated to achieve statistical power of 80% or more (continuous endpoint, $\alpha = 0.05$). The study included 202 diabetic cases (63 males and 139 females) and 445 healthy control subjects (114 males and 331 females) of the same ethnicity. The study included more females than males just because of more accessibility to female participants. People with T2DM were diagnosed by a physician for at least 6 months prior to their recruitment, and were on oral antihyperglycemic agents and/or insulin medication. Newly diagnosed persons with hyperglycemia (FPG > 7.0 mmol/l) were also included in the diabetic group (see below). All participants were on unrestricted diets. Any person suffering from an acute disease was excluded from the study. Pregnant women, patients with Type 1 diabetes and history of cancer were excluded. All participants were tested for renal, hepatic and cardiovascular diseases, and participants with clinically significant renal, hepatic and cardiovascular diseases were excluded. The participants did not have any history of chronic alcohol consumption. Underweight persons (body mass index, BMI < 18.5 kg/m$^2$) were also excluded.

The Institutional Review Board (IRB) of Harvard Biotech BD Ltd. (Dhaka, Bangladesh) reviewed and approved the study. An informed consent form to participate in the study was signed by each participant.

### 2.2. Laboratory Tests, Physical Examination and Socio-Medical History

Laboratory tests were performed using biochemical assay kits from Linear Chemicals S.L. (Barcelona, Spain) and an automatic biochemistry analyzer from Sinnowa Medical Science & Technology Co., Ltd. (Nanjing, Jiangsu, China; Model: Sinnolab MT 5000, Version 5.00). The diabetes status of each participant (diabetes patients and healthy controls) was confirmed by measuring fasting (at least 10 h) plasma glucose (FPG). An FPG level > 7.0 mmol/l (126 mg/dl), was considered diagnostic for diabetes (American Diabetes Association, 2014). Any newly diagnosed diabetes patient (FPG > 7.0 mmol/l) was included in the diabetic group. All participants were also subjected to serum biochemical tests for cholesterol, low-density lipoproteins (LDL), high-density lipoprotein (HDL), triglycerides, creatinine and alanine aminotransferase (ALT). Height and weight of each participant were measured to calculate the BMI defined as weight in kg divided by the square of height in meter (kg/m$^2$). Participants were asked for the history of alcohol consumption. Medical history was used to discover participants with kidney, liver and heart diseases. The status of cardiovascular disease was also evaluated by measuring blood pressure.

### 2.3. Homogenization of Stool

The supernatant of a homogenized stool suspension was used for alkaline phosphatase (AP) assay. A small amount of fresh stool (mgs) was measured and then the 'stool dilution buffer' (10 mM Tris–HCl, pH 8.0, 1 mM magnesium chloride, 10 μM zinc chloride) at a defined ratio was added. Usually, 50 μl of stool dilution buffer was added to 1 mg of stool. The sample was vigorously vortexed to prepare a homogenized stool suspension, which was then centrifuged at 10,000 × *g* for 20 min, and the supernatant containing AP was collected and assayed for AP concentration. It is to point out that stool suspended in water shows a bit lower AP activity compared to stool suspended in stool dilution buffer.

### 2.4. Alkaline Phosphatase Assay

The stool supernatant was assayed for alkaline phosphatase (AP) following an established protocol using the automatic biochemistry analyzer mentioned above (Nanjing, Jiangsu, China). In brief, 20 μl of supernatant was added to 1 ml of enzyme assay buffer (1.25 M diethanolamine (DEA) buffer, pH 10.2, 0.6 mM magnesium chloride) containing 10 mM p-nitrophenyl phosphate (pNPP), and the reaction mixture was incubated for one min at 37 °C followed by measuring the AP concentration by the analyzer pre-calibrated with AP standards. To determine the major isoform among stool APs, prior to assaying for AP activity an aliquot of stool sample was treated for 10 min with L-phenylalanine (L-Phe, 10 mM final conc.), a specific inhibitor of IAP, and another aliquot with L-homoarginine (L-Arg, 10 mM final conc.), a specific inhibitor of tissue-nonspecific alkaline phosphatase (TNAP) (Kaliannan et al., 2013; Sergienko et al., 2009). Each aliquot treated with an inhibitor was then mixed with the reaction buffer containing an equal concentration (10 mM) of the respective inhibitor, and assayed for AP activity using the analyzer. Because most of the AP activity in stool is due to IAP (see Results) the stool AP values are expressed as units of IAP/g stool. All AP assays were performed by a single laboratory technologist who was blinded to the diagnoses of participants.

### 2.5. Statistical Analysis

The SAS System (SAS Institute, Cary, North Carolina) was used for statistical analysis. Mean and standard errors were calculated for T2DM cases and non-T2DM controls stratified by sex. The correlation between IAP levels and various risk factors for T2DM was assessed via Pearson correlation coefficient stratified by sex and T2DM status (T2DM patients or non-T2DM controls). Mean differences in IAP levels between T2DM cases and non-T2DM controls were assessed via linear regression models controlling for the effects of age, sex, FPG and BMI on IAP levels and T2DM status. The statistical significance of the variance associated with independent variables were assessed from sum of square III using GLM procedure in SAS. Multiple logistic regression using Proc Logist procedure in SAS assessed association between T2DM cases with independent risk factors including IAP. Regression coefficients and odds ratios were used to express the independent risk contribution of IAP to T2DM status. The statistical significance of the difference between two groups was determined using unpaired two-tailed Student's t-Test. The difference between two groups was considered significant when the *p* value was < 0.05. Student's t-Test was performed using Microsoft Excel program. Post-hoc statistical power analysis of two independent groups was performed using an online program (http://clincalc.com/Stats/Power.aspx).

### 2.6. Role of the Funding Source

The funding source (Harvard Biotech BD Ltd., Dhaka, Bangladesh) played no role in the development of theory and study design. Harvard Biotech BD Ltd. was also not involved in the collection, analyses, and interpretation of data, or writing and submission of the manuscript. The corresponding author had full access to all the data in the study, and took the decision for publication of the data.

## 3. Results

### 3.1. Stool Alkaline Phosphatase Activity Is Mostly Due to Intestinal Alkaline Phosphatase

Intestinal alkaline phosphatase (IAP) knockout (*Akp3*$^{-/-}$) mice develop T2DM (Kaliannan et al., 2013), and based on this observation we hypothesized that IAP might play a protective role against T2DM in humans. Accordingly, we decided to measure IAP concentrations in the stools of diabetic and control non-diabetic healthy people. However,