SUPPLEMENTARY MATERIALS

Intestinal alkaline phosphatase deficiency increases the risk of diabetes

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Short Title: IAPD increases the risk of diabetes

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Abbreviations: AP, alkaline phosphatase; BMI, body mass index; FPG, fasting plasma glucose; IAP, intestinal alkaline phosphatase; LPS, lipopolysaccharides; LTA, lipoteichoic acids; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

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Appendix A

SUPPLEMENTARY STATISTICS ON DIABETES

(Refs: 1-9)

PREVALENCE

- Global number of people having diabetes in 2019: Approx. 463 million (9.3% of world adult population)
- Global number of people projected to have diabetes in 2030: Approx. 578 million (10.2% of world adult population)
- Global number of people projected to have diabetes in 2045: Approx. 700 million (10.8% of world adult population)
- Number of people in the USA having diabetes in 2018: Approx. 34.2 million (9.8% of total adult population)

DEATH

- Global death directly from diabetes in 2016: Approx.1.6 million
- Global death associated with hyperglycemia in 2012: Approx. 2.3 million
- U.S. death from diabetes in 2017: Approx. 270,700

HEALTH EXPENDITURE

- Global health expenditure on T2DM in 2017: Approx. $727 billion US dollars
- U.S. health expenditure on T2DM in 2017: Approx. $327 billion US dollars
- U.S. health expenditure on T2DM projected in 2030: Approx. $622 billion US dollars

Appendix B

INTESTINAL ALKALINE PHOSPHATASE

GENE

- Human: ALPI alkaline phosphatase, intestinal [Homo sapiens (human)] (synonymous to intestinal alkaline phosphatase, IAP)
- Gene ID: 248 (https://www.ncbi.nlm.nih.gov/gene/248)
- Location: Chromosome 2q37.1
- mRNA: 2259 bases CDS: 31-1617 (GenBank: BC132678.1)
- Protein: 528 aa (https://www.ncbi.nlm.nih.gov/nuccore/BC132678.1)

FUNCTION

IAP is exclusively expressed in the villus-associated enterocytes of small intestine and hence known as enterocyte differentiation marker. The enzyme is bidirectionally secreted into intestinal lumen as well as systemic circulation.[10] The luminal IAP travels downwards from the proximal small intestine to the distal large intestine and then excreted with stool.[11]

The physiological and pharmacological properties of IAP have been extensively reviewed.[12-16] Physiologically, IAP exerts an anti-inflammatory effects by detoxifying various bacterial toxins, such as lipopolysaccharides (LPS), lipoteichoic acid (LTA), CpG DNA, flagellin and uridine diphosphate (UDP), and IAP inactivates these targets by dephosphorylation (phosphohydrolysis).[17-21] Also, IAP maintains the normal homeostasis of intestinal microbiota and promotes the gut bacterial growth by reducing the concentrations of intestinal luminal

nucleotide triphosphates (ATP, etc.) that have toxic effect on bacterial growth.[22] Further, IAP maintains the gut mucosal integrity, regulates intestinal luminal pH, and limits fat absorption .[23-25]

Pharmacologically, in different animal and human models IAP has been used to treat the metabolic syndrome, enterocolitis, inflammatory bowel disease, peritonitis, acute kidney injury, enteropathogenic infections and ageing.[7,11,26-34]

Appendix C

SUPPLEMENTARY METHODS

STUDY POPULATION

In 2015, we conducted a case-control study (Case: Diabetes patients, Control: Healthy participants (no diabetes)), which established that intestinal alkaline phosphatase (IAP) deficiency is directly associated with T2DM.[7] When the average IAP level was 65 U/g stool in healthy people, it was 35 U/g stool in T2DM patients. In the context of defining a causative role of IAPD in the development of T2DM, we followed-up the health status of 671 participants of the control group for almost 5 years, wherein the baseline physical and biochemical profiles, including body mass index (BMI), fasting plasma glucose (FPG) and IAP values, lipid profile, of each individual participant was known. The follow-up data (second visit) were not available for 97 participants (19 males, 78 females; excluded group) because of migration, pregnancy, refusal, death, etc. Accordingly, the current prospective cohort study included 574 participants (168 males and 406 females). The participants, 30 to 60 years old, were stratified into two groups based on baseline IAP levels (Low IAP Group (IAPD Group): <65.0 U/g stool; High IAP Group (No IAPD Group): ≥65.0 U/g stool). Because of easy accessibility, the original case-control study included more females than males, and as a consequence, the number of females is also higher in this current prospective study. A participant was diagnosed as having diabetes when the FPG level was ≥7.0 mmol/l (126 mg/dl) or HbA1c level ≥6.5%.[1] All participants were on unrestricted diets, and had no history of chronic alcohol consumption.

The National Research Ethics Committee (NREC) of Bangladesh Medical Research Council (BMRC), Government of the People's Republic of Bangladesh (Dhaka, Bangladesh) reviewed and approved the study (BMRC Registration Number:150 02 10 2018; Ref.: BMRC/NREC/2016-2019/28). Each participant consented to participate in the study and signed an informed consent form approved by the NREC.

LABORATORY PROCEDURES

The procedures for physical examination, socio-medical history and biochemical tests have been previously described.[7] Physical examination of a participant included measuring height, weight and blood pressure. Body mass index (BMI) was calculated as weight in kg divided by the square of height in meter (kg/m^2). Each participant was inquired for the history of diabetes, heart, kidney and liver diseases, and also alcohol consumption. Laboratory tests were completed using biochemical assay reagents 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 was confirmed by measuring fasting (at least 10 hours) plasma glucose (FPG) concentration. An FPG level of ≥7.0 mmol/l (126 mg/dl) or HbA1c level ≥6.5% was considered diagnostic for diabetes.[1] Each participant was also subjected to serum biochemical tests for cholesterol, low-density lipoproteins (LDL), high-density lipoprotein (HDL), triglycerides, creatinine and alanine aminotransferase (ALT) as previously described.[7]

STOOL ALKALINE PHOSPHATASE (STAP) ASSAY

Alkaline phosphatase (AP) assay was performed on the supernatant of a homogenized stool suspension as previously described.[7] In brief, 100 mg of fresh stool was suspended in 5 ml of 'stool dilution buffer' (10 mM Tris-HCl, pH 8.0, 1 mM magnesium chloride, 10 μM zinc chloride) and homogenized by rigorous vortexing. The stool suspension was then centrifuged at 10,000 x g for 20 min, and the supernatant containing AP was collected and used for measuring AP concentration. AP in the stool supernatant was measured using the automatic biochemistry analyzer mentioned above (Nanjing, Jiangsu, China). Briefly, 20 μl of stool supernatant was mixed with 1 ml of AP assay buffer (1.25 M diethanolamine (DEA) buffer, pH 10.2, 0.6 mM magnesium chloride) containing 10 mM p-nitrophenylphosphate (pNPP). The reaction mixture was incubated for one min at 37^{o}C, and then AP concentration was measured by the analyzer pre-calibrated with AP standards. Because approx. 80% of the AP activity in stool is due to IAP, the stool AP (STAP) values are expressed as units of IAP per gram stool as previously defined.[7] AP assays were performed by laboratory technologists who were blinded to the diagnoses of participants.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SAS 9.4 software (SAS Institute, Inc., Cary, North Carolina). Mean and standard deviation were determined for controls and T2DM cases stratified by gender. Depending on the normality of the data, unpaired two-tailed Student's *t*-test or Wilcoxon-Mann–Whitney U test was used to examine the statistical significance of difference between two groups (control and T2DM). The correlation between IAP and various risk factors for T2DM was evaluated by Pearson's correlation coefficients stratified by gender and T2DM

status (T2DM patients or non-T2DM controls). Adjusted mean difference in IAP level between T2DM cases and non-T2DM controls was calculated by generalized linear model (GLM) of regression analysis controlling for the effects of sex, age, BMI, creatinine, Cholesterol, HDL, LDL, TG, ALT, SBP and DBP. The statistical significance of the variance associated with independent variables were determined from sum of square III using GLM procedure in SAS. Chi-square test was used to examine the association between the IAPD group and T2DM status. Log-binomial regression analysis was conducted using PROC GENMOD procedure in SAS to calculate the unadjusted and adjusted risk ratios (RR). The independent risk contribution of IAP to T2DM status was evaluated based on the adjusted RR. We performed repeated measure ANOVA (i.e., repeated measure generalized linear regression analysis) to examine the association of the IAP level with T2DM adjusted for other independent variables. A p value of <0.05 was considered statistically significant for all statistical tests. An online program was used to perform post-hoc statistical power analysis of two independent groups (http://clincalc.com/Stats/Power.aspx).

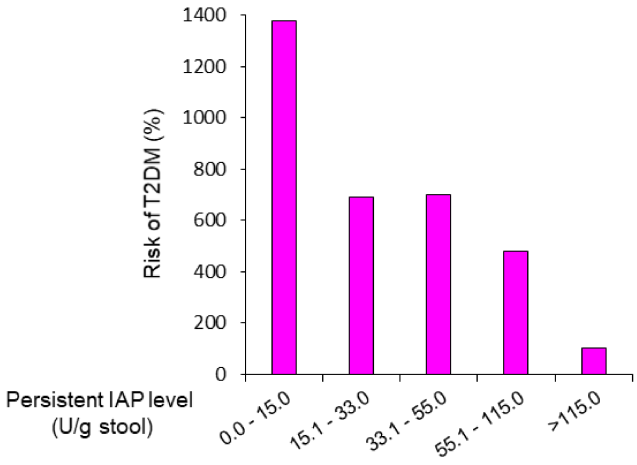

Fig. S1. A 5-year prospective cohort study shows that persistent intestinal alkaline phosphatase deficiency (IAPD) increases the risk of developing type 2 diabetes mellitus (T2DM). **Note:** The lower the persistent IAPD the higher the risk of T2DM.

Table S1. The participants included in or excluded from the study have similar physical and biochemical characteristics.

Characteristic	Total participants		Males		Females	
	Included	Excluded	Included	Excluded	Included	Excluded
No. of participants	574	97	168	19	406	78
Age group (years)	30 - 60	30 - 60	30 - 60	30 - 60	30 - 60	30 - 60
Average age (years)	41.7 ± 9.1	40.8 ± 8.9	42.8 ± 9.0	44.5 ± 8.8	41.2 ± 9.1	39.9 ± 8.8
Weight (kg)	58.4 ± 11.2	59.1 ± 11.7	63.0 ± 10.0	63.3 ± 12.0	56.6 ± 11.2	58.0 ± 11.5
Height (m)	1.5 ± 0.2	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.2	1.5 ± 0.1
BMI (kg/m^2)	25.0 ± 4.5	25.5 ± 4.6	23.8 ± 3.6	24.3 ± 4.8	25.5 ± 4.8	25.8 ± 4.5
Systolic blood pressure (mmHg)	129.7 ± 20.9	131.4 ± 19.4	130.5 ± 17.8	136.4 ± 14.4	129.3 ± 22.1	130.2 ± 20.4
Diastolic blood pressure (mmHg)	77.2 ± 12.5	76.0 ± 10.5	78.8 ± 12.4	81.1 ± 7.1	76.6 ± 12.5	74.7 ± 10.9
Creatinine (mg/dl)	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Cholesterol (mg/dl)	160.7 ± 25.2	158.4 ± 23.1	160.1 ± 26.9	161.4 ± 31.3	161.0 ± 24.5	157.7 ± 20.8
HDL (mg/dl)	37.8 ± 7.5	38.4 ± 7.0	37.7 ± 7.7	35.2 ± 7.7	37.9 ± 7.4	39.2 ± 6.7
LDL (mg/dl)	93.2 ± 20.8	89.7 ± 16.8	91.5 ± 23.0	97.2 ± 26.0	94.0 ± 19.9	87.9 ± 13.2
Triglycerides (mg/dl)	150.0 ± 44.9	152.9 ± 45.3	152.5 ± 58.3	145.9 ± 26.5	149.0 ± 38.0	154.5 ± 48.7
ALT (U/l)	43.8 ± 13.1	46.7 ± 26.5	46.3 ± 14.8	53.3 ± 53.8	42.7 ± 12.2	45.2 ± 13.7
FPG (mmol/l)	4.4 ± 0.8 (79.2 ± 14.4)	4.5 ± 0.8 (81.0 ± 14.4)	4.4 ± 0.7 (79.2 ± 12.6)	4.4 ± 0.9 (79.2 ± 16.2)	4.3 ± 0.8 (77.4 ± 14.4)	4.5 ± 0.8 (81.0 ± 14.4)
IAP (U/g stool)	73.2 ± 91.5	56.9 ± 71.7	66.1 ± 92.1	32.9 ± 25.3	76.2 ± 91.2	62.4 ± 77.6

The participants of this prospective cohort study were selected from the non-diabetic healthy control group described in a previous study.[7] Because of migration, refusal, pregnancy or death some participants were excluded from the study (see Fig. 1). A comparison of the first visit physical and biochemical characteristics of included or excluded participants is provided. The data on 4 dead participants have been excluded from the analysis. Data values are summarized as mean (average) ± SD for each variable. The statistical significance of difference between two respective groups (Included and Excluded) was examined using the unpaired two-tailed Student's *t*-test. $p<0.05$ is considered significant. **Note:** The physical and biochemical parameters were similar between the respective included and excluded groups.

Table S2. The physical and biochemical characteristics of participants included in the 5-year prospective cohort study.

Characteristic	Total participants		Males		Females	
	First visit	Second visit	First visit	Second visit	First visit	Second visit
No. of participants	574	574	168	168	406	406
Age group (years)	30 - 60	-	30 - 60	-	30 - 60	-
Average age (years)	41.7 ± 9.1	-	42.8 ± 9.0	-	41.2 ± 9.1	-
Weight (kg)	58.4 ± 11.2	59.8 ± 9.9*	63.0 ± 10.0	63.3 ± 9.1	56.6 ± 11.2	58.3 ±9.9*
Height (m)	1.5 ± 0.2	-	1.6 ± 0.1	-	1.5 ± 0.2	-
BMI (kg/m^2)	25.0 ± 4.5	25.6 ± 4.4*	23.8 ± 3.6	24.1 ± 3.6	25.5 ± 4.8	26.3 ± 4.6*
Systolic BP (mmHg)	129.7 ± 20.9	130.4 ± 16.9	130.5 ± 17.8	131.1 ± 14.6	129.3 ± 22.1	130.0 ± 17.8
Diastolic BP (mmHg)	77.2 ± 12.5	79.8 ± 8.9	78.8 ± 12.4	80.5 ± 8.4	76.6 ± 12.5	79.5 ±9.1
Creatinine (mg/dl)	0.8 ±0.2	0.9 ± 0.3***	0.9 ± 0.2	1.0 ± 0.3***	0.8 ± 0.2	0.9 ± 0.2***
Cholesterol (mg/dl)	160.7 ± 25.2	180.1 ± 34.1***	160.1 ± 26.9	174.0 ± 34.3***	161.0 ± 24.5	182.6 ± 33.7***
HDL (mg/dl)	37.8 ± 7.5	42.6 ± 8.7***	37.7 ± 7.7	40.8 ± 7.9***	37.9 ± 7.4	43.3 ± 8.9***
LDL (mg/dl)	93.2 ± 20.8	104.9 ± 28.6***	91.5 ± 23.0	99.9 ± 29.2**	94.0 ± 19.9	107.0 ± 28.1***
Triglycerides (mg/dl)	150.0 ± 44.9	162.1 ± 66.5***	152.5 ± 58.3	165.1 ± 69.5*	149.0 ± 38.0	160.8 ± 65.2**
ALT (U/l)	43.8 ± 13.1	37.1 ± 18.1***	46.3 ± 14.8	43.1 ± 17.8*	42.7 ± 12.2	34.6 ± 17.6***
FPG (mmol/l) (mg/dl)	4.4 ± 0.8 (79.2 ± 14.4)	6.0 ± 1.9*** (108.0 ± 34.2)	4.4 ± 0.7 (79.2 ± 12.6)	5.8 ± 0.9*** (104.4 ± 16.2)	4.3 ± 0.8 (77.4 ± 14.4)	6.1 ± 2.2*** (109.8 ± 39.6)
IAP (U/g stool)	73.2 ± 91.5	70.4 ± 54.3	66.1 ± 92.1	66.2 ± 44.5	76.2 ± 91.2	72.1 ± 57.8

Data values are summarized as mean (average) ± SD for each variable. The statistical significance of difference between two respective groups was determined using the paired two-tailed Student's *t*-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Table S3. Baseline distribution of potential confounding factors of participants grouped by the status of intestinal alkaline phosphatase deficiency (IAPD) from 2015 to 2020.

Baseline Characteristic	Persistent no IAPD	Incident IAPD	Persistent IAPD	Remittent IAPD
IAP level (U/g stool) (for defining IAPD status)	Baseline: ≥65.0 Follow-up: ≥65.0	Baseline: ≥65.0 Follow-up: <65.0	Baseline: <65.0 Follow-up: <65.0	Baseline: <65.0 Follow-up: ≥65.0
No. of participants (n = 574)	125 Male:31; Female 94	63 Male:16; Female 47	266 Male:80; Female 186	120 Male:41; Female 79
Age group (years)	30 - 60	30 - 60	30 - 60	30 - 60
Average age (years)	40.2 ± 8.9	42.1 ± 10.1	43.2 ± 8.8**	39.8 ± 9.1
Weight (kg)	59.3 ± 11.9	56.0 ± 10.6	58.1 ± 10.6	59.8 ± 12.2
Height (m)	1.5 ± 0.1	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
BMI (kg/m^2)	25.4 ± 5.1	24.4 ± 4.2	24.9 ± 4.1	25.2 ± 4.9
Systolic BP (mmHg)	128.0 ± 20.9	136.3 ± 26.9	129.8 ± 19.7	127.7 ± 19.7
Diastolic BP (mmHg)	76.4 ± 13.6	81.3 ± 15.4*	76.9 ± 11.8	76.6 ± 11.8
Creatinine (mg/dl)	0.77 ± 0.20	0.82 ± 0.22	0.80 ± 0.18*	0.81 ± 0.22
Cholesterol (mg/dl)	162.2 ± 26.1	160.6 ± 32.9	162.1 ± 24.9	156.4 ± 19.5
HDL (mg/dl)	37.8 ± 7.1	37.3 ±8.3	38.5 ± 7.7	36.7 ± 7.0
LDL (mg/dl)	93.7 ± 23.1	93.4 ± 24.9	93.7 ± 20.4	91.7 ± 16.8
Triglycerides (mg/dl)	148.5 ± 50.1	157.5 ± 50.5	152.7 ± 45.6	142.0 ± 31.7
ALT (U/l)	43.6 ± 13.4	42.8 ± 9.2	44.2 ± 13.1	43.5 ± 14.7
FPG (mmol/l)	4.3 ± 0.8	4.5 ± 0.8	4.4 ± 0.8	4.3 ± 0.7

Data values are summarized as mean (average) ± SD for each variable. The statistical significance of difference between two respective groups was examined using the unpaired two-tailed Student's *t*-test (Reference group: Persistent no IAPD). *, $p<0.05$; **, $p<0.01$.

Table S4. Pearson's correlation coefficients between IAP level and different risk factors of T2DM.

Risk factors	ALL participants		Male		Female	
	Healthy	T2DM	Healthy	T2DM	Healthy	T2DM
Age (year)	-0.10692	-0.06351	-0.18413	0.03667	-0.07733	-0.07966
BMI (kg/m^2)	0.09033	0.13732	0.09281	0.12749	0.07438	0.11775
Creatinine (mg/dl)	-0.07520	-0.14441	-0.09369	-0.13514	-0.05376	-0.14011
Cholesterol (mg/dl)	0.08481	0.04135	-0.05258	-0.42217	0.12494	0.16316
HDL (mg/dl)	0.12047	-0.11407	0.14480	-0.48713	0.10671	-0.04188
LDL (mg/dl)	0.07580	0.00605	-0.01547	-0.28390	0.10035	0.07638
Triglycerides (mg/dl)	-0.02536	0.24158	-0.15956	-0.38766	0.04490	0.31354
ALT (U/l)	-0.02509	-0.01383	-0.01193	0.05983	-0.01697	-0.00283
FPG (mmol/l)	-0.06700	0.16712	-0.11275	-0.47673	-0.04718	0.20493
Systolic blood pressure(mmHg)	-0.12104	-0.00835	-0.08053	0.01622	-0.12916	-0.01814
Diastolic blood pressure(mmHg)	-0.03241	0.00119	-0.02409	0.17715	-0.03136	-0.03988

A Pearson correlation coefficient close to +1 or -1 indicates that the two variables are highly correlated (positively or negatively, respectively). A correlation coefficient between 0 and +0.50 or between 0 and -0.50 was considered of having no correlation between the two variables.

Table S5. Repeated measure analysis shows that T2DM status is highly associated with intestinal alkaline phosphatase (IAP).

Source	DF	Type III SS	Mean Square	F Value	Pr > F
T2DM status	1	77605.144	77605.144	11.13	0.0009
SEX	1	4061.209	4061.209	0.58	0.4458
AGE	1	4975.518	4975.518	0.71	0.3987
BMI at Visit 1	1	619.880	619.880	0.09	0.7657
BMI at Visit 2	1	709.754	709.754	0.10	0.7499
CREATININE at Visit 1	1	15697.090	15697.090	2.25	0.1342
CREATININE at Visit 2	1	3512.831	3512.831	0.50	0.4782
CHOLESTEROL at Visit 1	1	9491.264	9491.264	1.36	0.2439
CHOLESTEROL at Visit 2	1	16623.520	16623.520	2.38	0.1233
HDL at Visit 1	1	2523.506	2523.506	0.36	0.5478
HDL at Visit 2	1	29982.986	29982.986	4.30	0.0386
LDL at Visit 1	1	22057.293	22057.293	3.16	0.0759
LDL at Visit 2	1	17881.810	17881.810	2.56	0.1100
TG at Visit 1	1	4775.418	4775.418	0.68	0.4084
TG at Visit 2	1	25075.565	25075.565	3.60	0.0585
SGPT at Visit 1	1	642.319	642.319	0.09	0.7617
SGPT at Visit 2	1	10511.694	10511.694	1.51	0.2201
SBP at Visit 1	1	3341.958	3341.958	0.48	0.4891
SBP at Visit 2	1	2915.203	2915.203	0.42	0.5183
DBP at Visit 1	1	23947.197	23947.197	3.43	0.0645
DBP at Visit 2	1	56.761	56.761	0.01	0.9282

Legend: SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table S6. The rate of increase of fasting plasma glucose (FPG) is not dependent on age.

Age group (years)	**No. of participants**	**Percentage (%) of increase of FPG level**
30 - 39	243	35.6 ± 2.3^{ns}
40 - 49	177	40.2 ± 3.0^{ns}
50 - 60	151	36.7 ± 2.5^{ns}

Participants included were males and females having diabetes or no diabetes. The percentage of increase of FPG from first visit (baseline value) to second visit (follow-up value) during the 5-year period was calculated. Data values are summarized as mean (average) ± SEM. Statistical significance of the difference between two groups was tested using the paired two-tailed Student's *t*-test. ns, not significant.

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