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RESEARCH ARTICLE

An evaluation of the effects of mixed heavy metals on prediabetes and type 2 diabetes: epidemiological and toxicogenomic analysis

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Abstract

The link between mixed heavy metals (mercury, lead, and cadmium), prediabetes, and type 2 diabetes mellitus (T2DM), especially molecular mechanisms, is poorly understood. Thus, we aimed to identify the association between mixed heavy metals and T2DM and its components using a data set from the Korean National Health and Nutrition Examination Survey. We further analyzed the main molecular mechanisms implicated in T2DM development induced by mixed heavy metals using in-silico analysis. Our findings observed that serum mercury was associated with prediabetes, elevated glucose, and ln2-transformed glucose when using different statistical methods. "AGE-RAGE signaling pathway in diabetic complications", "non-alcoholic fatty liver disease", "metabolic Syndrome X", and three miRNAs (hsa-miR-98-5p, hsa-let-7a-5p, and hsa-miR-34a-5p) were listed as the most important molecular mechanisms related to T2DM development caused by mixed heavy metals. These miRNA sponge structures were created and examined, and they may be beneficial in the treatment of T2DM. The predicted cutoff values for three heavy metal levels linked to T2DM and its components were specifically identified. Our results imply that chronic exposure to heavy metals, particularly mercury, may contribute to the development of T2DM. To understand the changes in the pathophysiology of T2DM brought on by a combination of heavy metals, more research is required.

Keywords Type 2 diabetes · Prediabetes · Chemical mixture · Threshold · Molecular mechanisms

Introduction

Diabetes is a chronic metabolic condition characterized by increased blood glucose levels that cause catastrophic damage to various organs such as the cardiovascular system, kidneys, eyes, and nerves over time. Diabetes affects approximately 422 million people worldwide, the vast majority of whom live in low- and middle-income countries, and is responsible for 1.5 million deaths each year (WHO 2021). Therefore, it is important that risk factors and diabetes be controlled to improve quality of life and minimize the societal disease burden.

Genetics, ethnicity, age, acanthosis nigricans, overweight, obesity, prediabetes, dyslipidemia, physical inactivity, depression, and gestational diabetes have been identified as the risk factors contributing to the development of diabetes. Many of the causes of diabetes, such as being overweight, being obese, and being physical inactive, can be prevented and reversed. Nevertheless, both the incidence and prevalence of diabetes have been gradually increasing, especially type 2 diabetes

Highlights
- Mixed chemicals were related to the risk of prediabetes and elevated glucose.
- 18 genes were related to mixed chemicals and T2DM development.
- Key molecular mechanisms affected by T2DM-related combined chemicals were described.
- Chemicals' cutoff thresholds related to the risk of T2DM were provided.
- miRNAs implicated in T2DM development were reported.

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(T2DM), over the last few decades. This condition can be explained for a variety of reasons. First, as worldwide diets have altered over the previous decades, consumption of energy-dense foods high in fat and free sugars has surged (Duc et al. 2021b; Duc et al. 2021c; Nguyen and Kim 2021; Nguyen et al. 2021a; Nguyen 2022a). Second, the percentage of the aging population is increasing in parallel with the prevalence of non-communicable diseases (Organization 2021). Third, the changing nature of various types of jobs, the increasing availability of transportation, and increased urbanization have all contributed to a reduction in physical exercise (WHO 2021). Last but not least, as global urbanization and industrialization have accelerated, heavy metal exposure has surged (Duc et al. 2021a; Nguyen and Kim 2021; Nguyen et al. 2021a; Nguyen et al. 2021d).

Heavy metals play a vital role in the pathogenesis of obesity, metabolic syndrome, and diabetes (Tinkov et al. 2017a, 2017b; Duc et al. 2021b; Nguyen et al. 2021a; Nguyen et al. 2021c; Nguyen et al. 2021d). Heavy metals (such as cadmium, lead, and mercury) are chronic environmental pollutants; thus, humans are directly exposed to them through eating, drinking, and breathing (Duc et al. 2021a; Nguyen et al. 2021a; Nguyen et al. 2021c; Nguyen et al. 2021d). Therefore, it is unavoidable in reality that prevalent environmental pollutants can be exposed at the same time. However, most previous research concentrated on the effects of a single heavy metal on the etiology of diabetes (Asif 2017; Tinkov et al. 2017a, 2017b; Ji et al. 2021a, 2021b). Single metals should correspond to mixed heavy metals, and diverse statistical approaches are necessary when studying chemical exposure in recent years (Keil Alexander et al. n.d.; Bobb 2015).

The link between heavy metals and diabetes is still controversial. Several studies reported a positive association between heavy metals and diabetes, but others found a negative association or no association between heavy metals and diabetes (Moon 2013; Rotter et al. 2015; Wu et al. 2017; Wang et al. 2018; Ji et al. 2021a, 2021b). Though numerous researchers have attempted to evaluate the link between heavy metals and diabetes, little is known about the molecular mechanisms related to diabetes induced by heavy metals. Therefore, the environmental predisposing factors and molecular mechanisms for diabetes should be documented to help in the prevention and early control of the disease. The present study aimed to (1) identify the relationship between a mixture of serum heavy metals, including cadmium, lead, and mercury, and T2DM and its components in Korean individuals aged ≥18 years and (2) determine the possible molecular mechanisms of mixed heavy metal-induced T2DM.

Material and Methods

Study participants

A data set from the Korean National Health and Nutrition Examination Survey (KNHANES), from 2009-2013 to 2016-2017, was used to examine the association between a mixture of heavy metals and T2DM (Duc et al. 2021b; Welfare 2021). The KNHANES surveys, which are a national surveillance system, used a multi-stage, stratified cluster-sampling method that considered the geographic region, the level of urbanization, the stage of economic growth, and the distribution of age and gender conducted by the Korean Ministry of Health and Welfare and the Korea Centers for Disease Control and Prevention (KCDC). These surveys, which are nationally representative cross-sectional studies, recruit approximately 10,000 participants each year. These surveys include three sections: a health examination, a health interview, and a nutrition survey, which were used to obtain information on health-related behaviors, biochemical and clinical characteristics for dietary intakes and common diseases, socioeconomic status, quality of life, healthcare utilization, and anthropometric measures. These surveys were conducted at a mobile evaluation center or participants' homes, by trained workers, including health interviewers, doctors, and medical technicians (Kweon et al. 2014). In total, 60,362 individuals participated in these surveys from 2009 to 2017. We removed 13,281 participants less than 18 years old and 41,477 without data, including serum heavy metals (28,783), diabetes (3,474), glycated hemoglobin (HbA1c, 3652), energy intake (893), body mass index (BMI, 17), family history of diabetes (135), and urine cotinine (4,820). Finally, a total of 5,304 participants were eligible for data analysis (Fig. S1) (Nguyen et al. 2021a).

Serum heavy metal measurement

Analyses of mercury, lead, and cadmium have been previously reported (Nguyen, 2021; Nguyen et al, 2021b, e). Briefly, after an eight-hour fast, blood samples were evaluated in accordance with protocol during a medical checkup. Blood samples were rapidly prepared, refrigerated, and sent to the main testing center in cold storage (NeoDin Medical Institute, Seoul, South Korea). All samples underwent 24-hour analyses. Graphite furnace atomic absorption spectrometry (model AAnalyst 600; Perkin Elmer, Turku, Finland) was used to quantify serum lead and cadmium levels. A direct mercury analyzer (type DMA-80 Analyzer; Bergamo, Italy) and gold amalgam

(Korea Centers for Disease Control and Prevention) were used to measure the serum total mercury levels. Commercial standards (Lyphochek Whole Blood Metals, Bio-Rad, CA, USA) were used as reference materials for internal quality assurance and control. The limits of detection for these heavy metals were 0.223 µg/dL for lead, 0.05 µg/L for mercury, and 0.087 µg/L for cadmium.

Covariates

Sociodemographic information (such as cotinine levels, age group, etc.,) as well as detailed information on laboratory data were available elsewhere (Duc et al. 2021b; Duc et al. 2021c; Yun et al. 2021). Potential covariates in the present study were applied based on the previous study (Duc et al. 2021a). Continuous variables were energy consumption (kcal), BMI (kg/m^2), age (years), and ln2-transformed cotinine levels. Other covariates included: family history of diabetes (yes, no), smoking (non/ex-smoker, current smoker), educational level (≤ middle school, high school, ≥ college), physical activity (yes, no), monthly household incomes (< 2,000, ≥ 2,000 and < 4,000, ≥ 4,000 and < 6,000, ≥ 6,000), sex (males, females).

Outcomes

The HbA1c levels (%) were determined using high performance liquid chromatography-723G7 (Tosoh, Tokyo, Japan). A Hitachi automatic analyzer 7600 was used to measure fasting glucose levels (mg/dL) (Hitachi, Tokyo, Japan). The intra- and inter-assay coefficients of variation for HbA1c were 3.12% and 2.80%, respectively, and 2.93% and 2.41 percent for fasting glucose levels (Nguyen et al. 2021d). T2DM, elevated glucose, and elevated HbA1c were identified according to the American Diabetes Association criteria. Serum HbA1c of ≥6.0% was considered elevated. Elevated fasting glucose was defined as a fasting glucose level of ≥100 mg/dL or the use of a drug to treat elevated fasting glucose. Prediabetes was defined as having a fasting glucose ranging from 100-125 mg/dL, or an HbA1c ranging from 5.7 to 6.4% without a previous diabetes diagnosis. T2DM was defined as having a HbA1c of ≥ 6.5%, fasting plasma glucose of ≥126 mg/dl, or being on anti-diabetic medication (Association 2021).

Statistical analysis

The statistical analysis was performed by using STATA (version 16.0; StataCorp, Texas, USA) and R (version 4.1.0) (Nguyen et al. 2021c; Nguyen 2022a). Heavy metal levels were ln2 transformed in this investigation because the range of heavy metals was right-skewed (Nguyen et al. 2021c; Nguyen 2022b).

Logistic and linear regression approaches

First, we used multivariate logistic regression to compare the higher quartiles to the lowest quartile of studied heavy metals to examine the link between each heavy metal and T2DM and its components (Duc et al. 2021b). Second, we looked at multivariate linear regression using the ln2-transformed levels of each heavy metal and the ln2-transformed glucose, and ln2-HbA1c as continuous outcome variables. Third, we further analyzed how these heavy metals interacted with T2DM and its components. Fourth, we analyzed threshold regression (Duc Nguyen et al. 2022b).

Secondary analysis approaches

The three most popular novel methodologies used to assess the effects of a chemical combination are well known: Bayesian kernel machine regression (BKMR), quantile g-computation (qgcomp), and weighted quantile sum (WQS) regression (Bobb et al. 2015; Renzetti et al. 2016; Keil et al. 2020). However, each approach has a unique set of drawbacks (Duc Nguyen et al. 2022b; Nguyen 2022c; Nguyen 2022d). Thus, we evaluated the impact of mixed heavy metals on diabetes and its components using these approaches to ensure the results are reliable (Nguyen 2022c; Nguyen 2022e).

Weighted quantile sum (WQS) regression model

This approach has previously been reported (Duc et al. 2021a; Nguyen et al. 2021c). In summary, as part of the strategy, the study population was randomly split into a training dataset (40 percent, n = 2,212) and a validation dataset (60 percent, n = 3,182). Bootstrapping was employed to determine empirical weights for each heavy metal in the mixture using the training dataset. In this study, heavy metals with estimated weights greater than 0.333 (1/3) were found to have a significant impact on the WQS score (Duc et al. 2021b). Because the WQS technique predicts that all mixture components will act in the same directionality on T2DM and its components, we developed and examined both a positive and a negative WQS score. gWQS, a R package, was used to do the analysis (Nguyen 2022d).

Quantile G-Computation (qgcomp)

The purpose and process of this technique have been described elsewhere (Duc et al. 2021a; Nguyen et al. 2021c). In brief, the gqcomp.noboot function, which separates all heavy metals into quintiles, gives a positive or

negative weight to each heavy metal, and fits a linear model for continuous outcomes using Bayesian variable penalization, was used to assess exposure effects. In the current study, heavy metals with an estimated weight greater than 0.05 were determined to have a significant impact on the gqcomp score. gqcomp.boot was also used to assess the linearity of the overall exposure effect. To represent the joint intervention levels of heavy metal exposure to T2DM and its components, the plot was created using g-computation and bootstrap variance with B up to 200. The analysis was carried out using the qgcomp package.

Bayesian kernel machine regression (BKMR) model

This approach's aim and process have been discussed elsewhere (Duc et al. 2021b; Nguyen et al. 2021c). In this study, a Gaussian kernel function was applied with a component-wise variable technique to set a Gaussian kernel function. After setting the final model with the Markov Chain Monte Carlo sampler for 10,000 iterations, the posterior inclusion probabilities (PIPs) for each heavy metal were measured, and estimations of the exposure-outcome function were established (Duc et al. 2021a). The analysis was carried out using the R package bkmr (Nguyen 2022e).

In silico analysis for mixed heavy metals and T2DM

The purpose of this method and its methodology have been described elsewhere (Nguyen 2022f, 2022g, 2022h). The link between T2DM and mixed heavy metals was determined by using data from the Comparative Toxicogenomics Database (CTD, (http://CTD.mdibl.org) (Duc Nguyen et al. 2022a; Nguyen 2022h, 2022i). The data downloaded on July 21, 2022, was used for the analysis reported in this study. Then, it was determined which genes contributed to the development of T2DM and heavy metal toxicity. We developed a network of overlapping genes activated by the examined heavy metals as well as other relevant genes associated with T2DM using GeneMANIA (http://geneMANIA.org/plug-in/) (Nguyen 2022c). The ToppGeneSuite portal (https://toppgene.cchmc.org) and its ToppFun function (https://toppgene.cchmc.org/enrichment.jsp) were used to connect T2DM-related molecular mechanisms (e.g., diseases, biological processes, and signaling pathways) to heavy metal mixture-induced genes. miRNA-target interaction networks and miRNA sponge structure were constructed and analyzed using MIENTURNET (http://userver.bio.uniroma1.it/apps/mienturnet/), and miRNAsong (http://www.med.muni.cz/histology/miRNAsong), respectively (Licursi et al. 2019; Duc Nguyen et al. 2022a; Nguyen 2022i).

Results

Study participant characteristics

This study comprised 912 adult individuals with T2DM, 2,134 individuals with prediabetes, 1950 individuals with elevated glucose, and 1,222 individuals with elevated HbA1c. Table 1 shows demographic information stratified by the presence or absence of T2DM, elevated glucose, and elevated HbA1c. Participants with T2DM, elevated glucose, and elevated HbA1c were more likely to be older, married, live in rural areas, be unemployed, be less educated, come from low-income families, usually drink, and have a family history of diabetes and dyslipidemia. Also, compared to their counterparts, they had significantly higher body mass index, waist circumference, triglycerides, high-sensitivity C-reaction protein, systolic and diastolic blood pressure, aspartate aminotransferase, and alanine aminotransferase.

Characteristics of heavy metal exposure

Table 2 shows the mean and geometric mean levels stratified by the presence or absence of T2DM, prediabetes, elevated glucose, and elevated HbA1c of three heavy metals. Serum levels of the studied heavy metals were more likely to be higher in subjects with T2DM, prediabetes, elevated glucose, and elevated HbA1c compared with those that did not.

The Pearson correlation coefficients (r) between serum heavy metals and cardiometabolic risk variables are shown in Fig. 1 (P value<0.001, r ranged from -0.36 to 0.85). ln2-transformed fasting glucose and ln2-transformed HbA1c (r= 0.80), body mass index and waist circumference (r= 0.85), and diastolic blood pressure and systolic blood pressure (r= 0.63) all had a strong correlation. The rest of the relationships were weak to moderate. For instance, the link between ln2-transformed serum lead and cadmium (r=0.32) and ln2-transformed serum mercury and cadmium (r =0.14).

Findings from multivariate logistic and linear regression models

Serum cadmium showed a significant trend (P for trend <0.001) with prediabetes, ln2-transformed glucose, and ln2-transformed HbA1c) in the upper two quartiles.

Table 1. Demographic distribution of individuals ≥18 years of age by type 2 diabetes and its components, (n = 5,304), KNHANES, Korean, 2009–2017.

Variables	Diabetes			Prediabetes			Elevated glucose			Elevated HbA1c		
	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value
	n=4,392	n=912		n=3,170	n=2,134		*n=3,354*	*n=1,950*		*n=4,082*	*n=1,222*	
Demographics												
Age (year) *	47.13 (16.06)	60.70 (11.97)	<0.001	45.29 (16.32)	55.65 (14.04)	<0.001	45.0 (16.2)	57.1 (13.3)	<0.001	46.2 (16.0)	60.3 (11.8)	<0.001
Age group *(%)*												
18-29	723 (16.5)	16 (1.8)	<0.001	648 (20.4)	91 (4.3)	<0.001	682 (20.3)	57 (2.9)	<0.001	723 (17.7)	16 (1.3)	<0.001
30-39	847 (19.3)	31 (3.4)		653 (20.6)	225 (10.6)		718 (21.4)	160 (8.2)		831 (20.4)	47 (3.9)	
40-49	875 (19.9)	108 (11.8)		615 (19.4)	368 (17.2)		652 (19.4)	331 (17.0)		823 (20.2)	160 (13.1)	
50-59	858 (19.5)	238 (26.1)		564 (17.8)	532 (24.9)		597 (17.8)	499 (25.6)		782 (19.1)	314 (25.7)	
60-69	691 (15.7)	296 (32.5)		421 (13.3)	566 (26.5)		441 (13.2)	546 (28.0)		576 (14.1)	411 (33.6)	
≥70	398 (9.1)	223 (24.4)		269 (8.5)	352 (16.5)		264 (7.9)	357 (18.3)		347 (8.5)	274 (22.4)	
Sex (%)												
Males	1,957 (44.6)	503 (55.2)	<0.001	1,447 (45.7)	1,013 (47.5)	0.192	1399 (41.7)	1061 (54.4)	<0.001	1839 (45.1)	621 (50.8)	<0.001
females	2,435 (55.4)	409 (44.9)		1,723 (54.3)	1,121 (52.5)		1955 (58.3)	889 (45.6)		2243 (54.9)	601 (49.2)	
Marital status *(%)*												
Married	3,499 (79.7)	875 (95.9)	<0.001	2,405 (75.9)	1,969 (92.3)	<0.001	2544 (75.9)	1830 (93.8)	<0.001	3,194 (78.3)	1,180 (96.6)	<0.001
Living alone	893 (20.3)	37 (4.1)		765 (24.1)	165 (7.7)		810 (24.1)	120 (6.2)		888 (21.7)	42 (3.4)	
Residential areas *(%)*												
Urban	3,626 (82.6)	693 (76.0)	<0.001	2,649 (83.6)	1,670 (78.3)	<0.001	2820 (84.1)	1499 (76.9)	<0.001	3381 (82.8)	938 (76.8)	<0.001
Rural	766 (17.4)	219 (24.0)		521 (16.4)	464 (21.7)		534 (15.9)	451 (23.1)		701 (17.2)	284 (23.2)	
Occupation *(%)*												
Blue-collar	1,693 (38.6)	193 (21.2)	<0.001	1,231 (38.8)	655 (30.7)	<0.001	1337 (39.9)	549 (28.1)	<0.001	1601 (39.2)	285 (23.3)	<0.001
White-collar	1,063 (24.2)	291 (31.9)		694 (21.9)	660 (30.9)		713 (21.3)	641 (32.9)		958 (23.5)	396 (32.4)	
Unemployed	1,636 (37.2)	428 (46.9)		1,245 (39.3)	819 (38.4)		1304 (38.9)	760 (39.0)		1523 (37.3)	541 (44.3)	
Education level *(%)*												
≤ Middle school	1,192 (27.1)	498 (54.6)	<0.001	803 (25.3)	887 (41.6)	<0.001	805 (24.0)	885 (45.4)	<0.001	1041 (25.5)	649 (53.1)	<0.001
High school	1,527 (34.8)	264 (29.0)		1,116 (35.2)	675 (31.6)		1192 (35.5)	599 (30.7)		1440 (35.3)	351 (28.7)	
≥ College	1,673 (38.1)	150 (16.4)		1,251 (39.5)	572 (26.8)		1357 (40.5)	466 (23.9)		1601 (39.2)	222 (18.2)	
Monthly household income *(%)*‡												
< 2,000	1,065 (24.3)	423 (46.4)	<0.001	757 (23.9)	731 (34.3)	<0.001	769 (22.9)	719 (36.9)	<0.001	974 (23.9)	514 (42.0)	<0.001
≥ 2,000 and < 4,000	1,295 (29.5)	249 (27.3)		948 (29.9)	596 (27.9)		997 (29.7)	547 (28.1)		1206 (29.5)	338 (27.7)	
≥ 4,000 and < 6,000	993 (22.6)	122 (13.4)		722 (22.8)	393 (18.4)		786 (23.5)	329 (16.8)		931 (22.8)	184 (15.1)	
≥ 6,000	1,039 (23.6)	118 (12.9)		743 (23.4)	414 (19.4)		802 (23.9)	355 (18.2)		971 (23.8)	186 (15.2)	

Table 1. (continued)

Variables	Diabetes		p-value	Prediabetes		p-value	Elevated glucose		p-value	Elevated HbA1c		p-value
	No	Yes		No	Yes		No	Yes		No	Yes	
	n=4,392	n=912		n=3,170	n=2,134		*n=3,354*	*n=1,950*		*n=4,082*	*n=1,222*	
Smoking status *(%)*												
Non/ex-smoker	3,513 (80.0)	664 (72.8)	0.003	2,498 (78.8)	1,679 (78.7)	0.915	2705 (80.7)	1472 (75.5)	<0.001	3254 (79.7)	923 (75.5)	0.002
Current smoker	879 (20.0)	248 (27.2)		672 (21.1)	455 (21.3)		649 (19.3)	478 (24.5)		828 (20.3)	299 (24.5)	
Drinking status *(%)*												
Often	1,043 (23.8)	319 (35.0)	<0.001	732 (23.1)	630 (29.5)	<0.001	792 (23.6)	570 (29.2)	<0.001	926 (22.7)	436 (35.6)	<0.001
Occasionally	2,351 (53.5)	365 (40.0)		1,721 (54.3)	995 (46.6)		1882 (56.1)	834 (42.8)		2202 (53.9)	514 (42.1)	
Never or rarely	998 (22.7)	228 (25.0)		717 (22.6)	509 (23.9)		680 (20.3)	546 (28.0)		954 (23.4)	272 (22.3)	
Physical activity *(%)*												
Not regular	3,311 (75.4)	694 (76.1)	0.650	2,389 (75.4)	1,616 (75.7)	0.763	2512 (74.9)	1493 (76.6)	0.173	3064 (75.1)	941 (77.0)	0.166
Regular	1,081 (24.6)	218 (23.9)		781 (24.6)	518 (24.3)		842 (25.1)	457 (23.4)		1018 (24.9)	281 (23.0)	
Family history of CVDs *(%)*												
No	2,673 (61.0)	641 (70.4)	<0.001	2,009 (63.5)	1,305 (61.3)	0.110	2069 (61.7)	1257 (64.5)	0.044	2503 (61.3)	823 (67.4)	<0.001
Yes	1,709 (39.0)	269 (29.6)		1,155 (36.5)	823 (38.7)		1285 (38.3)	693 (35.5)		1579 (38.7)	399 (32.7)	
Family history of diabetes *(%)*												
No	3,556 (81.0)	623 (68.3)	<0.001	2,528 (79.8)	1,651 (77.4)	0.038	2766 (82.5)	1413 (72.5)	<0.001	3331 (81.6)	848 (69.4)	<0.001
Yes	836 (19.0)	289 (31.7)		642 (20.2)	483 (22.6)		588 (17.5)	537 (27.5)		751 (18.4)	374 (30.6)	
Family history of hyperlipidemia *(%)*												
No	4,104 (93.4)	872 (95.6)	0.013	2,950 (93.1)	2,026 (94.9)	0.005	3121 (93.1)	1855 (95.1)	0.002	3807 (93.3)	1169 (95.7)	*0.*002
Yes	288 (6.6)	40 (4.4)		220 (6.9)	108 (5.1)		233 (6.9)	95 (4.9)		275 (6.7)	53 (4.3)	
Laboratory measurement												
BMI *(Kg/m^2)*	23.73 (3.44)	25.39 (3.68)	<0.001	23.54 (3.54)	24.71 (3.41)	<0.001	23.32 (3.36)	25.20 (3.51)	<0.001	23.56 (3.38)	25.52 (3.64)	<0.001
WC *(cm)*	81.34 (9.92)	88.36 (9.32)	<0.001	80.94 (10.39)	84.93 (9.33)	<0.001	79.97 (9.81)	86.98 (9.19)	<0.001	80.88 (9.79)	88.11 (9.39)	<0.001
Total cholesterol *(mg/dL)*	192.96 (35.98)	184.92 (43.85)	<0.001	188.30 (36.00)	196.45 (39.29)	<0.001	190.91 (34.93)	192.74 (41.71)	0.087	192.03 (35.63)	190.06 (43.40)	0.107
LDL-C *(mg/dL)*	115.94 (32.75)	111.26 (37.82)	0.004	111.33 (32.74)	119.85 (35.23)	<0.001	115.09 (31.95)	114.50 (36.86)	0.677	114.73 (32.45)	115.14 (37.69)	0.802
Triglyceride *(mg/dL)* †	104 (44-293)	143.50 (59-413)	<0.001	101 (42-310)	124 (51-341)	<0.001	98 (42-271)	136 (56-398)	0.001	102 (44-291)	139 (56-399)	<0.001

Table 1. (continued)

Variables	Diabetes		p-value	Prediabetes		p-value	Elevated glucose		p-value	Elevated HbA1c		p-value
	No	Yes		No	Yes		No	Yes		No	Yes	
	n=4,392	n=912		n=3,170	n=2,134		*n=3,354*	*n=1,950*		*n=4,082*	*n=1,222*	
HDL-C *(mg/dL)*	51.62 (13.03)	45.04 (11.32)	<0.001	51.52 (13.10)	48.95 (12.68)	<0.001	52.36 (13.0)	47.27 (12.33)	<0.001	51.85 (13.04)	45.93 (11.72)	<0.001
hs-CRP *(mg/L)* †	0.57 (0.20-4.11)	0.80 (0.30-6.39)	<0.001	0.51 (0.20-4.10)	0.70 (0.26-5.16)	<0.001	1.11 (1.93)	1.49 (2.39)	<0.001	1.15 (2.03)	1.64 (2.41)	<0.001
Energy intake *(Kcal)*	2037.23 (899.25)	1897.23 (865.02)	<0.001	2030.61 (901.68)	1987.23 (884.40)	0.083	2017.92 (896.15)	2004.97 (893.03)	0.611	2041.09 (902.00)	1919.86 (864.76)	<0.001
Systolic BP *(mmHg)*	117.10 (16.18)	127.31 (16.25)	<0.001|	116.40 (16.03)	122.49 (16.86)	<0.001|	115.28 (15.62)	125.00 (16.55)	<0.001	116.66 (16.14)	126.18 (16.18)	<0.001|
Diastolic BP *(mmHg)*	75.68 (10,04)	76.95 (10.57)	<0.001|	75.11 (10.13)	77.06 (10.05)	<0.001|	74.76 (9.78)	77.85 (10.45)	<0.001	75.56 (10.11)	77.02 (10.17)	<0.001|
AST *(IU/L)* †	20 (13-37)	22.5 (14-56)	<0.001	19 (13-39)	21 (15-42)	<0.001	19 (13-35)	22 (14-49)	<0.001	20 (13-37)	22 (15-52)	<0.001
ALT *(IU/L)* †	17 (8-49)	23 (11-68)	<0.001	17 (8-51)	19 (10-55)	<0.001	16 (8-43)	21 (11-64)	<0.001	17 (8-48)	23 (11-67)	<0.001
Urine cotinine *(mg/dL)* †	1.13 (0.16-1864)	2.86 (0.13-1695.7)	<0.001	1.35 (0.16-1838.2)	1.26 (0.15-1830.61)	0.3187	1.15 (0.15-1850.89)	1.60 (0.16-1770)	<0.001	1.15 (0.16-1237)	2.11 (0.13-1711)	<0.001

*: mean±SD; ‡: thousand won; †: median (IQR) and P-value using Wilcoxon rank-sum test; |: two-sample t test with equal variances (others are two-sample t test with unequal variances); BMI: body mass index; WC: waist circumference; CVDs: Cardiovascular disease; BP: blood pressure; AST: aspartate aminotransferase; ALT: alanine aminotransferase, hs-CRP: high-sensitivity C-reaction protein; HDL-C: High-density lipoprotein cholesterol; elevated HbA1c (≥6.0%); elevated glucose (≥ 100mg/dL).

Table 2. Distribution of heavy metal exposure in type 2 diabetes and its components (n = 5,304), KNHANES, Korean, 2009–2017.

Variables	Indicators	Serum cadmium (μg/L)	p-value*	Serum lead (μg/dL)	p-value*	Serum mercury (μg/L)	p-value*
Study population (n=5,304)	GM 95%CI	0.96 (0.94-0.97)	--	1.86 (1.84-1.88)	--	3.34 (3.28-3.40)	--
	Mean (SD)	1.13 (0.66)		2.06 (1.05)		4.12 (3.22)	
T2DM (n=912)	GM 95%CI	1.11 (1.08-1.15)		2.14 (2.08-2.20)		3.64 (3.48-3.81)	
	Mean (SD)	1.27 (0.68)	<0.001	2.36 (1.27)	<0.001	4.62 (3.66)	<0.001
Without T2DM (n=4,392)	GM 95%CI	0.93 (0.91-0.94)		1.80 (1.70-1.83)		3.28 (3.22-3.34)	
	Mean (SD)	1.10 (0.65)		1.99 (0.99)		4.02 (3.11)	
Prediabetes (n=2,134)	GM 95%CI	1.09 (1.06-1.11)		2.01 (1.97-2.04)		3.59 (3.50-3.69)	
	Mean (SD)	1.24 (0.66)	<0.001	2.20 (1.10)	<0.001	4.46 (3.62)	<0.001
Without T2DM (n=3,170)	GM 95%CI	0.88 (0.86-0.90)		1.76 (1.74-1.79)		3.18 (3.11-3.25)	
	Mean (SD)	1.05 (0.65)		1.96 (1.01)		3.89 (2.90)	
Elevated glucose (*n=1,950*)	GM 95%CI	1.06 (1.04-1.09)		2.06 (2.02-2.10)		3.77 (3.66-3.88)	
	Mean (SD)	1.22 (0.66)	<0.001	2.28 (1.21)	<0.001	4.72 (3.76)	<0.001
Non-elevated glucose (*n=3,354*)	GM 95%CI	0.90 (0.88-0.92)		1.75 (1.72-1.78)		3.11 (3.05-3.18)	
	Mean (SD)	1.08 (0.65)		1.93 (0.92)		3.77 (2.80)	
Elevated HbA1c (n=1,222)	GM 95%CI	1.13 (1.10-1.17)		2.09 (2.04-2.14)		3.72 (3.58-3.86)	
	Mean (SD)	1.29 (0.66)	<0.001	2.31 (1.21)	<0.001	4.65 (3.64)	<0.001
Non-elevated HbA1c (*n=4,082*)	GM 95%CI	0.91 (0.89-0.93)		1.79 (1.77-1.82)		3.23 (3.17-3.30)	
	Mean (SD)	1.08 (0.65)		1.98 (0.99)		3.96 (3.07)	

*P value using Wilcoxon rank-sum test; GM: geometric mean 95% confidence intervals; SD: standard deviation; elevated HbA1c (≥6.0%); elevated glucose (≥ 100mg/dL).

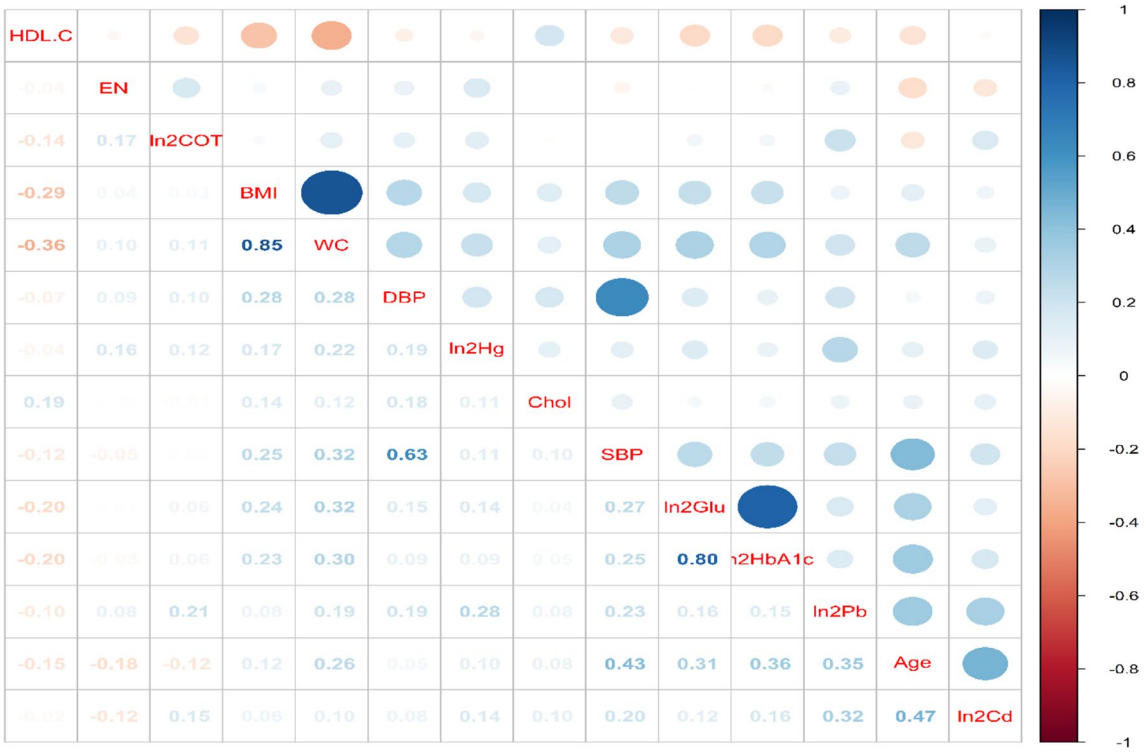


Fig. 1. Pairwise Pearson correlations among cardiometabolic risk factors and ln2-transformed levels of heavy metals (n = 5,304), KNHANES, Korean, 2009–2017. BMI: body mass index, WC: waist circumference, DBP: diastolic blood pressure, SBP: systolic blood pressure, EN: energy, HDL-C: High-density lipoprotein cholesterol, ln2Glu: ln2-transformed glucose, ln2COT: ln2-transformed cotinine; Chol: cholesterol.

There were significant links between serum mercury, prediabetes, elevated HbA1c, elevated glucose, and ln2-transformed glucose, with a significant trend (P for trend <0.001). Serum lead, on the other hand, was linked to prediabetes and ln2-transformed HbA1c in the fourth quartiles (P for trend <0.001).. Next, we analyzed the relationship between heavy metals and T2DM and its components when the studied heavy metals were considered continuous variables. Serum cadmium was linked to T2DM, elevated glucose, prediabetes, ln2-transformed glucose, and ln2-HbA1c. Prediabetes, elevated glucose, HbA1c, and ln2-glucose were found to be associated with serum mercury. We further investigated how heavy metals interact with T2DM and its components. After adjusting for potential variables, we found an interaction between serum lead and mercury levels and elevated glucose levels, as well as between mixed three heavy metals and prediabetes and elevated glucose levels (Tables S1A and 1B).

Findings from the WQS models

The WQS indices were found to be associated with prediabetes, elevated glucose, and ln2-transformed glucose (Table S2 and Fig. 2A-F). In fully adjusted models, the WQS indexes were found to be associated with prediabetes (OR: 1.92, 95%CI: 1.22–3.01), elevated glucose (OR: 1.86, 95%CI: 1.14–3.02), and ln2-transformed glucose (OR: 1.03, 95%CI: 1.02–1.05). In almost all models, serum mercury had the highest weight. The serum cadmium was then given a medium weight, while the serum lead was given the lightest (Table S3).

Findings from the gqcomp models

The gqcomp indices were found to be significantly linked with prediabetes. In the fully adjusted models, a quartile increase in the gpcomp index was significantly related to

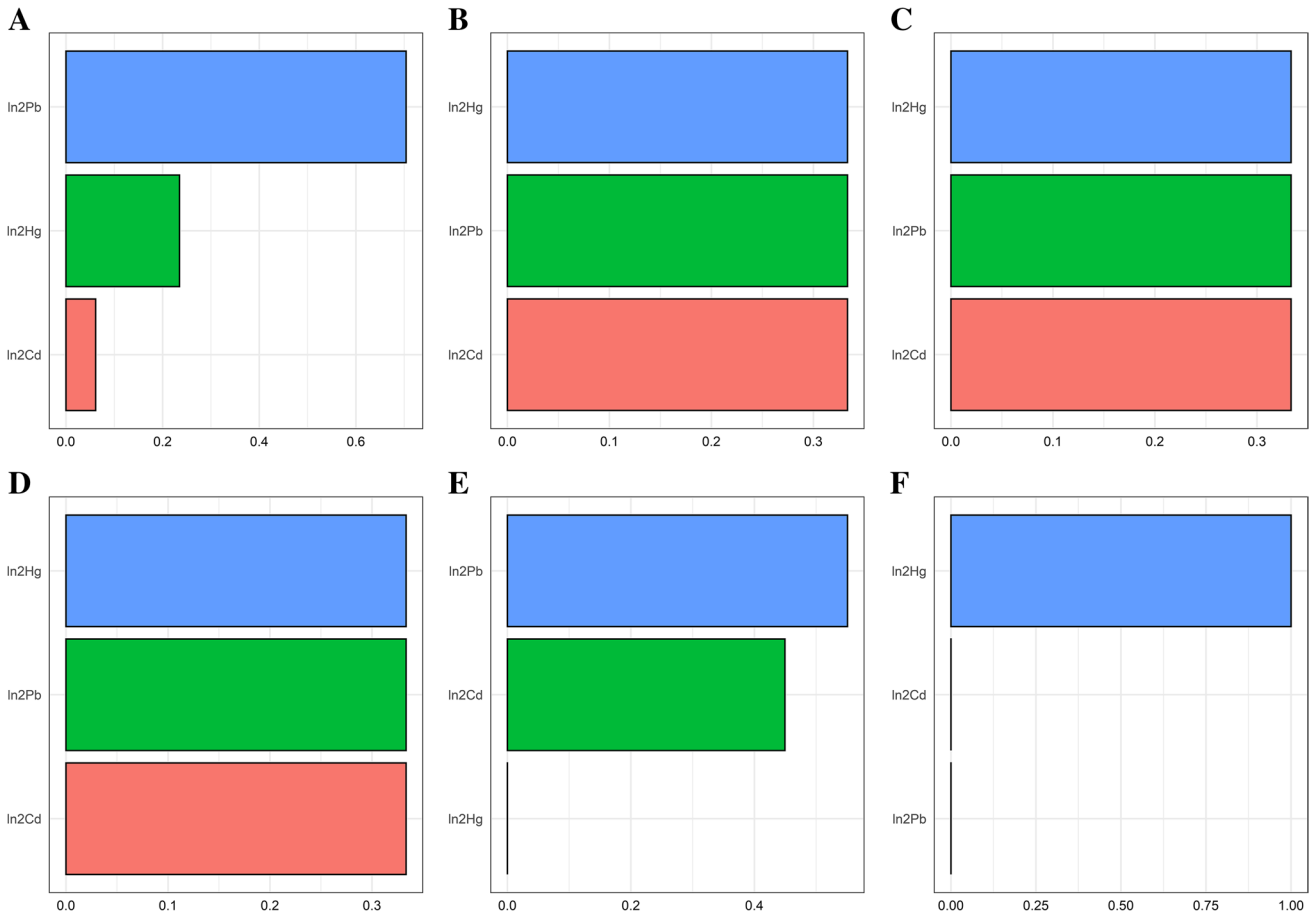


Fig. 2. WQS model regression index weights for (**A**) type 2 diabetes (positive weights), (**B**) prediabetes (positive weights), (**C**) elevated glucose (positive weights), (**D**) elevated HbA1c (positive weights), (**E**) ln2 glucose (positive weights), and (**F**) ln2 HbA1c (negative weights). Models were adjusted for sex (males, females), BMI (kg/m^2), age (years), energy intake (kcal), family history of diabetes (yes, no), physical activity (yes, no), smoking (non/ex-smoker, current smoker), ln2 cotinine (mg/dL), educational level (≤ middle school, high school, ≥ college), monthly household incomes (< 2,000, ≥ 2,000 and < 4,000, ≥ 4,000 and < 6,000, ≥ 6,000).

prediabetes (OR = 1.53, 95%CI: 1.25–1.88) (Table S4). The predicted weights of heavy metals for each gqcomp index, as well as the joint effect of mixed heavy metals on T2DM and its components, are shown in Table S5 and Fig. 3A-F. In almost all models, serum mercury had the largest positive weight, similar to the WQS model. After that, serum cadmium observed a moderately negative weight, while serum Pb observed the lowest weight.

Fig. 3. gqcomp model regression index weights and Joint effects (95% CI) of the mixture on (**A**) type 2 diabetes, (**B**) prediabetes, (**C**) elevated glucose, (**D**) elevated HbA1c, (**E**) ln2 glucose, and (**F**) ln2 HbA1c. Models were adjusted for sex (males, females), BMI (kg/m^2), age (years), energy intake (kcal), family history of diabetes (yes, no), physical activity (yes, no), smoking (non/ex-smoker, current smoker), ln2 cotinine (mg/dL), educational level (≤ middle school, high school, ≥ college), monthly household incomes (< 2,000, ≥ 2,000 and < 4,000, ≥ 4,000 and < 6,000, ≥ 6,000).

Findings from the BKMR models

In order to further analyze the effects of combining three heavy metals, we applied the BKMR method due to the linearity and interaction limitations of the earlier techniques. In all models in the current study, the PIPs of serum mercury were shown to be greater than those of other heavy metals in all models (Table S6).

Figures 4 A-F show the overall relationships between the mixed heavy metals, T2DM, and its components. When mixed heavy metals were at or above the 60th percentile compared to the 50th percentile, prediabetes, elevated glucose, and ln2-transformed glucose increased considerably, demonstrating significant positive associations with prediabetes, elevated glucose, and ln2-transformed glucose, respectively. Despite the lack of statistical significance between the T2DM, elevated HbA1c, and ln2-transformed HbA1c models, there was an increased and decreased inclination, respectively.

On the other hand, we investigated the univariate (independently heavy metal) exposure-response functions of T2DM and its components after being exposed to heavy metal (Fig. S2 A-E). Serum mercury, cadmium, and lead revealed growing associations with T2DM and its components at the highest levels when these heavy metals were at their median levels. Three heavy metals were observed to have a positive relationship with prediabetes, elevated glucose, and ln2-transformed glucose, respectively. Furthermore, the investigated heavy metals in this study were found to interact (Fig. S3 A-F). Table 3 presents the results of four distinct statistical models. In these models, we observed that mercury and cadmium had the strongest negative or positive associations.

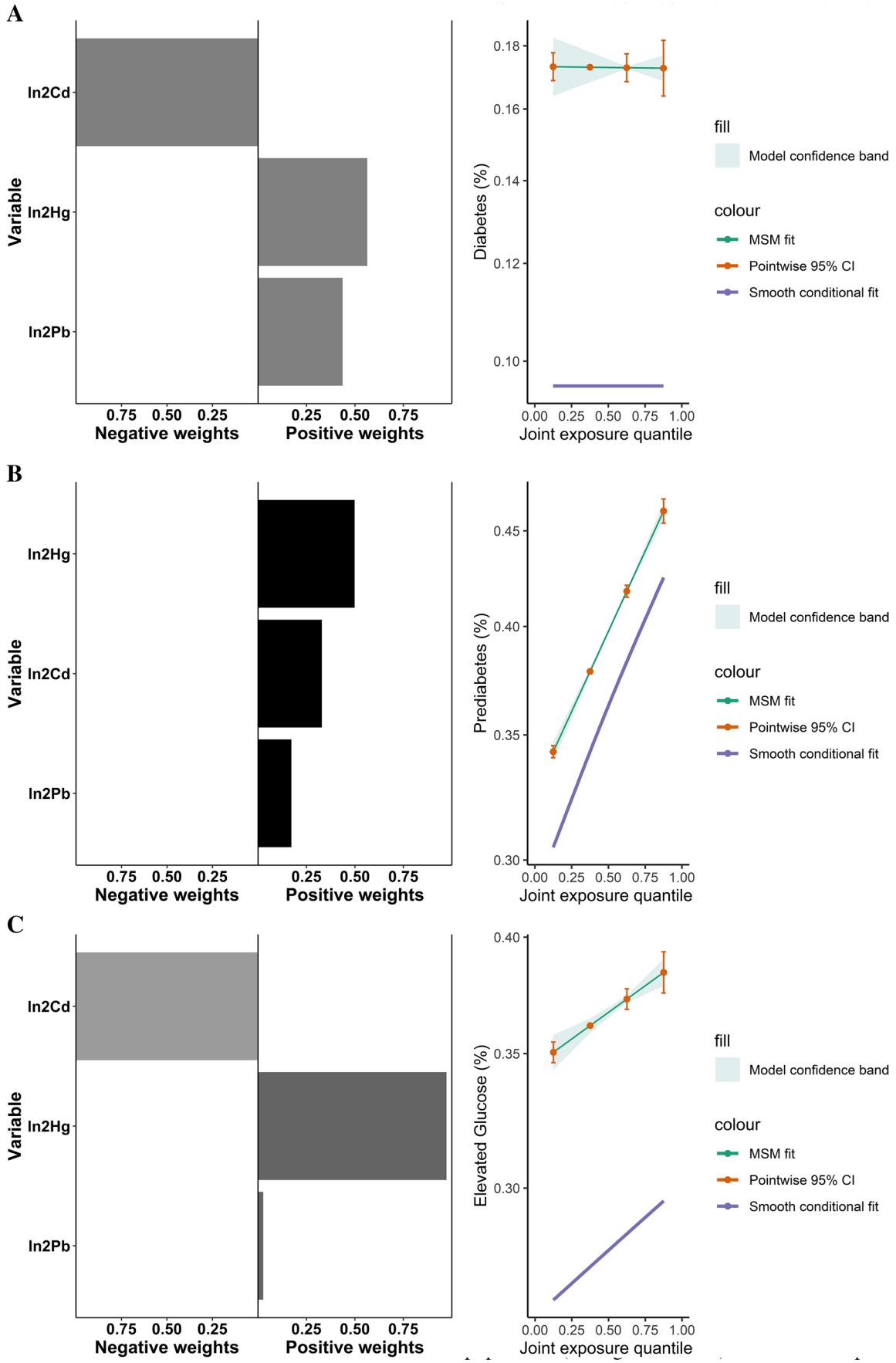
Molecular mechanisms related to mixed heavy metals and type 2 diabetes

As shown in Table 4A, cadmium, mercury, and lead altered 60, 35, and 39 genes that were involved in the pathogenesis of T2DM, respectively (Table 4A). Eighteen genes were impacted by mixed heavy metals, and this interaction was linked to the development of T2DM (Fig. 5A). The most prominent interactions between T2DM genes were identified as “physical interactions” (32.8%), “co-expressions” (28.9%), and “predicted by the server” (26.2%) (Fig. 5B). "Apoptosis", "AGE-RAGE signaling pathway in diabetic complications", "oxidative stress", and "IL-18 signaling pathway" were key signaling pathways implicated in combined heavy metals and T2DM. "Oxidative stress" and "apoptosis" were found to be the two main biological processes that were related to the etiology of T2DM induced by mixed heavy metals. The most prevalent condition associated with combined heavy metals was "diabetes" (Table 4B).

We next assessed the association between single and mixed heavy metals in the pathophysiology of T2DM. As shown in Fig. S4, cadmium altered two key miRNAs (hsa-miR-155-5p and hsa-miR-34a-5p), lead altered three key miRNAs (hsa-miR-34a-5p, hsa-miR-155a-5p, and hsa-miR-21-5p), whereas mercury altered five key miRNAs (hsa-miR-34a-5p, hsa-miR-98-5p, hsa-let-7a-5p, has-miR-9-5p, and hsa-miR-155-5p). "AGE-RAGE pathway”, "non-alcoholic fatty liver disease”, "gestational diabetes”, and “type 2 diabetes mellitus” were listed as the most important signaling pathways and related diseases induced by cadmium, lead, and mercury (Fig. S5 and Table S7). In terms of mixed heavy metals, the key miRNAs associated with T2DM and mixed heavy metals were identified as hsa-miR-98-5p, hsa-let-7a-5p, and hsa-miR-34a-5p (Fig. 5 C-D). "AGE-RAGE signaling pathway in diabetic complications" and "non-alcoholic fatty liver disease”, "metabolic syndrome X", "gestational diabetes”, and “type 2 diabetes mellitus” were listed as key signaling pathways associated with T2DM and mixed heavy metals (Fig. 5 E-F). The template of the miRNA sponges for these miRNAs was then generated and analyzed (Fig. 5 G and Table S8) (Barta et al. 2016).

Discussion

Four different statistical models were used in this study to explore the impact of heavy metal combinations on T2DM and its components in Korean people aged ≥18 years. We found that serum mercury was the most powerful predictor


A
In2Cd
In2Hg
In2Pb
Variable
0.75 0.50 0.25
Negative weights
0.25 0.50 0.75
Positive weights
Diabetes (%)
0.18
0.16
0.14
0.12
0.10
0.00 0.25 0.50 0.75 1.00
Joint exposure quantile
fill
Model confidence band
colour
MSM fit
Pointwise 95% CI
Smooth conditional fit
B
In2Hg
In2Cd
In2Pb
Variable
0.75 0.50 0.25
Negative weights
0.25 0.50 0.75
Positive weights
Prediabetes (%)
0.45
0.40
0.35
0.30
0.00 0.25 0.50 0.75 1.00
Joint exposure quantile
fill
Model confidence band
colour
MSM fit
Pointwise 95% CI
Smooth conditional fit
C
In2Cd
In2Hg
In2Pb
Variable
0.75 0.50 0.25
Negative weights
0.25 0.50 0.75
Positive weights
Elevated Glucose (%)
0.40
0.35
0.30
0.00 0.25 0.50 0.75 1.00
Joint exposure quantile
fill
Model confidence band
colour
MSM fit
Pointwise 95% CI
Smooth conditional fit

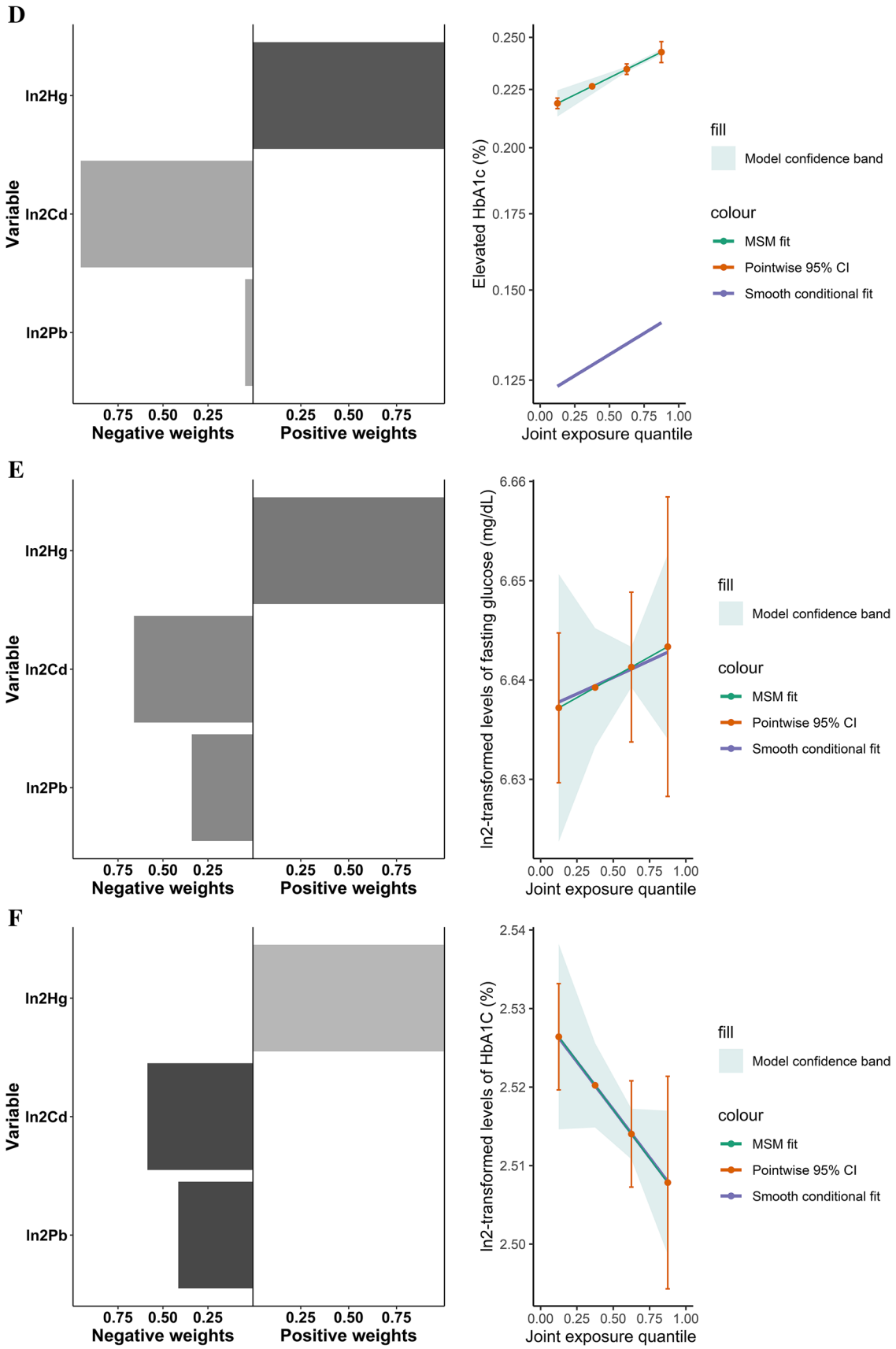


Fig. 3. (continued)

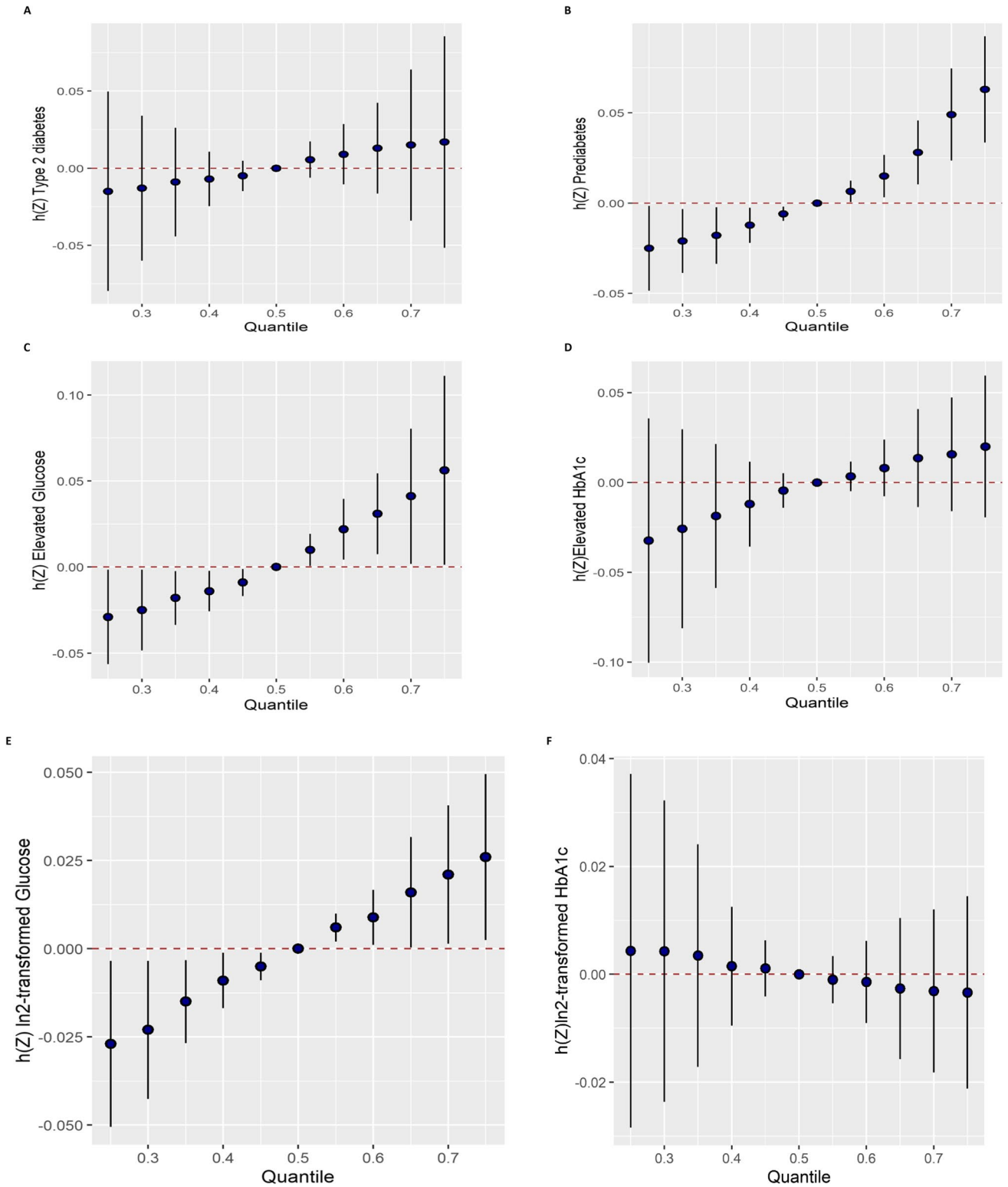


Fig. 4. Cumulative effect (95% CI) of the heavy metal mixture on (**A**) type 2 diabetes, (**B**) elevated glucose, (**C**) elevated HbA1c, (**D**) ln2 glucose, and (**E**) ln2 HbA1c, when all the heavy metals at particular percentiles were compared to all the chemicals at their 50th percentile. The results were assessed by the BKMR models, adjusted for sex (males, females), BMI (kg/m^2), age (years), energy intake (kcal), family history of diabetes (yes, no), physical activity (yes, no), smoking (non/ex-smoker, current smoker), ln2 cotinine (mg/dL), educational level (≤ middle school, high school, ≥ college), monthly household incomes (< 2,000, ≥ 2,000 and < 4,000, ≥ 4,000 and < 6,000, ≥ 6,000).

Table 3. A summary of results using the four approaches to evaluate the link between mixed heavy metals and type 2 diabetes and its components, (n = 5,304), KNHANES, Korean, 2009–2017.

Approaches	Indicators	T2DM	Prediabetes	Elevated glucose	Elevated HbA1c	ln2 glucose	ln2 HbA1c
Linear regression model	Strong negative associations (lightest β indicators)	Cadmium	--	Cadmium	Cadmium	Cadmium	Cadmium, and lead
	Strong positive associations (highest β indicators)	Mercury	Mercury, cadmium, and lead	Mercury	Mercury	Mercury	Mercury
QWS model	Highest negative weights		--	--	NS	--	NS
	Highest positive weights	NS	Mercury, cadmium, and lead	Mercury, cadmium, and lead	NS	Mercury	NS
Qgcomp model	Highest negative weights	NS	--	NS	NS	NS	NS
	Highest positive weights		Mercury	NS	NS	NS	NS
BKMR model	Negative trend (highest PIPs)	NS	--	Cadmium	NS	Cadmium	NS
	Positive trend (highest PIPs)		Mercury	Mercury	NS	Mercury	NS

NS: not significant; PIPs: posterior inclusion probabilities; QWS: weighted quantile sum; qgcomp: quantile g-computation; qgcomp; BKMR: Bayesian kernel machine regression. T2DM: type 2 diabetes.

low levels of heavy metal exposure may not be linked with HbA1c.

Chemicals, including heavy metals, from the environment are metabolized in the liver and then discharged into the intestines through bile (Klaassen 2013; Duc Nguyen et al. 2022b). Only around 5% of environmental chemicals are eliminated in the feces, while 90–95% could be reabsorbed through the enterohepatic circulation (Dawson 2018). The etiology of T2DM is implicated in a disruption in hepatic glucose homeostasis. Furthermore, elevated serum glucose levels are caused by impaired liver and kidney functions, as well as diminished pancreatic and muscle function. When heavy metals enter the human body, they accumulate in the liver, kidneys, and pancreas, where they disrupt glucose metabolism and its interactions with other metabolic pathways, particularly glycolysis, glycogenesis, and gluconeogenesis, by changing and affecting the specific activity of important enzymes and by damaging the pancreas and adrenal glands. Therefore, heavy metals play an important role in the pathogenesis of T2DM (Javaid et al. 2021).

There is a potential biological link between heavy metals and the development of T2DM. Although the majority of cadmium is deposited in the kidney, chronic exposure has been linked to cadmium accumulation in the pancreas, particularly in the beta islets. Cadmium poisoning can impair the antioxidant system and energy metabolism, as well as cause inflammation and mitochondrial damage in pancreatic beta cells (Buha et al. 2020). Furthermore, cadmium may activate gluconeogenesis through decreased insulin sensitivity by changing glucose transporter expression and increasing the activity of gluconeogenic enzymes, resulting in decreased glucose uptake (Edwards and Ackerman 2016). An elevation in blood glucose and a reduction in insulin levels could be caused by damage to the pancreas (Tinkov et al. 2017a, 2017b). Although the precise mechanism linking mercury to diabetes is uncertain, mercury is a well-known oxidative stress-causing toxin. A literature review found that mercury may cause T2DM by causing hyperglycemia and disrupting pancreatic function through oxidative stress on the mitochondria or stimulation of the c-JunN-terminal kinase signaling pathway (Schumacher and Abbott 2017). An in vivo study revealed that mercury-induced oxidative stress produces apoptosis in cells and in the isolated mouse pancreas, in addition to pancreatic beta-cell failure (Chen et al. 2006b). Another in vivo study observed that mercury could induce the activation of phosphoinositide 3-kinase and the production of reactive oxygen species, causing inhibition of insulin secretion and pancreatic beta-cell dysfunction via the Akt signaling pathway (Chen et al. 2006a). On the other hand, lead can cause oxidative stress, which is a risk factor for T2DM (Fridlyand and Philipson 2006). Lead elevates resting intracellular Ca^{2+}, which could have a direct impact on calcineurin function and, as a result, alter calcineurin-dependent cellular processes like insulin-producing pancreatic beta-cells (Soleimanpour et al. 2010). Lead exposure can also cause increased gluconeogenesis by lowering the

Table 4. Characteristics of molecular mechanisms involved in the etiology of type 2 diabetes and the investigated heavy metals.

A. Genes					
Chemical Name	Chemical ID	Disease Name	Disease ID	Inference Network	No. Genes
Cadmium (Cd)	D002104	Diabetes Mellitus, Type 2	MESH:D003924	ADAMTS9|AKT1|ATF3|ATP2A3|BAX|BCL2|BCL2L1|BCL2L11 |BRAF|C3|CASP3|CASP8|CAT|CBS|CD36|CISD2|CPT1A|EDN1 |EGFR|FAS|GCLC|GCLM|GPX1|GSTM1|HHEX|HK1|HMOX1 |HP|HPX|ICAM1|IL13RA1|IL6|INS|INS1|IRS1|IRS2|KL|MIR10B |MIR140|MIR142|MIR144|MIR195|MIR221|MIR222|MIR27A |MIR30A|MIR375|MIR377|MIR423|MIR532|MIR628|MIR92B |MIR98|NFKB1|NOS2|NOS3|OGG1|PAX6|PIK3CG|PPARA|PPARG |PPARGC1A|PSMD6|RELA|S100A6|SCTR|SHBG|SIRT1|SLC2A1 |SLC2A2|SLC30A8|SOD1|SOD2|TGFB1|TIMP1|TNF|TNFRSF1A |TNFRSF1B|UCP2	60
Mercury (Hg)	D008628	Diabetes Mellitus, Type 2	MESH:D003924	AKT2|BAX|BCL2|BCL2L11|BRAF|C3|CASP3|CAT|CYP1A2 |ENPP1|FAS|GCLC|GCLM|GPX1|GSTM1|HMOX1|HNF1A |HPX|IL6|IRS1|LEPR|MIR151A|MIR423|MIRLET7D|NFKB1 |NOS2|OGG1|RELA|SLC2A4|SOD1|TIMP1|TNF|TNFRSF1A |ZFAND3	35
Lead (Pb)	D007854	Diabetes Mellitus, Type 2	MESH:D003924	AKT1|AKT2|AUTS2|BAX|BCL2|BCL2L1|C3|CASP3|CAT|CMIP |CYP1A2|EGFR|FAS|FTO|GCLM|GLIS3|GPD2|GPX1|GSTM1 |HMOX1|HP|HPX|IGF2BP2|IL6|INS1|JADE2|JAZF1|LEP|MIR204 |MIR222|MIR4516|NFKB1|NOS2|NOS3|NOTCH2|OGG1|PAX6 |PPARG|PRKCB|RELA|RNF6|SIRT1|SLC1A2|SLC2A1|SNAP25 |SOD1|SOD2|TNF|TNFRSF1B|UCP2|WFS1|ZFAND3	39
Mixed (Cd+Hg+Pb)		Diabetes Mellitus, Type 2	MESH:D003924	GSTM1 BAX GCLM BCL2 RELA HMOX1 IL6 NOS2 TNF HPX GPX1 NFKB1 OGG1 SOD1 CASP3 CAT FAS C3	18
Molecular mechanisms					
ID	Name			pValue	Genes from Input
Biological processes					
GO:0006979	Response to oxidative stress			1.017E-15	CASP3, OGG1, GCLM, CAT, IL6, RELA, BCL2, TNF, NFKB1, SOD1, HMOX1, GPX1

Table 4. (continued)

GO:0043066	Negative regulation of apoptotic process		2.681E-15	FAS, CASP3, BAX, OGG1, GCLM, CAT, IL6, RELA, BCL2, TNF, NFKB1, SOD1, HMOX1, GPX1
GO:0043069	Negative regulation of programmed cell death		3.755E-15	FAS, CASP3, BAX, OGG1, GCLM, CAT, IL6, RELA, BCL2, TNF, NFKB1, SOD1, HMOX1, GPX1
GO:0060548	Negative regulation of cell death		1.804E-14	FAS, CASP3, BAX, OGG1, GCLM, CAT, IL6, RELA, BCL2, TNF, NFKB1, SOD1, HMOX1, GPX1
GO:0043067	Regulation of programmed cell death		8.668E-14	FAS, CASP3, BAX, OGG1, GCLM, NOS2, CAT, IL6, RELA, BCL2, TNF, NFKB1, SOD1, HMOX1, GPX1
Pathways				
M39818	IL-18 signaling pathway	4.179E-13		FAS, CASP3, BAX, NOS2, IL6, RELA, BCL2, TNF, NFKB1, HMOX1
1319988	AGE-RAGE signaling pathway in diabetic complications	2.569E-11		CASP3, BAX, IL6, RELA, BCL2, TNF, NFKB1
M39654	Apoptosis	9.358E-12		FAS, CASP3, BAX, RELA, BCL2, TNF, NFKB1
P00006	Apoptosis signaling pathway	3.180E-11		FAS, CASP3, BAX, RELA, BCL2, TNF, NFKB1
M39645	Oxidative Stress	5.956E-10		CAT, NFKB1, SOD1, HMOX1, GPX1
Diseases				
C0011860	Diabetes Mellitus, Non-Insulin-Dependent	3.545E-27		GSTM1, FAS, CASP3, OGG1, NOS2, C3, CAT, RELA, BCL2, TNF, NFKB1, SOD1, HMOX1, GPX1, HPX
C0011853	Diabetes Mellitus, Experimental	8.688E-24		FAS, CASP3, BAX, NOS2, CAT, IL6, RELA, BCL2, TNF, SOD1, HMOX1, GPX1
C0002152	Alloxan Diabetes	8.688E-24		FAS, CASP3, BAX, NOS2, CAT, IL6, RELA, BCL2, TNF, SOD1, HMOX1, GPX1
C0038433	Streptozotocin Diabetes	8.688E-24		FAS, CASP3, BAX, NOS2, CAT, IL6, RELA, BCL2, TNF, SOD1, HMOX1, GPX1

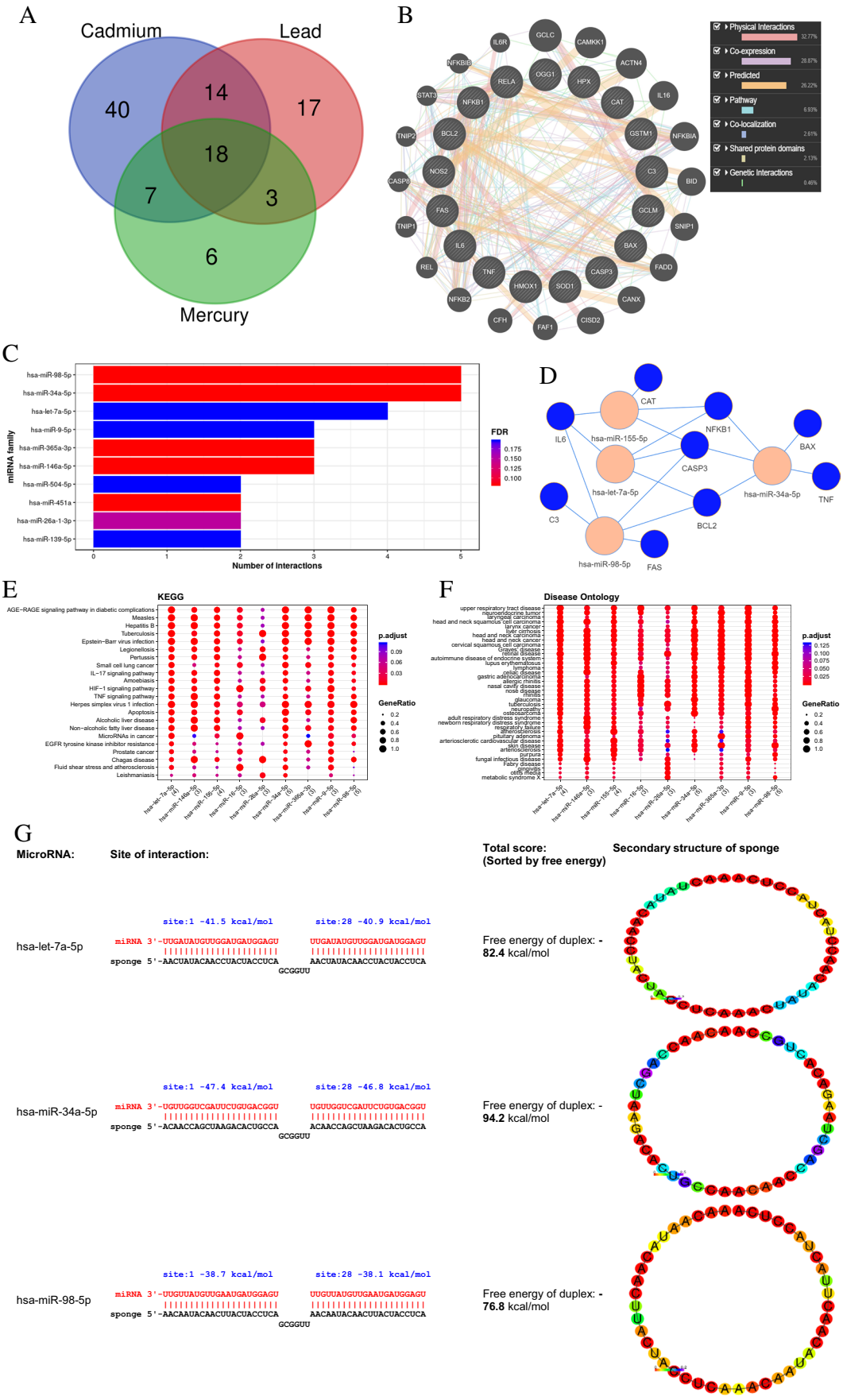
inhibitory effect of Rev-erb-alpha on gluconeogenic gene expression (Leff et al. 2018).

As mentioned above, oxidative stress and apoptosis play important roles in the etiology of T2DM. According to our in-silico analysis, the main pathways that mixed heavy metals induce in relation to the development of T2DM are oxidative stress and apoptosis. Furthermore, we observed the "AGE-RAGE signaling pathway in the diabetic complication pathway" was one of the key pathways caused by mixed heavy metals linked with T2DM development. The AGE/RAGE signaling pathway has been extensively researched in a variety of disease conditions, including T2DM. It has been known that AGEs may be a crucial component in the development of diabetes complications. These AGEs can cause an inflammatory response and raise oxidative stress in the body through numerous pathways, which has a significant impact on the onset and worsening of diabetic vascular problems (Rhee and Kim 2018). A literature review reported that through activation of TGF-beta-mediated fibrosis, Nox-1, ERK1/2 pathways, NFkB, and decreased SOD-1 expression, the AGE/RAGE signaling pathway has been linked to oxidative stress associated with diabetes-induced vascular calcification (Kay et al. 2016). On the other hand, the IL-18 signaling pathway, and non-alcoholic fatty liver disease were involved in the pathogenesis of T2DM induced by mixed heavy metals. A case-control study of Bulgarian adults observed that the serum level of IL-18 in T2DM patients was higher than in healthy controls (Zaharieva et al. 2018). In an in vivo study, systemic IL-18 treatment was found to increase diabetes development in young nonobese diabetic mice (Oikawa et al. 2003). T2DM risk in patients with nonalcoholic fatty liver disease is five times higher than in those without (Hazlehurst et al. 2016).

In terms of miRNAs, we observed that hsa-miR-98-5p, hsa-let-7a-5p, and hsa-miR-34a-5p had the highest expression and interactions induced by mixed heavy metals and were related to T2DM development. Khan et al. indicated that miR-98-5p was significantly downregulated in five adult T2DM subjects and that miR-98-5p may stimulate apoptosis and inhibit proliferation by targeting PPP1R15B in keratinocytes (Khan et al. 2020). An in vivo study found the level of miR98 expression in SW480 cells cultured under high glucose conditions was considerably lower than in frequently cultured colon cancer SW480 cells. Colon cancer cell growth and invasion are inhibited by increased expression of miR98. By targeting the target gene IGF1R, miR98 can prevent colon cancer cells from proliferating and invading (Liu et al. 2020). Mononen et al. reported that hsa-let-7a-5p level was linked with glycemic status in Young Finns Study participants (n = 871) (Mononen et al. 2019). Let-7a-5p was also found to be downregulated in diabetic nephropathy by Wang et al., suggesting that it may play a role in diabetic nephropathy pathogenesis through modulating high-mobility group AT-hook 2 expression and the PI3K-AKT signaling pathway (Wang et al. 2019). MiR-34a-5p expression was increased in Zucker diabetic fatty rats fed a high-fat diet. In comparison to Zucker lean rats, the authors suggested that miR-34a-5p could inhibit pancreatic cell proliferation by interacting with the Wnt signaling pathway. MiR-34a-5p was also observed to affect blood glucose levels via regulating insulin secretion via the insulin signaling system (Su et al. 2021). Furthermore, silencing miR-34a-5p in hepatocyte HepG2 cells reduced the formation of cellular triglycerides caused by high glucose + oleic acid/palmitic acid combination (Lee et al. 2022). Having in mind that sponges play an important role in the process of miRNA regulation, we designed and tested a miRNA sponge structure. These miRNA sponges can suppress all seed family members and the entire miRNA cluster, making them potentially useful in T2DM therapy (Barta et al. 2016; Nguyen 2022e, 2022f).

Limitations

To our knowledge, this is the first large-scale study in Korea to investigate the cumulative effects of heavy metals on T2DM and its components in participants aged 18 and older. Our findings were supported by the secondary analyses, which employed three unique mixture modeling methodologies. This study, however, has several drawbacks. First, the cross-sectional technique cannot determine whether heavy metals and T2DM are causally related. Second, a single serum sample was used to assess heavy metal exposure. Thus, the evaluations may not have accurately reflected long-lasting exposure circumstances because T2DM is a long-term illness (Nguyen et al. 2021c; Duc Nguyen et al. 2022b). Third, this study only focused on the mixed effects of three common heavy metals that were available in the KNHANES database; other potential heavy metals that were also related to TD2M (such as arsenic, nickel, chromium, etc.) were not analyzed. Fourth, the in silico toxicogenomic assessment used in the present study to determine the molecular processes involved in the etiology of combined heavy metals and diabetes should primarily be viewed as preliminary screening results. More work (in vivo or in vitro) is required to confirm our findings. The miRNA sponges developed in this study can only be useful in some cases; therefore, these findings should be seen as a precursor to more comprehensive in-vitro and in-vivo laboratory testing (Nguyen 2022g, 2022h). Fifth, even though the CTD database is different from the KNHANES database, the findings from the CTD database partly support the link between heavy metal exposure and T2DM.


A
Cadmium
Lead
Mercury
40
14
17
18
7
3
6
B
Physical Interactions
Co-expression
Predicted
Pathway
Co-localization
Shared protein domains
Genetic Interactions
C
miRNA family
Number of interactions
FDR
D
E
KEGG
p.adjust
GeneRatio
F
Disease Ontology
p.adjust
GeneRatio
G
MicroRNA:
Site of interaction:
Total score:
(Sorted by free energy)
Secondary structure of sponge
hsa-let-7a-5p
site:1 -41.5 kcal/mol
site:28 -40.9 kcal/mol
Free energy of duplex: -82.4 kcal/mol
hsa-miR-34a-5p
site:1 -47.4 kcal/mol
site:28 -46.8 kcal/mol
Free energy of duplex: -94.2 kcal/mol
hsa-miR-98-5p
site:1 -38.7 kcal/mol
site:28 -38.1 kcal/mol
Free energy of duplex: -76.8 kcal/mol

◂**Fig. 5.** Key molecular mechanisms implicated in type 2 diabetes induced by mixed heavy metals. Venn diagram for the differentially expressed genes induced by studied heavy metals (**A**). A network of overlapping genes induced by mixed heavy metals (Pb, Hg, and Cd) was created, as well as 18 type-2-diabetes-related genes (**B**). miRNA–target interaction network for miRNAs derived from the list of genes associated with type 2 diabetes and the mixed heavy metals (**C**-**D**), pink dots represent miRNA targets, blue dots represent target genes (CTD Database (http://CTD.mdibl.org). signaling pathways and diseases related to type 2 diabetes and mixed heavy metals (**E**-**F**). miRNA sponge structures for four selected miRNAs (**G**). FDR: False Discovery Rate.

Conclusions

The combined effect of heavy metals was found to be substantially associated with prediabetes and elevated glucose levels. The most important component related to prediabetes and elevated glucose was found to be serum mercury, which indicated positive trends. In silico assessment reveal that mixed heavy metals interacted with 18 genes and were linked to T2DM. Among T2DM-related genes, physical interactions were found to be the most common (32.8 percent). “AGE-RAGE signaling pathway in diabetic complications”, "non-alcoholic fatty liver disease," apoptosis, and the “IL-18 signaling pathway”), "type 2 diabetes”, "metabolic Syndrome X", hsa-miR-98-5p, hsa-let-7a-5p, and hsa-miR-34a-5p have been identified as key molecular mechanisms associated with heavy metals and T2DM development. The cutoff thresholds for exposure levels associated with T2DM and its components, in particular, were described. In summary, our findings suggest that long-term exposure to heavy metals, particularly mercury, may play a role in the progression of T2DM.

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Authors' contributions Hai Duc Nguyen: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Availability of data and materials The datasets examined during this study are available on request at the Korea Centers for Disease Control and Prevention (https://knhanes.kdca.go.kr/knhanes/main.do).

Declarations

Ethical Approval The KNHANES investigation commission accepted this study (IRB Approval numbers: 2009-01CON-03-2C, 2010-02CON-21-C, 2011-02CON-06-C, 2012-01EXP-01-2C, 2013-07CON-03-4C, 2013-12EXP-03-5C). KNHANES was excluded from assessment on research ethics under the Bioethics and Safety Act from 2016 to 2017.

Consent to Participate All KNHANES participants submitted written informed permission prior to the investigations, which were carried out by the Korea Centers for Disease Control and Prevention's Health and Nutrition Examination Department.

Consent to Publish Not applicable.

Competing interests The authors have no conflict of interest to declare.

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