Association between arsenic exposure and biomarkers of type 2 diabetes mellitus in a Croatian population: a comparative observational pilot study

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None to declare.

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Abstract:

Chronic exposure to high inorganic As levels in drinking water has been related to many diseases, including type 2 diabetes mellitus (T2D). The association with low and moderate As levels, however, remains controversial and has yet not been studied in European populations. This study aimed to investigate possible association between As exposure and biomarkers of T2D in Croatian population. Observation recruited 86 adults from Eastern Croatia, where groundwater is contaminated with inorganic As, and 116 adults from Western Croatia, where As levels in drinking water are low. Both populations were divided in patient groups (T2D or prediabetes) and healthy controls. Exposure was assessed by determining total As in blood and urine and As metabolites in urine.

Eastern Croatian population had a significantly higher content of As in urine than Western, whereas the opposite was true for arsenobetain. Total As and As metabolites in urine positively correlated with hemoglobin A1c (HbA1c) and negatively with albuminuria.

This study provides important preliminary data on the levels of As in urine and blood and their association with biomarkers of T2D in Croatian population exposed to low or moderate levels of As through drinking water as a solid basis for further research of the pathophysiological effects of such As exposure on the status and complications of diabetes.

Keywords: arsenic exposure, type 2 diabetes mellitus, drinking water, biomarkers of T2D

1. INTRODUCTION

The majority of human exposure to inorganic As comes from natural geological sources that contaminate groundwater. One such source in Europe are the deeper sediments of the Pannonian Basin from the Middle and Upper Pleistocene with As levels as high as 653 mg/kg (Ujević Bošnjak et al. 2012). Part of this Basin is in the Eastern Croatia (Romić et al. 2011, Ujević Bošnjak et al. 2010). In the dissolved fraction of untreated groundwater, As is mainly detected as arsenite [As (III)] (Ujević Bošnjak et al. 2012), which is more difficult to remove and more toxic than arsenate [As (V)] (De Marco et al. 2003). Since Croatia entered the EU, much has been done to remove As from drinking water to meet the limit of 10 μg/L in drinking water, but more than 200,000 people in Eastern region are still exposed to As levels above the EU limit (American Diabetes Association 2011, Habuda Stanić et al. 2007, Lintschinger et al. 1998).

In the human epidemiological studies, exposure to high As levels through food and drinking water has been associated with a wide variety of serious chronic conditions, including various cancers, vascular and cardiovascular disease, diabetes, developmental and reproductive problems, and neurologic and cognitive problems (Davey et al. 2008, Naujokas et al. 2013). No other element has so many diverse health effects (Davey et al. 2008).

Inorganic As increases glucose and insulin levels *in vivo*, decreases glucose uptake in insulin-sensitive cells, and interferes with transcription involved in insulin signal transduction and insulin sensitivity *in vitro* (Navas-Acien et al. 2008). In humans, high chronic exposure to inorganic As (>100 μg/L in drinking water) in occupational settings has been associated with higher risk of type 2 diabetes mellitus (T2D) (Wang et al. 2014; Jovanović et al. 2013; Makris et al. 2012) and higher HbA1c, a marker of glycaemia (Jensen and Hansen 1998). However, the effects of low and moderate chronic As exposure are still inconclusive. Absorbed or ingested, inorganic As compounds undergo methylation in the human body to form

monomethylarsonate (MMA) and dimethylarsinate (DMA). Together with unchanged inorganic As, they are excreted in the urine. A recent prospective study has demonstrated that higher DMA and lower MMA in urine have been related to higher body mass index (BMI) and a higher risk of T2D (Grau-Perez et al. 2017).

Data on the association between As exposure and T2D in the European population are scarce (Bräuner et al. 2014, Grau-Perez et al. 2017, Jovanović et al. 2013). Health risk assessment of cumulative exposure to As through drinking water has pointed to increased toxic and carcinogenic risks among rural residents in Eastern Croatia (Ujević Bošnjak et al. 2012). However, there is no study to associate T2D and As exposure in Croatian population. According to the CroDiab registry (Croatian Institute of Public Health 2017; Ministry of Health of the Republic of Croatia 2015), T2D is the 8th leading cause of death in Croatia, while the total number of patients with T2D registered in 2015 was 260,092. Given the widespread exposure to As from drinking water worldwide and its adverse health effects, more information on the possible association between As exposure and T2D prevalence is required.

This preliminary study intended to fill that gap by investigating firstly, the effect of exposure to elevated levels of As (>10 μg/L in drinking water) on As levels in blood and urine. Then, the association of As levels in blood and urine and As metabolites in urine with biomarkers of T2D in Croatian population including patient groups (T2D or prediabetes) and healthy controls.

2. MATERIALS AND METHODS

2.1.Participants

This observational study recruited 202 adults urban participants: 86 from the city of Osijek in Eastern Croatia (area of 170 km^2, coordinates 45°33'18"N, 18°41'44"E), where As levels in

drinking water exceed the EU limit of 10 μg/L, and 116 from the city of Zagreb in Western Croatia (area of 640 km^2, coordinates 45°48'52"N, 15°58'41"), where As levels ~~keep~~ is below the EU limit. The participants were further divided into normoglycemic or healthy (H), those characterized with prediabetes (PreD), and the T2D group (D) ~~that fits~~according to the American Diabetes Association criteria (American Diabetes Association 2011). The study protocol was accepted and approved by the Ethics Committee of the Clinical Hospital Osijek, Osijek, Croatia, the Ethics Committee of the Merkur University Hospital, Zagreb, Croatia, and the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia. Informed consents were obtained from all participants before any other action.

General exclusion criteria were acute infection, renal insufficiency (eGFR < 30 mL/min/1.73 m^2), cardiovascular disease, and malignant diseases. Of the T2D participants, only those taking oral hypoglycemic drugs but no insulin were included in the study.

Sociodemographic, lifestyle and other general information were collected about all participants, including gender, age, education level, smoking, family history of diabetes, physical activity, dietary recall interviews covering the past 5 days (including seafood intake), daily intake of fluids, and origin of water used for drinking. Anthropometric data, which included height, weight, waist and hips circumference were also collected by trained nurses.

2.2. Blood and urine collection and analysis

Exposure was assessed by determining total As in blood and urine samples and by determining As (III), As (V), arsenobetaine (AsBet), DMA, and MMA in urine samples of each participant. All participants were clinically examined and tested for biomarkers relevant for T2D diagnosis and monitoring (Table 1). A single collection of one urine and one blood sample was carried out for each study participant.

Fasting blood samples were taken in the morning after at least 8-hour fast. Spot urine samples were collected before blood sampling in polypropylene tubes, frozen within 1 hour of collection, shipped on dry ice, and stored at -80 °C until As analysis. Fasting serum glucose, HbA1c, and urinary albumin and creatinine were determined in the laboratories of the Clinical Hospital Osijek and Merkur University Hospital, Zagreb. Urinary albumin was measured in spot urine samples with an immunoturbidimetric method, while creatinine was determined with a compensated alkaline picrate Jaffè assay on an automated analyzer (AU680, Beckman Coulter, Brea, USA). The albumin-to-creatinine ratio (ACR) was calculated by dividing urinary albumin (mg/L) with urinary creatinine (mmol/L), and the results expressed as mg/mmol.

Fasting serum insulin for the entire study population was assayed with a chemiluminiscent immunoassay traceable to the World Health Organization (WHO) 1st IRP 66/304 standard (Advia Centaur-XP, Siemens Siemens Healthcare GmbH, Erlangen, Germany). Fasting glucose was assayed with a UV spectrophotometric hexokinase method using dedicated reagents and automated analyzer with a limit of detection 0.04 mmol/L (AU680, Beckman Coulter, Brea, USA).

Estimated glomerular filtration rate (eGFR) was obtained from serum creatinine values measured with the IDMS-traceable compensated Jaffé method (KDIGO Board 2013) using the 4-variable CKD-EPI equation. In addition, the Homeostasis Model Assessment (HOMA) was applied to estimate steady state function of pancreatic β cells (HOMA%B) and insulin resistance (HOMA-IR) (Levy et al. 1998). HOMA-IR and HOMA%B were estimated from fasting glucose and insulin levels using HOMA calculator (https://www.dtu.ox.ac.uk/homacalculator/).

HbA1c was assessed in whole blood, anticoagulated with K_3EDTA, using an automated immunoturbidimetric method traceable to both NGSP- and IFCC-standards with a limit of

detection of 4.2% and 23 mmol/mol, respectively. The results are expressed in dual units (% and mmol/mol) according to traceability towards respective standards.

T2D status was established if the HbA1c level was above 6.5%, as defined by the diagnostic criteria of the American Diabetes Association (American Diabetes Association 2011). Participants with the HbA1c level from 5.6% to 6.4% were considered prediabetic, while participants with HbA1c below 5.6% were considered normoglycemic.

2.3.Determination of As levels and species

Total As and As species were determined using the method described by Lintschinger et al. (1998). Standard stock solutions for As (III) (100 mg As/L) and As (V) (100 mg As/L) were prepared by dissolving the appropriate amount of sodium metaarsenite (Merck (Sigma), 97%) and sodium arsenate heptahydrate (Merck (Sigma)) in water. Standards for MMA, DMA, and AsBet were kindly donated by the Service Central d'Analyse (CNRS, Vernaison, France) in the form of aqueous standard stock solutions, each containing 400 mg As/L. All stock solutions were stored in the dark at 4 °C. Working standard solutions of lower concentrations were prepared daily by dilution with water. All chemicals used for the preparation of mobile phases were of analytical reagent grade or higher purity. Mobile phases for HPLC separation of As species were prepared from a tetramethylammonium hydroxide (TMAH) stock solution (TAMA Chemicals, Osaka, Japan) and sodium carbonate (Merck, Darmstadt, Germany). The solutions were prepared by dissolving the compounds in ultrapure water obtained from a special cartridge deionization unit (Milli-Q Water Purification System). Diluted nitric acid (Merck, Suprapur®, purified by sub-boiling distillation) was used for dilution of urine samples (1:5) before determination of total As concentrations.

Total As was determined by inductively coupled plasma-sector field-mass spectrometry (ICP-sf-MS) (Element II, Thermo Scientific Bremen, Germany) operating in a high resolution

mode. Instrumental parameters were as follows: forward power of 1300 W, plasma gas at 15.0 L Ar/min, nebulizer gas at 0.80 L Ar/min (daily optimized), auxiliary gas at 0.80 L Ar/min, mass resolution of 10000, sample introduction by ESI-FAST system at 0.8 ml/min connected to a sea-spray nebulizer/cyclon spray chamber.

For As speciation we hyphenated ion exchange chromatography to ICP-sf-MS. As species were separated according to a slightly modified method of Lintschinger et al. (1998) using a HPLC system Beckman System Gold 127NM Solvent Module (Beckman Coulter Biomedical, Munich, Germany) equipped with 9725i PEEK injection valve from Rheodyne (Sigma-Aldrich, CT, USA) and a degasser Degassex™ Model D6-4400 (Phenomenex, Darmstadt, Germany). Chromatographic parameters were as follows: Thermo (Dionex) IonPac AG14 50 x 4 mm as pre-column, Thermo (Dionex) IonPac AS14 250 x 4 mm as column, mobile phase A consisting of 1 mmol/L TMAH and mobile phase B consisting of 10 mmol/L ammonium carbonate at pH 10.0. Flow rate was set to 1.0 mL/min, and the injection volume was 100 µL. The gradient program used a slower eluent change from eluent A to B compared to Davey et al. (2008) to ensure separation of DMA and As (III) and was set as follows: 0–4 min 0% B; 4–9 min 0-100% B; 9–16 min 100% B. After each run the column was purged with 5 mM EDTA solution for 10 minutes and then re-equilibrated with eluent A. Typical chromatograms are shown in Figure S1 in the Supporting Information (SI).

Isotope ^{75}As was detected with ICP-sf-MS by direct PEEK tube connection from column outlet to sea spray nebulizer. The ICP-sf-MS parameters were the same as for total As determination. The limit of quantification was 0.13 - 0.145 µg/L for As species and was uniformly set to 0.15 µg/L. AsBet, MMA, DMA, As (III), and As (V) were quantified by comparing As species peak areas with respective calibration curves. The obtained IEC-ICP-sf-MS data were processed and peak areas calculated with the PeakFit™ software v4.12 for Windows® (SeaSolve Software Inc., San Jose, CA, USA).

2.4.Statistical Analysis

All statistics were run on a SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Before data modelling, all variables with non-normal distribution of the residuals were log-transformed. Variables with a value below the limit of detection (LOD) were excluded from the analysis.

To estimate the correlation between As species and HOMA-IR, HOMA%B, fasting glucose, insulin level, HbA1c, creatinine, eGFR, and albuminuria we used linear regression analysis adjusted for potential confounding factors such as gender, education level, and age. For each regression equation, we estimated the β value, standard error, and 95% interval of confidence (CI). In addition, based on the t-distribution we presented the relative p-values.

Differences in fasting glucose, insulin levels, HbA1c, and albuminuria (dependent variables) between healthy, prediabetes, and T2D participants, as well as between Eastern and Western Croatian participants (independent variables) were analyzed with the multivariate analysis of variance (MANOVA). Type III Sum of Squares was used to test the health status and geographic origin and interactions between the two. The model was adjusted for the same confounding factors used for regression analysis. We calculated the least squares mean (LS-mean) due to unbalanced experiment.

Scheffé's test was applied for multiple comparisons. It provided detailed information about the differences between LS-means. These multiple comparisons for different interactions are presented by a diffogram, that is, a mean-mean scatter diagram (Adesemoye et al. 2008).

In addition, two general linear models (GLM) were used to study the relationship between urine As with the geographic origin and all the variables related to T2D and As exposure. The first model had age, gender, and education as confounding factors, and the second included all As forms and T2D variables.

The relationships between all variables were analyzed with principal component analysis (PCA) (Law and Jolliffe 1987) using a SIMCA version 13.03 software (Umetrics, Umeå, Sweden).

3. RESULTS

3.1.General demographic and health characteristics of study participants

General participant demographics are shown in Table 1. The participants are divided by geographical origin to Eastern (Osijek) and Western (Zagreb) Croatia and by health status to healthy participants with normal glucose tolerance (H), prediabetic participants (PreD), and T2D patients (D).

The loading plot presented in Figure 1 and the score plots presented in Figure 2 show all relationships between the variables obtained with PCA analysis. The main variance (represented by the first component, X-axis) is absorbed by the health status, and the second component (Y-axis) shows the differentiation between the Eastern and Western regions. As expected, the D group (T2D patients) is characterized by a high level of insulin fasting glucose, HOMA-IR, waist and hip circumference, and BMI (Figure 1). Most of these parameters highly correlated with the level of HbA1c and age. The MANOVA analysis (Table S1 in SI) revealed that the T2D patients differed from healthy and prediabetic participants in the following dependent variables: fasting glucose, HbA1c, and insulin. The correction for confounding factors revealed no significant differences in albumin. No significant differences were found between healthy and prediabetic participants. Geographical origin had no significant impact on insulin and fasting glucose. The interactions between geographical origin and health status are shown in Figure S2, and diffogram plots for each interaction in Figure S3 (both in SI). Interestingly, T2D patients from Eastern Croatia

significantly differed in HbA1c values from all participants (healthy, prediabetic, and T2D) from Western Croatia. They also significantly differed from western T2D patients in albumin levels (Figure S3 in the SI).

As for the second component (geographic origin), participants from Eastern Croatia are positioned in the upper part of both plots (Figures 1 and 2), which is related to higher levels of total As in urine. Higher urine As levels, in turn, may reflect higher exposure through municipal tap water as the major source of water intake (more than 85% of participants with a mean intake of > 1 L/day at enrolment) (Table 1). Reports for As levels in municipal drinking in Osijek (Eastern Croatia) range from 46 μg/L in 2000 to 31 μg/L in 2017 (Vodovod Osijek 2016). In Zagreb, As ranged between 0.50 and 7.68 μg/L in 2016 and 2017, respectively.

Table 3 shows blood and urine findings of total As, As (III), As (V), AsBet, MMA, and DMA. AsBet prevailed in the urine of study participants from Western Croatia, while inorganic As species prevailed in the urine of participants from the Eastern region (Table 3). Even though we did not evaluate exposure to organic As through diet (fish, shellfish, rice, and wine), it is quite safe to say that the consumption of seafood as the main source of AsBet is much higher in Western than Eastern Croatia, which could explain the findings. Values for As correlated with the glycemic status and were higher in T2D than PreD participants from both regions (Table 3). Regional clustering of As(V), MMA, and DMA in turn, confirms the expected high association between these As species and Eastern Croatia (Figures 1 and 2). This association is further analyzed below.

3.2.**Arsenic exposure and metabolism and their association with health status**

Table 4 shows the relationships of total As and other urine As forms with HbA1c, insulin, creatinine, eGFR, and albuminuria adjusted for age, education level, and gender). Total urine As positively correlated with HbA1c, while total blood As negatively correlated with urine

albumin. A positive correlation was also observed between HOMA-IR and MMA, and a negative correlation between AsBet and insulin.

The first general linear model confirmed that the participants from Eastern Croatia had significantly higher ($p<0.0001$) urine As levels when adjusted for age and gender as confounding factors (Table 5). However, the second, extended model, which included more independent variables, showed no significant difference between the regions when controlled for all the confounders, even though the estimation was still high (Table 5).

After a stepwise selection, we established that only the As variables (except for As(III)) and BMI significantly differed between the Eastern and Western regions. All the covariates positively correlated with urine As levels. However, there was no clear evidence of an association between geographic origin and exposure to As in relation to glycemic status with all variables included in the model (Table 5).

4. DISCUSSION

In this study, urinary As was higher in study participants from Eastern than Western Croatia, but organic (dietary) AsBet prevailed among Western participants, while inorganic As prevailed in the participants from the East, mostly in the form of As (V). Higher concentrations of As (V), MMA, and DMA in urine were associated with geographical origin (Table 5), but no correlation was found between these As forms and HbA1c or fasting glucose levels.

In line with earlier studies (Coronado et al. 2007; Faseke et al. 2015; Gribble et al. 2012; Navas-Ancien et al. 2008; Rhee et al. 2013), we found a positive association between total urinary As and HbA1c as biomarker of T2D, regardless of the region and glycemic status (Table 4). However, our general linear model analysis showed no clear evidence of an association between exposure to As through drinking water and T2D when all variables were

included in the model (Table 5). This may have to do with the fact that in addition to exposure levels, the toxicity of As depends on its metabolism, which is characterized by a series of methylation steps (Drobna et al. 2009). Inorganic As found in drinking water and its main metabolites in urine As (III), As (V), MMA, and DMA are considered highly toxic to human cells (James et al. 2013, Vega et al. 2001). Recent cross-sectional studies from Mexico and Bangladesh (Mendez et al. 2016, Nizam et al. 2013) and a prospective study from the United States (Kuo et al. 2015) demonstrated an increased risk of T2D in study participants with a metabolic profile characterized by lower MMA and higher DMA levels in urine. In our study, we did not find a significant association between MMA and T2D, but did between MMA and HOMA-IR biomarker. Before that, only a few epidemiologic studies evaluated the association between HOMA-IR and As exposure (Del Razo et al. 2011, Gribble et al. 2012, Lin et al. 2014, Park et al. 2016), but found none, even though animal studies pointed to the opposite (Fu et al. 2010, Palacios et al. 2012).

We also did not observe any significant association of HOMA-IR and HOMA%B with total As in urine and blood, unlike a US family study (Grau-Perez et al. 2017), which also reported inverse correlation between MMA and HOMA2-IR when either inorganic As or DMA were decreased. Instead, our regression analysis (Table 4) showed positive correlations between total urinary As and HbA1c and between MMA and HOMA-IR ($p = 0.01$ and $p = 0.02$, respectively).

Although some recent studies reported a positive correlation between albuminuria and total urinary As (Chen et al. 2011, Zheng et al. 2013), our findings pint to the opposite in the sense that the correlation was negative (Table 4) and do not support the hypothesis that higher exposure to inorganic As increases the risk of diabetic nephropathy as one of the T2D complications (Brownlee 2001). Robles-Osorio et al. (2012), in turn, did not find any association between urinary As and albuminuria but did find an association between total

urinary As and urinary excretion of αl-microglobulin as a marker of early tubular injury. Obviously, more prospective research is needed to resolve whether As induces nephrotoxicity in humans.

5. Limitations of this study

In spite of all the interesting findings reported in this study, some limitations should be taken into account. Most importantly, our study was limited by a relatively small sample size. In addition, our population groups (Eastern and Western) were characterized by variations in age, exposure time, diet and lifestyle that further limit the interpretation of our results. Therefore, our study should be considered a pilot collecting with preliminary data for future, larger, multicentric epidemiological studies in European populations. Our sample size was also insufficient to evaluate dose–response relationships for each exposure and outcome in detail. Furthermore, As levels in blood and urine (including urinary As species) were measured at a single time point, which fails to give an insight into the variability in typical exposure levels. Future studies evaluating As metabolism in relation to As exposure and health status in larger study populations are needed.

6. CONCLUSION

This is the first Croatian, but also European, study on levels of As species in urine of healthy volunteers vs. diabetic patients chronically exposed to elevated As levels through drinking water. Its experimental design relied on a high-quality protocol and laboratory methods. The determination of blood and urinary concentrations of As and its metabolites allowed correlation and association analysis with regard to biomarkers of T2D. As expected, we found association between concentrations of As in drinking water with total As in urine. Positive

correlations were found between increased levels of As in urine and HbA1c, as well as between increased MMA in urine and HOMA-IR biomarker. Our study therefore provides important implications for future public health research in Europe. The role of As in the incidence of diabetes and diabetic complications (diabetic nephropathy in particular) should be clearly defined and the underlying pathophysiological mechanism(s) identified.

Conflicting interests

None to declare.

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References

Adesemoye TO, Torbert A, Kloepper HJ. 2008. Enhanced Plant Nutrient Use Efficiency with PGPR and AMF in an Integrated Nutrient Management System. **Can J Microbiol** 54(10):876-86, DOI: 10.1139/w08-081

American Diabetes Association. 2011. Diagnosis and classification of diabetes mellitus. **Diabetes Care** 34:S62-S69, DOI: 10.2337/dc11-S062

Bräuner EV, Nordsborg RB, Andersen ZJ, Tjønneland A, Loft S, Raaschou-Nielsen O. 2014. Long-term exposure to low-level arsenic in drinking water and diabetes incidence: a prospective study of the Diet, Cancer and Health cohort. **Environ Health Perspect** 122:1059–1065, DOI:10.1289/ehp.1408198

Brownlee M. 2001. Biochemistry and molecular cell biology of diabetic complications. **Nature** 414(6865):813–820, DOI: 10.1038/414813a

Chen Y, Parvez F, Liu M, Pesola GR, Gamble MV, Slavkovich V et al. 2011. Association between arsenic exposure from drinking water and proteinuria: results from the Health

Effects of Arsenic Longitudinal Study. **Int J Epidemiol** 40:828–835, DOI: 10.1093/ije/dyr022

Coronado-González JA, Del Razo LM, García-Vargas G, Sanmiguel-Salazar F, Escobedo-de la Peña J. 2007. Inorganic arsenic exposure and type 2 diabetes mellitus in Mexico. **Environ Res** 104:383-389, DOI:10.1016/j.envres.2007.03.004

Croatian Institute of Public Health. 2017. CroDiab registry. Available at: https://www.hzjz.hr/en/division-of-epidemiology-and-prevention-of-chronic-non-communicable-diseases/crodiab-registry/ Accessed May 31th 2018

Ćavar S, Klapec T, Grubešić RJ, Valek M. 2005. High exposure to arsenic from drinking water at several localities in eastern Croatia. **Sci Tot Environ** 339:277–282, DOI:10.1016/j.scitotenv.2004.12.013

Davey JC, Nomikos AP, Wungjiranirun M, Sherman JR, Ingram L, Batki C, Lariviere JP, Hamilton JW. 2008. Arsenic as an endocrine disruptor: Arsenic disrupts retinoic acid receptor–and thyroid hormone receptor–mediated gene regulation and thyroid hormone–mediated amphibian tail metamorphosis. **Environ Health Persp** 116(2):165-172, DOI:10.1289/ehp.10131

De Marco MJ, SenGupta AK, Greenleaf JE. 2003. Arsenic removal using a polymeric/inorganic hybrid sorbent. **Water Res** 37:164–176, DOI:10.1016/S0043-1354(02)00238-5

Del Razo LM, García-Vargas GG, Valenzuela OL, Castellanos EH, Sánchez-Peña LC, Currier JM et al. 2011. Exposure to arsenic in drinking water is associated with increased prevalence of diabetes: a cross-sectional study in the Zimapán and Lagunera regions in Mexico. **Environ Health** 10:72, DOI:10.1186/1476-069X-10-73

Drobna Z, Styblo M, Thomas DJ. 2009. An overview of arsenic metabolism and toxicity. **Curr Protoc Toxicol** 42(431):4.31.1–4.31.6, DOI:10.1002/0471140856.tx0431s42

EFSA (European Food Safety Authority). 2009. EFSA Panel on Contaminants in the Food Chain (CONTAM); scientific opinion on arsenic in food. EFSAJ 7(10):1351, DOI:10.2903/j.Efsa.2009.1351.

Feseke SK, St-Laurent J, Anassour-Sidi E, Ayotte P, Bouchard M, Levallois P. 2015. Arsenic exposure and type 2 diabetes: results from the 2007–2009 Canadian Health Measures Survey [in French]. **Health Promot Chronic Dis Prev Can** 35(4):63–72, DOI:10.24095/hpcdp.35.4.01.

Fu J, Woods CG, Yehuda-Shnaidman E, Zhang Q, Wong V, Collins S, et al. 2010. Low-level arsenic impairs glucose-stimulated insulin secretion in pancreatic beta cells: involvement of cellular adaptive response to oxidative stress. **Environ Health Perspect** 118(6):864–870, DOI:10.1289/ehp.0901608.

Grau-Pérez M, Kuo CC, Gribble MO, Balakrishnan P, Jones Spratlen M, Vaidya D et al. 2017. Association of low-moderate arsenic exposure and arsenic metabolism with incident diabetes and insulin resistance in the Strong Heart Family study. **Environ Health Persp** 125(12):127004, DOI:10.1289/EHP2566

Gribble MO, Howard BV, Umans JG, Shara NM, Francesconi KA, Goessler W, et al. 2012. Arsenic exposure, diabetes prevalence, and diabetes control in the Strong Heart Study. **Am J Epidemiol** 176(10):865–874, DOI:10.1093/aje/kws153.

Habuda-Stanić M, Kuleš M, Kalajdžić B, Romić Ž. 2007. Quality of groundwater in eastern Croatia. The problem of arsenic pollution. **Desalination** 210:157–162, DOI:10.1016/j.desal.2006.05.040

https://www.dtu.ox.ac.uk/homacalculator/ Accessed July 9 th 2018

James KA, Marshall JA, Hokanson JE, Meliker JR, Zerbe GO, Byers TE. 2013. A case-cohort study examining lifetime exposure to inorganic arsenic in drinking water and diabetes mellitus. **Environ Res** 123:33-38, DOI:10.1016/j.envres.2013.02.005

Jensen GE, Hansen ML. 1998. Occupational arsenic exposure and glycosylated haemoglobin. **Analyst** 123(1):77-80, DOI:10.1039/A705699K

Jovanović D, Rašić-Milutinović Z, Paunović K, Jakovljević B, Plavšić S, Milošević J. 2013. Low levels of arsenic in drinking water and type 2 diabetes in Middle Banat region, Serbia. **Int J Hyg Environ health** 216:50-55, DOI:10.1016/j.ijheh.2012.01.001

Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. 2013. KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. **Kidney Int Suppl** 3:1–150

Kuo CC, Howard BV, Umans JG, Gribble MO, Best LG, Francesconi KA, et al. 2015. Arsenic exposure, arsenic metabolism, and incident diabetes in the Strong Heart Study. **Diabetes Care** 38(4):620–627, DOI:10.2337/dc14-1641.

Lampron-Goulet É, Gagnon F, Langlois MF. 2017. Association between consumption of private well water contaminated by low levels of arsenic and dysglycemia in a rural region of Quebec, Canada. **Environ Res** 159:232-238, DOI:10.1016/j.envres.2017.07.049

Law J, Jolliffe IT. 1987. Principal Component Analysis. **The Statistician** 36, 37–52.

Levy JC, Matthews DR, Hermans MP. 1998. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. **Diabetes Care** 21(12):2191-2192, DOI: 10.2337/diacare.21.12.2191

Li Z, Piao F, Liu S, Wang Y, Qu S. 2009. Subchronic exposure to arsenic trioxide-induced oxidative DNA damage in kidney tissue of mice. **Exp Toxicol Pathol** 62(5):543-547, DOI:10.1016/j.etp.2009.07.003

Lin HC, Huang YK, Shiue HS, Chen LS, Choy CS, Huang SR, et al. 2014. Arsenic methylation capacity and obesity are associated with insulin resistance in obese children and adolescents. **Food Chem Toxicol** 74:60–67, DOI:10.1016/j.fct.2014.08.018.

Lintschinger J, Schramel P, Hatalak-Rauscher A, Wendler I, Michalke B. 1998. A new method for the analysis of arsenic species in urine by using HPLC-ICP-MS. **Fresen J Anal Chem** 362(3):313-318, DOI:10.1007/s002160051080

Makris KC, Christophi CA, Paisi M, Ettinger AS. 2012. A preliminary assessment of low level arsenic exposure and diabetes mellitus in Cyprus. **BMC Public Health** 12:334, DOI:10.1186/1471-2458-12-334

Mendez MA, González-Horta C, Sánchez-Ramírez B, Ballinas-Casarrubias L, Cerón RH, Morales DV, et al. 2016. Chronic exposure to arsenic and markers of cardiometabolic risk: a cross sectional study in Chihuahua, Mexico. **Environ Health Perspect** 124(1):104–111, DOI:10.1289/ehp.1408742.

Ministry of Health of the Republic of Croatia. 2015. National diabetes programme 2015 – 2018. Available at: https://www.hzjz.hr/wp-content/uploads/2013/11/National-Diabetes-Programme-2015_2020.pdf Accessed: May 31th 2018

Naujokas MF, Anderson B, Ahsan H, Aposhian HV, Graziano JH, Thompson C, Suk WA. 2013. The broad scope of health effects from chronic arsenic exposure: update on a worldwide public health problem. **Environ Health Perspec** 121:295-302, DOI:10.1289/ehp.1205875

Navas-Acién A, Silbergeld EK, Pastor-Barriuso R, Guallar E. 2008. Arsenic exposure and prevalence of type 2 diabetes in US adults. **J Am Med Assoc** 300(7):814–822, DOI:10.1001/jama.300.7.814

Nizam S, Kato M, Yatsuya H, Khalequzzaman M, Ohnuma S, Naito H, et al. 2013. Differences in urinary arsenic metabolites between diabetic and non-diabetic subjects in Bangladesh. **Int J Environ Res Public Health** 10(3):1006–1019, DOI:10.3390/ijerph10031006.

Pan WC, Seow WJ, Kile ML, Hoffman EB, Quamruzzaman Q, Rahman M, et al. 2013. Association of low to moderate levels of arsenic exposure with risk of type 2 diabetes in Bangladesh. **Am J Epidemiol** 178(10):1563–1570, DOI:10.1093/aje/kwt195.

Palacios J, Roman D, Cifuentes F. 2012. Exposure to low level of arsenic and lead in drinking water from Antofagasta City induces gender differences in glucose homeostasis in rats. **Biol Trace Elem Res** 148:224–231, DOI:10.1007/s12011-012-9355-3.

Park SK, Peng Q, Bielak LF, Silver KD, Peyser PA, Mitchell BD. 2016. Arsenic exposure is associated with diminished insulin sensitivity in non-diabetic Amish adults. **Diabetes Metab Res Rev** 32(6):565–571, DOI:10.1002/dmrr.2769.

Pravilnik o zdravstvenoj ispravnosti vode za piće [Drinking water regulation] (in croatian). 2008. **Offical Gazette** No.47

Rhee SY, Hwang YC, Woo JT, Chin SO, Chon S, Kim YS. 2013. Arsenic exposure and prevalence of diabetes mellitus in Korean adults. **J Korean Med Sci** 28:861-868, DOI:10.3346/jkms.2013.28.6.861

Robles-Osorio ML, Pérez-Maldonado IN, Martín del Campo D, Montero-Perea D, Avilés-Romo I, Sabath-Silva E, Sabath E. 2012. Urinary arsenic levels and risk of renal injury in a cross-sectional study in open population. **Rev Invest Clin** 64(6 Pt 2):609-614, PMID:23593778

Romić Ž, Habuda-Stanić M, Kalajdžić B, Kuleš M. 2011. Arsenic distribution, concentration and speciation in groundwater of the Osijek area, eastern Croatia **App Geochem** 26:37–44, DOI:10.1016/j.apgeochem.2010.10.013

Ujević Bošnjak M, Duić Ž, Casiot C, Sipos L, Santo V, Dadić Ž, Halamić J. 2010. Occurrence and geochemistry of arsenic in the groundwater of Eastern Croatia. **App Geochem** 25(7):1017–1029, DOI:10.1016/j.apgeochem.2010.04.008

Ujević Bošnjak M, Capak K, Jazbec A, Casiot C, Sipos L, Poljak V, Dadić Ž. 2012. Hydrochemical characterization of arsenic contaminated alluvial aquifers in Eastern Croatia using multivariate statistical techniques and arsenic risk assessment. **Sci Tot Environ** 420:100–110, DOI:10.1016/j.scitotenv.2012.01.021

Vega L, Styblo M, Patterson R, Cullen W, Wang C, Germolec D. 2001. Differential effects of trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in

normal human epidermal keratinocytes. **Toxicol Appl Pharmacol** 172:225-232, DOI:10.1006/taap.2001.9152

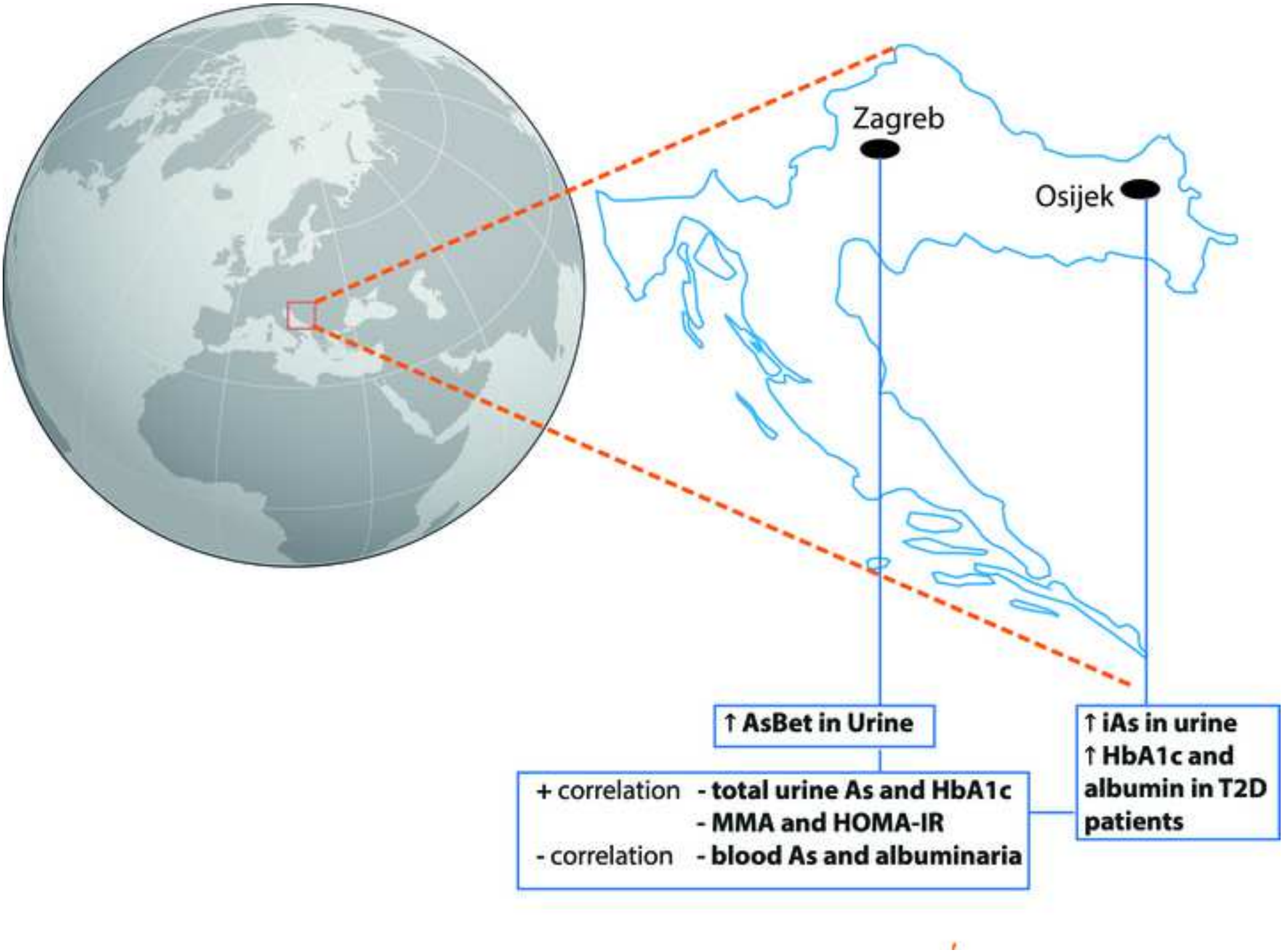
Vodovod Osijek 2016, https://vodovod.com/wp-content/uploads/2015/03/GODI%C5%A0NJE-IZVJE%C5%A0%C4%86E-O-KVALITETI-VODE-ZA-LJUDSKU-POTRO%C5%A0NJU-U-2016.-GODINI.pdf Accessed July 9 th 2018

Vodovod Osijek 2017, http://vodovod.com/wp-content/uploads/2015/03/GODI%C5%A0NJE-IZVJE%C5%A0%C4%86E-O-KVALITETI-VODE-ZA-LJUDSKU-POTRO%C5%A0NJU-U-2017.-GODINI.pdf Accessed July 9 th 2018

Wang W, Xie Z, Lin Y, Zhang D. 2014. Association of inorganic arsenic exposure with type 2 diabetes mellitus: a meta-analysis. **J Epidemiol Community Health** 68:176-184. PMID: 24133074 DOI: 10.1136/jech-2013-203114

Zheng LY, Umans JG, Tellez-Plaza M, Yeh F, Francesconi KA, Goessler W, et al. 2013. Urine arsenic and prevalent albuminuria: evidence from a population-based study. **Am J Kidney Dis** 61(3):385-394, DOI:10.1053/j.ajkd.2012.09.011

*Graphical Abstract



*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Association between arsenic exposure and biomarkers of type 2 diabetes mellitus in a Croatian population: a comparative observational pilot study

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Highlights:

- This is first study providing data on urinary levels of As species in Croatian population
- Correlation between As in urine and blood with biomarkers of diabetes were investigated.
- Higher concentrations of As species in urine were associated with geographical origin
- As levels in urine positively correlated with HbA1c and negatively with albuminuria

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Competing Financial Interests

None to declare.

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Abstract:

Chronic exposure to high inorganic As levels in drinking water has been related to many diseases, including type 2 diabetes mellitus (T2D). The association with low and moderate As levels, however, remains controversial and has yet not been studied in European populations. This study aimed to investigate possible association between As exposure and biomarkers of T2D in Croatian population. Observation recruited 86 adults from Eastern Croatia, where groundwater is contaminated with inorganic As, and 116 adults from Western Croatia, where As levels in drinking water are low. Both populations were divided in patient groups (T2D or prediabetes) and healthy controls. Exposure was assessed by determining total As in blood and urine and As metabolites in urine.

Eastern Croatian population had a significantly higher content of As in urine than Western, whereas the opposite was true for arsenobetain. Total As and As metabolites in urine positively correlated with hemoglobin A1c (HbA1c) and negatively with albuminuria.

This study provides important preliminary data on the levels of As in urine and blood and their association with biomarkers of T2D in Croatian population exposed to low or moderate levels of As through drinking water as a solid basis for further research of the pathophysiological effects of such As exposure on the status and complications of diabetes.

Keywords: arsenic exposure, type 2 diabetes mellitus, drinking water, biomarkers of T2D

1. INTRODUCTION

The majority of human exposure to inorganic As comes from natural geological sources that contaminate groundwater. One such source in Europe are the deeper sediments of the Pannonian Basin from the Middle and Upper Pleistocene with As levels as high as 653 mg/kg (Ujević Bošnjak et al. 2012). Part of this Basin is in the Eastern Croatia (Romić et al. 2011, Ujević Bošnjak et al. 2010). In the dissolved fraction of untreated groundwater, As is mainly detected as arsenite [As (III)] (Ujević Bošnjak et al. 2012), which is more difficult to remove and more toxic than arsenate [As (V)] (De Marco et al. 2003). Since Croatia entered the EU, much has been done to remove As from drinking water to meet the limit of 10 μg/L in drinking water, but more than 200,000 people in Eastern region are still exposed to As levels above the EU limit (American Diabetes Association 2011, Habuda Stanić et al. 2007, Lintschinger et al. 1998).

In the human epidemiological studies, exposure to high As levels through food and drinking water has been associated with a wide variety of serious chronic conditions, including various cancers, vascular and cardiovascular disease, diabetes, developmental and reproductive problems, and neurologic and cognitive problems (Davey et al. 2008, Naujokas et al. 2013). No other element has so many diverse health effects (Davey et al. 2008).

Inorganic As increases glucose and insulin levels *in vivo*, decreases glucose uptake in insulin-sensitive cells, and interferes with transcription involved in insulin signal transduction and insulin sensitivity *in vitro* (Navas-Acien et al. 2008). In humans, high chronic exposure to inorganic As (>100 μg/L in drinking water) in occupational settings has been associated with higher risk of type 2 diabetes mellitus (T2D) (Wang et al. 2014; Jovanović et al. 2013; Makris et al. 2012) and higher HbA1c, a marker of glycaemia (Jensen and Hansen 1998). However, the effects of low and moderate chronic As exposure are still inconclusive. Absorbed or ingested, inorganic As compounds undergo methylation in the human body to form

monomethylarsonate (MMA) and dimethylarsinate (DMA). Together with unchanged inorganic As, they are excreted in the urine. A recent prospective study has demonstrated that higher DMA and lower MMA in urine have been related to higher body mass index (BMI) and a higher risk of T2D (Grau-Perez et al. 2017).

Data on the association between As exposure and T2D in the European population are scarce (Bräuner et al. 2014, Grau-Perez et al. 2017, Jovanović et al. 2013). Health risk assessment of cumulative exposure to As through drinking water has pointed to increased toxic and carcinogenic risks among rural residents in Eastern Croatia (Ujević Bošnjak et al. 2012). However, there is no study to associate T2D and As exposure in Croatian population. According to the CroDiab registry (Croatian Institute of Public Health 2017; Ministry of Health of the Republic of Croatia 2015), T2D is the 8th leading cause of death in Croatia, while the total number of patients with T2D registered in 2015 was 260,092. Given the widespread exposure to As from drinking water worldwide and its adverse health effects, more information on the possible association between As exposure and T2D prevalence is required.

This preliminary study intended to fill that gap by investigating firstly, the effect of exposure to elevated levels of As (>10 μg/L in drinking water) on As levels in blood and urine. Then, the association of As levels in blood and urine and As metabolites in urine with biomarkers of T2D in Croatian population including patient groups (T2D or prediabetes) and healthy controls.

2. MATERIALS AND METHODS

2.1.Participants

This observational study recruited 202 adults urban participants: 86 from the city of Osijek in Eastern Croatia (area of 170 km^2, coordinates 45°33’18”N, 18°41’44”E), where As levels in

drinking water exceed the EU limit of 10 μg/L, and 116 from the city of Zagreb in Western Croatia (area of 640 km^2, coordinates 45°48'52"N, 15°58'41"), where As levels is below the EU limit. The participants were further divided into normoglycemic or healthy (H), those characterized with prediabetes (PreD), and the T2D group (D) according to the American Diabetes Association criteria (American Diabetes Association 2011). The study protocol was accepted and approved by the Ethics Committee of the Clinical Hospital Osijek, Osijek, Croatia, the Ethics Committee of the Merkur University Hospital, Zagreb, Croatia, and the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia. Informed consents were obtained from all participants before any other action.

General exclusion criteria were acute infection, renal insufficiency (eGFR < 30 mL/min/1.73 m^2), cardiovascular disease, and malignant diseases. Of the T2D participants, only those taking oral hypoglycemic drugs but no insulin were included in the study.

Sociodemographic, lifestyle and other general information were collected about all participants, including gender, age, education level, smoking, family history of diabetes, physical activity, dietary recall interviews covering the past 5 days (including seafood intake), daily intake of fluids, and origin of water used for drinking. Anthropometric data, which included height, weight, waist and hips circumference were also collected by trained nurses.

2.2. Blood and urine collection and analysis

Exposure was assessed by determining total As in blood and urine samples and by determining As (III), As (V), arsenobetaine (AsBet), DMA, and MMA in urine samples of each participant. All participants were clinically examined and tested for biomarkers relevant for T2D diagnosis and monitoring (Table 1). A single collection of one urine and one blood sample was carried out for each study participant.

Fasting blood samples were taken in the morning after at least 8-hour fast. Spot urine samples were collected before blood sampling in polypropylene tubes, frozen within 1 hour of collection, shipped on dry ice, and stored at -80 °C until As analysis. Fasting serum glucose, HbA1c, and urinary albumin and creatinine were determined in the laboratories of the Clinical Hospital Osijek and Merkur University Hospital, Zagreb. Urinary albumin was measured in spot urine samples with an immunoturbidimetric method, while creatinine was determined with a compensated alkaline picrate Jaffè assay on an automated analyzer (AU680, Beckman Coulter, Brea, USA). The albumin-to-creatinine ratio (ACR) was calculated by dividing urinary albumin (mg/L) with urinary creatinine (mmol/L), and the results expressed as mg/mmol.

Fasting serum insulin for the entire study population was assayed with a chemiluminiscent immunoassay traceable to the World Health Organization (WHO) 1st IRP 66/304 standard (Advia Centaur-XP, Siemens Siemens Healthcare GmbH, Erlangen, Germany). Fasting glucose was assayed with a UV spectrophotometric hexokinase method using dedicated reagents and automated analyzer with a limit of detection 0.04 mmol/L (AU680, Beckman Coulter, Brea, USA).

Estimated glomerular filtration rate (eGFR) was obtained from serum creatinine values measured with the IDMS-traceable compensated Jaffé method (KDIGO Board 2013) using the 4-variable CKD-EPI equation. In addition, the Homeostasis Model Assessment (HOMA) was applied to estimate steady state function of pancreatic β cells (HOMA%B) and insulin resistance (HOMA-IR) (Levy et al. 1998). HOMA-IR and HOMA%B were estimated from fasting glucose and insulin levels using HOMA calculator (https://www.dtu.ox.ac.uk/homacalculator/).

HbA1c was assessed in whole blood, anticoagulated with K_3EDTA, using an automated immunoturbidimetric method traceable to both NGSP- and IFCC-standards with a limit of

detection of 4.2% and 23 mmol/mol, respectively. The results are expressed in dual units (% and mmol/mol) according to traceability towards respective standards.

T2D status was established if the HbA1c level was above 6.5%, as defined by the diagnostic criteria of the American Diabetes Association (American Diabetes Association 2011). Participants with the HbA1c level from 5.6% to 6.4% were considered prediabetic, while participants with HbA1c below 5.6% were considered normoglycemic.

2.3.Determination of As levels and species

Total As and As species were determined using the method described by Lintschinger et al. (1998). Standard stock solutions for As (III) (100 mg As/L) and As (V) (100 mg As/L) were prepared by dissolving the appropriate amount of sodium metaarsenite (Merck (Sigma), 97%) and sodium arsenate heptahydrate (Merck (Sigma)) in water. Standards for MMA, DMA, and AsBet were kindly donated by the Service Central d'Analyse (CNRS, Vernaison, France) in the form of aqueous standard stock solutions, each containing 400 mg As/L. All stock solutions were stored in the dark at 4 °C. Working standard solutions of lower concentrations were prepared daily by dilution with water. All chemicals used for the preparation of mobile phases were of analytical reagent grade or higher purity. Mobile phases for HPLC separation of As species were prepared from a tetramethylammonium hydroxide (TMAH) stock solution (TAMA Chemicals, Osaka, Japan) and sodium carbonate (Merck, Darmstadt, Germany). The solutions were prepared by dissolving the compounds in ultrapure water obtained from a special cartridge deionization unit (Milli-Q Water Purification System). Diluted nitric acid (Merck, Suprapur®, purified by sub-boiling distillation) was used for dilution of urine samples (1:5) before determination of total As concentrations.

Total As was determined by inductively coupled plasma-sector field-mass spectrometry (ICP-sf-MS) (Element II, Thermo Scientific Bremen, Germany) operating in a high resolution

mode. Instrumental parameters were as follows: forward power of 1300 W, plasma gas at 15.0 L Ar/min, nebulizer gas at 0.80 L Ar/min (daily optimized), auxiliary gas at 0.80 L Ar/min, mass resolution of 10000, sample introduction by ESI-FAST system at 0.8 ml/min connected to a sea-spray nebulizer/cyclon spray chamber.

For As speciation we hyphenated ion exchange chromatography to ICP-sf-MS. As species were separated according to a slightly modified method of Lintschinger et al. (1998) using a HPLC system Beckman System Gold 127NM Solvent Module (Beckman Coulter Biomedical, Munich, Germany) equipped with 9725i PEEK injection valve from Rheodyne (Sigma-Aldrich, CT, USA) and a degasser Degassex™ Model D6-4400 (Phenomenex, Darmstadt, Germany). Chromatographic parameters were as follows: Thermo (Dionex) IonPac AG14 50 x 4 mm as pre-column, Thermo (Dionex) IonPac AS14 250 x 4 mm as column, mobile phase A consisting of 1 mmol/L TMAH and mobile phase B consisting of 10 mmol/L ammonium carbonate at pH 10.0. Flow rate was set to 1.0 mL/min, and the injection volume was 100 µL. The gradient program used a slower eluent change from eluent A to B compared to Davey et al. (2008) to ensure separation of DMA and As (III) and was set as follows: 0–4 min 0% B; 4–9 min 0-100% B; 9–16 min 100% B. After each run the column was purged with 5 mM EDTA solution for 10 minutes and then re-equilibrated with eluent A. Typical chromatograms are shown in Figure S1 in the Supporting Information (SI).

Isotope ^{75}As was detected with ICP-sf-MS by direct PEEK tube connection from column outlet to sea spray nebulizer. The ICP-sf-MS parameters were the same as for total As determination. The limit of quantification was 0.13 - 0.145 µg/L for As species and was uniformly set to 0.15 µg/L. AsBet, MMA, DMA, As (III), and As (V) were quantified by comparing As species peak areas with respective calibration curves. The obtained IEC-ICP-sf-MS data were processed and peak areas calculated with the PeakFit™ software v4.12 for Windows® (SeaSolve Software Inc., San Jose, CA, USA).

2.4.Statistical Analysis

All statistics were run on a SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Before data modelling, all variables with non-normal distribution of the residuals were log-transformed. Variables with a value below the limit of detection (LOD) were excluded from the analysis.

To estimate the correlation between As species and HOMA-IR, HOMA%B, fasting glucose, insulin level, HbA1c, creatinine, eGFR, and albuminuria we used linear regression analysis adjusted for potential confounding factors such as gender, education level, and age. For each regression equation, we estimated the β value, standard error, and 95% interval of confidence (CI). In addition, based on the t-distribution we presented the relative p-values.

Differences in fasting glucose, insulin levels, HbA1c, and albuminuria (dependent variables) between healthy, prediabetes, and T2D participants, as well as between Eastern and Western Croatian participants (independent variables) were analyzed with the multivariate analysis of variance (MANOVA). Type III Sum of Squares was used to test the health status and geographic origin and interactions between the two. The model was adjusted for the same confounding factors used for regression analysis. We calculated the least squares mean (LS-mean) due to unbalanced experiment.

Scheffé's test was applied for multiple comparisons. It provided detailed information about the differences between LS-means. These multiple comparisons for different interactions are presented by a diffogram, that is, a mean-mean scatter diagram (Adesemoye et al. 2008).

In addition, two general linear models (GLM) were used to study the relationship between urine As with the geographic origin and all the variables related to T2D and As exposure. The first model had age, gender, and education as confounding factors, and the second included all As forms and T2D variables.

The relationships between all variables were analyzed with principal component analysis (PCA) (Law and Jolliffe 1987) using a SIMCA version 13.03 software (Umetrics, Umeå, Sweden).

3. RESULTS

3.1.General demographic and health characteristics of study participants

General participant demographics are shown in Table 1. The participants are divided by geographical origin to Eastern (Osijek) and Western (Zagreb) Croatia and by health status to healthy participants with normal glucose tolerance (H), prediabetic participants (PreD), and T2D patients (D).

The loading plot presented in Figure 1 and the score plots presented in Figure 2 show all relationships between the variables obtained with PCA analysis. The main variance (represented by the first component, X-axis) is absorbed by the health status, and the second component (Y-axis) shows the differentiation between the Eastern and Western regions. As expected, the D group (T2D patients) is characterized by a high level of insulin fasting glucose, HOMA-IR, waist and hip circumference, and BMI (Figure 1). Most of these parameters highly correlated with the level of HbA1c and age. The MANOVA analysis (Table S1 in SI) revealed that the T2D patients differed from healthy and prediabetic participants in the following dependent variables: fasting glucose, HbA1c, and insulin. The correction for confounding factors revealed no significant differences in albumin. No significant differences were found between healthy and prediabetic participants. Geographical origin had no significant impact on insulin and fasting glucose. The interactions between geographical origin and health status are shown in Figure S2, and diffogram plots for each interaction in Figure S3 (both in SI). Interestingly, T2D patients from Eastern Croatia significantly differed in HbA1c values from all participants (healthy, prediabetic, and T2D)

from Western Croatia. They also significantly differed from western T2D patients in albumin levels (Figure S3 in the SI).

As for the second component (geographic origin), participants from Eastern Croatia are positioned in the upper part of both plots (Figures 1 and 2), which is related to higher levels of total As in urine. Higher urine As levels, in turn, may reflect higher exposure through municipal tap water as the major source of water intake (more than 85% of participants with a mean intake of > 1 L/day at enrolment) (Table 1). Reports for As levels in municipal drinking in Osijek (Eastern Croatia) range from 46 μg/L in 2000 to 31 μg/L in 2017 (Vodovod Osijek 2016). In Zagreb, As ranged between 0.50 and 7.68 μg/L in 2016 and 2017, respectively.

Table 3 shows blood and urine findings of total As, As (III), As (V), AsBet, MMA, and DMA. AsBet prevailed in the urine of study participants from Western Croatia, while inorganic As species prevailed in the urine of participants from the Eastern region (Table 3). Even though we did not evaluate exposure to organic As through diet (fish, shellfish, rice, and wine), it is quite safe to say that the consumption of seafood as the main source of AsBet is much higher in Western than Eastern Croatia, which could explain the findings. Values for As correlated with the glycemic status and were higher in T2D than PreD participants from both regions (Table 3). Regional clustering of As(V), MMA, and DMA in turn, confirms the expected high association between these As species and Eastern Croatia (Figures 1 and 2). This association is further analyzed below.

3.2. **Arsenic exposure and metabolism and their association with health status**

Table 4 shows the relationships of total As and other urine As forms with HbA1c, insulin, creatinine, eGFR, and albuminuria adjusted for age, education level, and gender). Total urine As positively correlated with HbA1c, while total blood As negatively correlated with urine

albumin. A positive correlation was also observed between HOMA-IR and MMA, and a negative correlation between AsBet and insulin.

The first general linear model confirmed that the participants from Eastern Croatia had significantly higher ($p<0.0001$) urine As levels when adjusted for age and gender as confounding factors (Table 5). However, the second, extended model, which included more independent variables, showed no significant difference between the regions when controlled for all the confounders, even though the estimation was still high (Table 5).

After a stepwise selection, we established that only the As variables (except for As(III)) and BMI significantly differed between the Eastern and Western regions. All the covariates positively correlated with urine As levels. However, there was no clear evidence of an association between geographic origin and exposure to As in relation to glycemic status with all variables included in the model (Table 5).

4. DISCUSSION

In this study, urinary As was higher in study participants from Eastern than Western Croatia, but organic (dietary) AsBet prevailed among Western participants, while inorganic As prevailed in the participants from the East, mostly in the form of As (V). Higher concentrations of As (V), MMA, and DMA in urine were associated with geographical origin (Table 5), but no correlation was found between these As forms and HbA1c or fasting glucose levels.

In line with earlier studies (Coronado et al. 2007; Faseke et al. 2015; Gribble et al. 2012; Navas-Ancien et al. 2008; Rhee et al. 2013), we found a positive association between total urinary As and HbA1c as biomarker of T2D, regardless of the region and glycemic status (Table 4). However, our general linear model analysis showed no clear evidence of an association between exposure to As through drinking water and T2D when all variables were

included in the model (Table 5). This may have to do with the fact that in addition to exposure levels, the toxicity of As depends on its metabolism, which is characterized by a series of methylation steps (Drobna et al. 2009). Inorganic As found in drinking water and its main metabolites in urine As (III), As (V), MMA, and DMA are considered highly toxic to human cells (James et al. 2013, Vega et al. 2001). Recent cross-sectional studies from Mexico and Bangladesh (Mendez et al. 2016, Nizam et al. 2013) and a prospective study from the United States (Kuo et al. 2015) demonstrated an increased risk of T2D in study participants with a metabolic profile characterized by lower MMA and higher DMA levels in urine. In our study, we did not find a significant association between MMA and T2D, but did between MMA and HOMA-IR biomarker. Before that, only a few epidemiologic studies evaluated the association between HOMA-IR and As exposure (Del Razo et al. 2011, Gribble et al. 2012, Lin et al. 2014, Park et al. 2016), but found none, even though animal studies pointed to the opposite (Fu et al. 2010, Palacios et al. 2012).

We also did not observe any significant association of HOMA-IR and HOMA%B with total As in urine and blood, unlike a US family study (Grau-Perez et al. 2017), which also reported inverse correlation between MMA and HOMA2-IR when either inorganic As or DMA were decreased. Instead, our regression analysis (Table 4) showed positive correlations between total urinary As and HbA1c and between MMA and HOMA-IR ($p = 0.01$ and $p = 0.02$, respectively).

Although some recent studies reported a positive correlation between albuminuria and total urinary As (Chen et al. 2011, Zheng et al. 2013), our findings pint to the opposite in the sense that the correlation was negative (Table 4) and do not support the hypothesis that higher exposure to inorganic As increases the risk of diabetic nephropathy as one of the T2D complications (Brownlee 2001). Robles-Osorio et al. (2012), in turn, did not find any association between urinary As and albuminuria but did find an association between total

317 urinary As and urinary excretion of αl-microglobulin as a marker of early tubular injury.
318 Obviously, more prospective research is needed to resolve whether As induces nephrotoxicity
319 in humans.
320

321 ## 5. Limitations of this study

322 In spite of all the interesting findings reported in this study, some limitations should be taken
323 into account. Most importantly, our study was limited by a relatively small sample size. In
324 addition, our population groups (Eastern and Western) were characterized by variations in
325 age, exposure time, diet and lifestyle that further limit the interpretation of our results.
326 Therefore, our study should be considered a pilot collecting with preliminary data for future,
327 larger, multicentric epidemiological studies in European populations. Our sample size was
328 also insufficient to evaluate dose–response relationships for each exposure and outcome in
329 detail. Furthermore, As levels in blood and urine (including urinary As species) were
330 measured at a single time point, which fails to give an insight into the variability in typical
331 exposure levels. Future studies evaluating As metabolism in relation to As exposure and
332 health status in larger study populations are needed.
333

334 ## 6. CONCLUSION

335 This is the first Croatian, but also European, study on levels of As species in urine of healthy
336 volunteers vs. diabetic patients chronically exposed to elevated As levels through drinking
337 water. Its experimental design relied on a high-quality protocol and laboratory methods. The
338 determination of blood and urinary concentrations of As and its metabolites allowed
339 correlation and association analysis with regard to biomarkers of T2D. As expected, we found
340 association between concentrations of As in drinking water with total As in urine. Positive

correlations were found between increased levels of As in urine and HbA1c, as well as between increased MMA in urine and HOMA-IR biomarker. Our study therefore provides important implications for future public health research in Europe. The role of As in the incidence of diabetes and diabetic complications (diabetic nephropathy in particular) should be clearly defined and the underlying pathophysiological mechanism(s) identified.

Conflicting interests

None to declare.

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References

Adesemoye TO, Torbert A, Kloepper HJ. 2008. Enhanced Plant Nutrient Use Efficiency with PGPR and AMF in an Integrated Nutrient Management System. **Can J Microbiol** 54(10):876-86, DOI: 10.1139/w08-081

American Diabetes Association. 2011. Diagnosis and classification of diabetes mellitus. **Diabetes Care** 34:S62-S69, DOI: 10.2337/dc11-S062

Bräuner EV, Nordsborg RB, Andersen ZJ, Tjønneland A, Loft S, Raaschou-Nielsen O. 2014. Long-term exposure to low-level arsenic in drinking water and diabetes incidence: a prospective study of the Diet, Cancer and Health cohort. **Environ Health Perspect** 122:1059–1065, DOI:10.1289/ehp.1408198

Brownlee M. 2001. Biochemistry and molecular cell biology of diabetic complications. **Nature** 414(6865):813–820, DOI: 10.1038/414813a

Chen Y, Parvez F, Liu M, Pesola GR, Gamble MV, Slavkovich V et al. 2011. Association between arsenic exposure from drinking water and proteinuria: results from the Health

371 Effects of Arsenic Longitudinal Study. **Int J Epidemiol** 40:828–835, DOI:
372 10.1093/ije/dyr022
373 Coronado-González JA, Del Razo LM, García-Vargas G, Sanmiguel-Salazar F, Escobedo-de
374 la Peña J. 2007. Inorganic arsenic exposure and type 2 diabetes mellitus in Mexico.
375 **Environ Res** 104:383-389, DOI:10.1016/j.envres.2007.03.004
376 Croatian Institute of Public Health. 2017. CroDiab registry. Available at:
377 https://www.hzjz.hr/en/division-of-epidemiology-and-prevention-of-chronic-non-
378 communicable-diseases/crodiab-registry/ Accessed May 31th 2018
379 Ćavar S, Klapec T, Grubešić RJ, Valek M. 2005. High exposure to arsenic from drinking
380 water at several localities in eastern Croatia. **Sci Tot Environ** 339:277–282,
381 DOI:10.1016/j.scitotenv.2004.12.013
382 Davey JC, Nomikos AP, Wungjiranirun M, Sherman JR, Ingram L, Batki C, Lariviere JP,
383 Hamilton JW. 2008. Arsenic as an endocrine disruptor: Arsenic disrupts retinoic acid
384 receptor–and thyroid hormone receptor–mediated gene regulation and thyroid
385 hormone–mediated amphibian tail metamorphosis. **Environ Health Persp**
386 116(2):165-172, DOI:10.1289/ehp.10131
387 De Marco MJ, SenGupta AK, Greenleaf JE. 2003. Arsenic removal using a
388 polymeric/inorganic hybrid sorbent. **Water Res** 37:164–176, DOI:10.1016/S0043-
389 1354(02)00238-5
390 Del Razo LM, García-Vargas GG, Valenzuela OL, Castellanos EH, Sánchez-Peña LC,
391 Currier JM et al. 2011. Exposure to arsenic in drinking water is associated with
392 increased prevalence of diabetes: a cross-sectional study in the Zimapán and Lagunera
393 regions in Mexico. **Environ Health** 10:72, DOI:10.1186/1476-069X-10-73
394 Drobna Z, Styblo M, Thomas DJ. 2009. An overview of arsenic metabolism and toxicity.
395 **Curr Protoc Toxicol** 42(431):4.31.1–4.31.6, DOI:10.1002/0471140856.tx0431s42
396 EFSA (European Food Safety Authority). 2009. EFSA Panel on Contaminants in the Food
397 Chain (CONTAM); scientific opinion on arsenic in food. EFSAJ 7(10):1351,
398 DOI:10.2903/j.Efsa.2009.1351.
399 Feseke SK, St-Laurent J, Anassour-Sidi E, Ayotte P, Bouchard M, Levallois P. 2015. Arsenic
400 exposure and type 2 diabetes: results from the 2007–2009 Canadian Health Measures
401 Survey [in French]. **Health Promot Chronic Dis Prev Can** 35(4):63–72,
402 DOI:10.24095/hpcdp.35.4.01.
403 Fu J, Woods CG, Yehuda-Shnaidman E, Zhang Q, Wong V, Collins S, et al. 2010. Low-level
404 arsenic impairs glucose-stimulated insulin secretion in pancreatic beta cells:
405 involvement of cellular adaptive response to oxidative stress. **Environ Health**
406 **Perspect** 118(6):864–870, DOI:10.1289/ehp.0901608.
407 Grau-Pérez M, Kuo CC, Gribble MO, Balakrishnan P, Jones Spratlen M, Vaidya D et al.
408 2017. Association of low-moderate arsenic exposure and arsenic metabolism with
409 incident diabetes and insulin resistance in the Strong Heart Family study. **Environ**
410 **Health Persp** 125(12):127004, DOI:10.1289/EHP2566
411 Gribble MO, Howard BV, Umans JG, Shara NM, Francesconi KA, Goessler W, et al. 2012.
412 Arsenic exposure, diabetes prevalence, and diabetes control in the Strong Heart Study.
413 **Am J Epidemiol** 176(10):865–874, DOI:10.1093/aje/kws153.

Habuda-Stanić M, Kuleš M, Kalajdžić B, Romić Ž. 2007. Quality of groundwater in eastern Croatia. The problem of arsenic pollution. **Desalination** 210:157–162, DOI:10.1016/j.desal.2006.05.040

https://www.dtu.ox.ac.uk/homacalculator/ Accessed July 9 th 2018

James KA, Marshall JA, Hokanson JE, Meliker JR, Zerbe GO, Byers TE. 2013. A case-cohort study examining lifetime exposure to inorganic arsenic in drinking water and diabetes mellitus. **Environ Res** 123:33-38, DOI:10.1016/j.envres.2013.02.005

Jensen GE, Hansen ML. 1998. Occupational arsenic exposure and glycosylated haemoglobin. **Analyst** 123(1):77-80, DOI:10.1039/A705699K

Jovanović D, Rašić-Milutinović Z, Paunović K, Jakovljević B, Plavšić S, Milošević J. 2013. Low levels of arsenic in drinking water and type 2 diabetes in Middle Banat region, Serbia. **Int J Hyg Environ health** 216:50-55, DOI:10.1016/j.ijheh.2012.01.001

Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. 2013. KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. **Kidney Int Suppl** 3:1–150

Kuo CC, Howard BV, Umans JG, Gribble MO, Best LG, Francesconi KA, et al. 2015. Arsenic exposure, arsenic metabolism, and incident diabetes in the Strong Heart Study. **Diabetes Care** 38(4):620–627, DOI:10.2337/dc14-1641.

Lampron-Goulet É, Gagnon F, Langlois MF. 2017. Association between consumption of private well water contaminated by low levels of arsenic and dysglycemia in a rural region of Quebec, Canada. **Environ Res** 159:232-238, DOI:10.1016/j.envres.2017.07.049

Law J, Jolliffe IT. 1987. Principal Component Analysis. **The Statistician** 36, 37–52.

Levy JC, Matthews DR, Hermans MP. 1998. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. **Diabetes Care** 21(12):2191-2192, DOI: 10.2337/diacare.21.12.2191

Li Z, Piao F, Liu S, Wang Y, Qu S. 2009. Subchronic exposure to arsenic trioxide-induced oxidative DNA damage in kidney tissue of mice. **Exp Toxicol Pathol** 62(5):543-547, DOI:10.1016/j.etp.2009.07.003

Lin HC, Huang YK, Shiue HS, Chen LS, Choy CS, Huang SR, et al. 2014. Arsenic methylation capacity and obesity are associated with insulin resistance in obese children and adolescents. **Food Chem Toxicol** 74:60–67, DOI:10.1016/j.fct.2014.08.018.

Lintschinger J, Schramel P, Hatalak-Rauscher A, Wendler I, Michalke B. 1998. A new method for the analysis of arsenic species in urine by using HPLC-ICP-MS. **Fresen J Anal Chem** 362(3):313-318, DOI:10.1007/s002160051080

Makris KC, Christophi CA, Paisi M, Ettinger AS. 2012. A preliminary assessment of low level arsenic exposure and diabetes mellitus in Cyprus. **BMC Public Health** 12:334, DOI:10.1186/1471-2458-12-334

Mendez MA, González-Horta C, Sánchez-Ramírez B, Ballinas-Casarrubias L, Cerón RH, Morales DV, et al. 2016. Chronic exposure to arsenic and markers of cardiometabolic risk: a cross sectional study in Chihuahua, Mexico. **Environ Health Perspect** 124(1):104–111, DOI:10.1289/ehp.1408742.

Ministry of Health of the Republic of Croatia. 2015. National diabetes programme 2015 – 2018. Available at: https://www.hzjz.hr/wp-content/uploads/2013/11/National-Diabetes-Programme-2015_2020.pdf Accessed: May 31th 2018

Naujokas MF, Anderson B, Ahsan H, Aposhian HV, Graziano JH, Thompson C, Suk WA. 2013. The broad scope of health effects from chronic arsenic exposure: update on a worldwide public health problem. **Environ Health Perspec** 121:295-302, DOI:10.1289/ehp.1205875

Navas-Acién A, Silbergeld EK, Pastor-Barriuso R, Guallar E. 2008. Arsenic exposure and prevalence of type 2 diabetes in US adults. **J Am Med Assoc** 300(7):814–822, DOI:10.1001/jama.300.7.814

Nizam S, Kato M, Yatsuya H, Khalequzzaman M, Ohnuma S, Naito H, et al. 2013. Differences in urinary arsenic metabolites between diabetic and non-diabetic subjects in Bangladesh. **Int J Environ Res Public Health** 10(3):1006–1019, DOI:10.3390/ijerph10031006.

Pan WC, Seow WJ, Kile ML, Hoffman EB, Quamruzzaman Q, Rahman M, et al. 2013. Association of low to moderate levels of arsenic exposure with risk of type 2 diabetes in Bangladesh. **Am J Epidemiol** 178(10):1563–1570, DOI:10.1093/aje/kwt195.

Palacios J, Roman D, Cifuentes F. 2012. Exposure to low level of arsenic and lead in drinking water from Antofagasta City induces gender differences in glucose homeostasis in rats. **Biol Trace Elem Res** 148:224–231, DOI:10.1007/s12011-012-9355-3.

Park SK, Peng Q, Bielak LF, Silver KD, Peyser PA, Mitchell BD. 2016. Arsenic exposure is associated with diminished insulin sensitivity in non-diabetic Amish adults. **Diabetes Metab Res Rev** 32(6):565–571, DOI:10.1002/dmrr.2769.

Pravilnik o zdravstvenoj ispravnosti vode za piće [Drinking water regulation] (in croatian). 2008. **Offical Gazette** No.47

Rhee SY, Hwang YC, Woo JT, Chin SO, Chon S, Kim YS. 2013. Arsenic exposure and prevalence of diabetes mellitus in Korean adults. **J Korean Med Sci** 28:861-868, DOI:10.3346/jkms.2013.28.6.861

Robles-Osorio ML, Pérez-Maldonado IN, Martín del Campo D, Montero-Perea D, Avilés-Romo I, Sabath-Silva E, Sabath E. 2012. Urinary arsenic levels and risk of renal injury in a cross-sectional study in open population. **Rev Invest Clin** 64(6 Pt 2):609-614, PMID:23593778

Romić Ž, Habuda-Stanić M, Kalajdžić B, Kuleš M. 2011. Arsenic distribution, concentration and speciation in groundwater of the Osijek area, eastern Croatia **App Geochem** 26:37–44, DOI:10.1016/j.apgeochem.2010.10.013

Ujević Bošnjak M, Duić Ž, Casiot C, Sipos L, Santo V, Dadić Ž, Halamić J. 2010. Occurrence and geochemistry of arsenic in the groundwater of Eastern Croatia. **App Geochem** 25(7):1017–1029, DOI:10.1016/j.apgeochem.2010.04.008

Ujević Bošnjak M, Capak K, Jazbec A, Casiot C, Sipos L, Poljak V, Dadić Ž. 2012. Hydrochemical characterization of arsenic contaminated alluvial aquifers in Eastern Croatia using multivariate statistical techniques and arsenic risk assessment. **Sci Tot Environ** 420:100–110, DOI:10.1016/j.scitotenv.2012.01.021

Vega L, Styblo M, Patterson R, Cullen W, Wang C, Germolec D. 2001. Differential effects of trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in

normal human epidermal keratinocytes. **Toxicol Appl Pharmacol** 172:225-232, DOI:10.1006/taap.2001.9152

Vodovod Osijek 2016, https://vodovod.com/wp-content/uploads/2015/03/GODI%C5%A0NJE-IZVJE%C5%A0%C4%86E-O-KVALITETI-VODE-ZA-LJUDSKU-POTRO%C5%A0NJU-U-2016.-GODINI.pdf Accessed July 9 th 2018

Vodovod Osijek 2017, http://vodovod.com/wp-content/uploads/2015/03/GODI%C5%A0NJE-IZVJE%C5%A0%C4%86E-O-KVALITETI-VODE-ZA-LJUDSKU-POTRO%C5%A0NJU-U-2017.-GODINI.pdf Accessed July 9 th 2018

Wang W, Xie Z, Lin Y, Zhang D. 2014. Association of inorganic arsenic exposure with type 2 diabetes mellitus: a meta-analysis. **J Epidemiol Community Health** 68:176-184. PMID: 24133074 DOI: 10.1136/jech-2013-203114

Zheng LY, Umans JG, Tellez-Plaza M, Yeh F, Francesconi KA, Goessler W, et al. 2013. Urine arsenic and prevalent albuminuria: evidence from a population-based study. **Am J Kidney Dis** 61(3):385-394, DOI:10.1053/j.ajkd.2012.09.011

Table 1. Distribution of demographic and health characteristics by geographical origin (Eastern vs. Western Croatia) and health status

Characteristics		East			West		
		Healthy, SD or *n* (%)	Prediabetes, SD or n (%)	T2D, SD or n (%)	Healthy, SD or n (%)	Prediabetes, SD or n (%)	T2D, SD or n (%)
Age		49 ± 14	64 ± 7	64 ± 10	45 ± 11	57 ± 6	57 ± 7
Man		10 (5.2)	3 (1.6)	18 (9.3)	13 (6.7)	14 (7.3)	30 (15.5)
Women		6 (3.1)	6 (3.1)	37 (19.2)	13 (6.7)	13 (6.7)	30 (15.5)
BMI (kg/m2)		29 ± 5	32 ± 5	32 ± 7	25 ± 4	31 ± 9	32 ± 5
Education[a]	High school or less	12 (6.4)	9 (4.8)	32 (17.1)	11 (5.9)	21 (11.2)	55 (29.4)
	College or higher	4 (2.1)	-	9 (4.8)	14 (7.5)	6 (3.2)	14 (7.5)
Smoking status[a]	Never	9 (4.8)	6 (3.2)	40 (21.3)	4 (2.1)	2 (1.1)	45 (23.4)
	Former	1 (0.5)	-	1 (0.5)	-	7 (3.7)	11 (5.9)
	Current	6 (3.2)	3 (1.6)	11 (5.9)	21 (11.7)	18 (9.6)	3 (1.6)
Daily intake of fluids[a]	< 1L	1 (0.5)	1 (0.5)	6 (3.2)	4 (2.1)	6 (3.2)	7 (3.7)
	> 1L	14 (7.5)	8 (4.3)	44 (23.4)	22 (11.7)	21 (11.2)	54 (28.7)
Origin of water used for drinking[a]	Municipal	15 (7.9)	9 (4.7)	47 (24.6)	24 (12.6)	22 (11.5)	51 (26.7)
	Well	-	-	3 (1.6)	3 (1.6)	4 (2.1)	7 (3.7)
	Bottled	-	-	1 (0.5)	1 (0.5)	1 (0.5)	3 (1.6)
Physical activity[a]	Regularly	8 (4.3)	5 (2.7)	31 (16.6)	16 (8.6)	23 (12.3)	41 (21.4)
	Occasionally	4 (2.1)	3 (1.6)	13 (6.9)	10 (5.3)	4 (2.1)	19 (9.6)
	None	3 (1.6)	1 (0.5)	6 (3.2)	-	-	2 (1.1)

Means ± standard deviation; T2D, Diabetes mellitus type 2. [a]Characteristics in the table do not sum to total; 193 answers to some questions were missing from the questionnaire

Table 2. Values of biomarkers of type 2 diabetes mellitus (T2D) among study participants by geographical origin (West vs. East Croatia) and health status (healthy, prediabetes and T2D) Following biomarkers of T2D are presented: fasting glucose, insulin, "Homeostasis Model Assessment of Insulin Resistance" (HOMA-IR), "Homeostasis Model Assessment of β-cell function" (HOMA%B), hemoglobin A1c (HbA1c), albumin/creatinine ratio (Alb/Cre), and estimated glomerular filtration rate (eGFR).

Biomarkers of T2D	East			West		
	Healthy	Prediabetes	T2D	Healthy	Prediabetes	T2D
Fasting glucose (mmol/L)	5.5±2.6 (3.4-5.8)	5.3±1.4 (3.5-8.3)	8.5±3.3 (3.7-18.4)	5.7±1.0 (3.8-9.1)	7.3±1.7 (4.7-13.3)	9.9±3.0 (5.9-18.8)
Insulin (pmol / L)	95.1±57.3 (28.7-34.3)	44.0±18.2 (15.8-71.3)	164.0±141.1 (15.1-88.1)	94.0±19.5 (9.7-24.6)	119.5±97.6 (41.3-21.2)	153.5±156.8 (17.4-99)
HOMA - IR	2.0±1.2 (0.7-4.8)	1.0±0.4 (0.4-1.6)	3.7±2.6 (0.5-10.1)	2.0±2.4 (0.2-12.2)	2.8±2.2 (1.0-11.6)	4.5±4.2 (0.4-17.2)
HOMA %B	161.9±94.9 (23.3-334.6)	95.0±47.0 (25.2-176.1)	113.8±107.4 (7.1-594.4)	108.1±59.8 (33.4-340.2)	94.6±49.1 (32.2-224.0)	64.5±42.6 (10.6-10.1)
HbA1c (%)	5.2±0.4 (4.9-5.6)	6.0±0.3 (5.6-6.4)	9.3±1.4 (7.1-13.9)	4.9±0.5 (4.2-5.5)	6.1±0.3 (5.6-6.5)	7.9±1.3 (6.6-11.9)
HbA1c (mmol/mol)	32.9±4.7 (30-38)	41.9±3.1 (38-46)	9.3±1.4 (54-128)	30.3±4.6 (22-36)	42.5±3.2 (37-47)	61.7±14.4 (47-106)
Alb/ Cre (mg/mmol)	1.7±2.8 (0.01-5.1)	0.3±0.3 (0.02-0.8)	6.0±24.3 (0.01-56.9)	1.5±1.5 (0.3-6.1)	1.4±2.5 (0.3-13.5)	15.9±48.4 (0.2-274.1)
eGFR (mL/min/1.73 m^2)	91.8±33.2 (29.7-27.5)	84.0±17.7 (47.8-104.7)	84.1±26.2 (37-143.1)	98.7±11.5 (71.9-19.1)	96.9±15.0 (68-123.3)	92.3±16.8 (60-153.3)

Parameters are given as means ± standard deviation, while ranges are given in parenthesis

Table 3. Descriptive information on As exposure (levels of As in blood and urine) and As metabolism (levels of arsenobetaine (AsBet), dimethylarsinate (DMA), As(III), monomethylarsonate (MMA) and As(V) in urine) by geographical origin (West vs. East Croatia) and health status (healthy, prediabetes and type 2 diabetes mellitus (T2D)).

As level (µg/L)	East			West		
	Healthy	Prediabetes	T2D	Healthy	Prediabetes	T2D
Total As in blood	0.74±0.43 (0.09-1.86)	0.55±0.43 (0.14-1.40)	0.58±0.39 (0.14-1.64)	1.42±1.02 (0.11-5.38)	0.69±1.07 (0.13-5.10)	1.05±3.49 (0.06-25.80)
Total urine As	20.5±12.1 (1.3-36.6)	14.2±17.0 (1.3-55.7)	23.4±19.0 (1.2-82.3)	8.6±10.9 (1.4-35.7)	11.5±39.0 (0.60-206)	12.8±48.3 (0.5-361)
AsBet in urine	5.2±5.2 (0.1-16.3)	5.8±10.5 (0.1-29.4)	5.2±7.7 (0.2-38.3)	9.1±6.6 (1.9-20.2)	27.7±61.9 (1.4-167.9)	32.6±82.4 (0.4-334.1)
DMA in urine	1.7±1.3 (0.2-3.8)	1.1±1.2 (0.2-3.5)	2.2±2.7 (0.2-14.3)	1.6±1.2 (0.3-4.1)	0.5±0.3 (0.2-0.8)	2.9±6.3 (0.2-19.7)
As (III) in urine	0.8 ± 0.5 (0.2-1.6)	0.8 ± 0.9 (0.3-2.2)	1.2±1.8 (0.2-10.1)	3.4±3.7 (0.6-13.0)	2.4±1.6 (0.7-4.5)	2.7±2.1 (0.6-8.1)
MMA in urine	2.4±2.1 (0.3-9.1)	1.7±2.9 (0.2-8.8)	3.0±3.1 (0.2-14.9)	2.9±2.4 (0.29-7.16)	1.7±1.6 (0.5-4.7)	1.8±1.3 (0.2-4.0)
As (V) in urine	3.7±2.4 (0.2-7.7)	2.2±1.4 (0.9-5.0)	4.4±4.0 (0.4-23.2)	1.6±2.4 (0.1-6.3)	0.7±0.70 (0.2-1.5)	2.3±4.0 (0.1-14.1)

Parameters are given as means ± standard deviation, while ranges are given in parenthesis.

Table 4. Significant parameter correlations obtained with regression analysis

Arsenic exposure	Biomarker	Beta	SE	95%CI	p-value
Total As in urine	HbA1c	0.24	0.10	(0.05, 0.43)	0.01
Total As in blood	Albumin	-0.37	0.15	(-0.67, -0.07)	0.01
MMA	HOMA-IR	0.15	0.11	(-0.02, 0.33)	0.02
AsBet	Insulin	-0.01	0.01	(-0.02, -0.001)	0.04

Beta – relative β-values; SE – standard error; CI – 95% confidence interval

Table 5. Results of the general linear models. The first includes age and gender as confounding factors. The second provides results after stepwise selection of the fully adjusted model and includes arsenic metabolites, diabetic variables, and the biological variables are all included. Only significant values are shown

Model	GLM parameters	Estimate	SE	Pr > |t|
First model	Geographical origin (East)	14.59	2.32	<.0001
	Age	0.06	0.11	0.58
	Gender (Female)	-1.48	2.20	0.50
Second model	Geographical origin (East)	2.48	2.62	0.35
	AsBet	0.47	0.13	0.0005
	DMA	2.67	0.38	<.0001
	As (V)	1.18	0.34	0.001
	MMA	3.52	0.45	<.0001
	BMI	0.36	0.15	0.018

SE – standard error (SE)

FIGURE CAPTIONS

Figure 1. PCA loadings plot. Bold characters designate variables related to arsenic exposure, health status, and geographic origin.

Figure 2. PCA score plot. Participants from Eastern Croatia are clustered in the upper section. Diabetic patients are clustered on the right section, whereas healthy and prediabetes participant on the left. The total variance absorbed by the two components is 20%.

Figure 1

Click here to download high resolution image

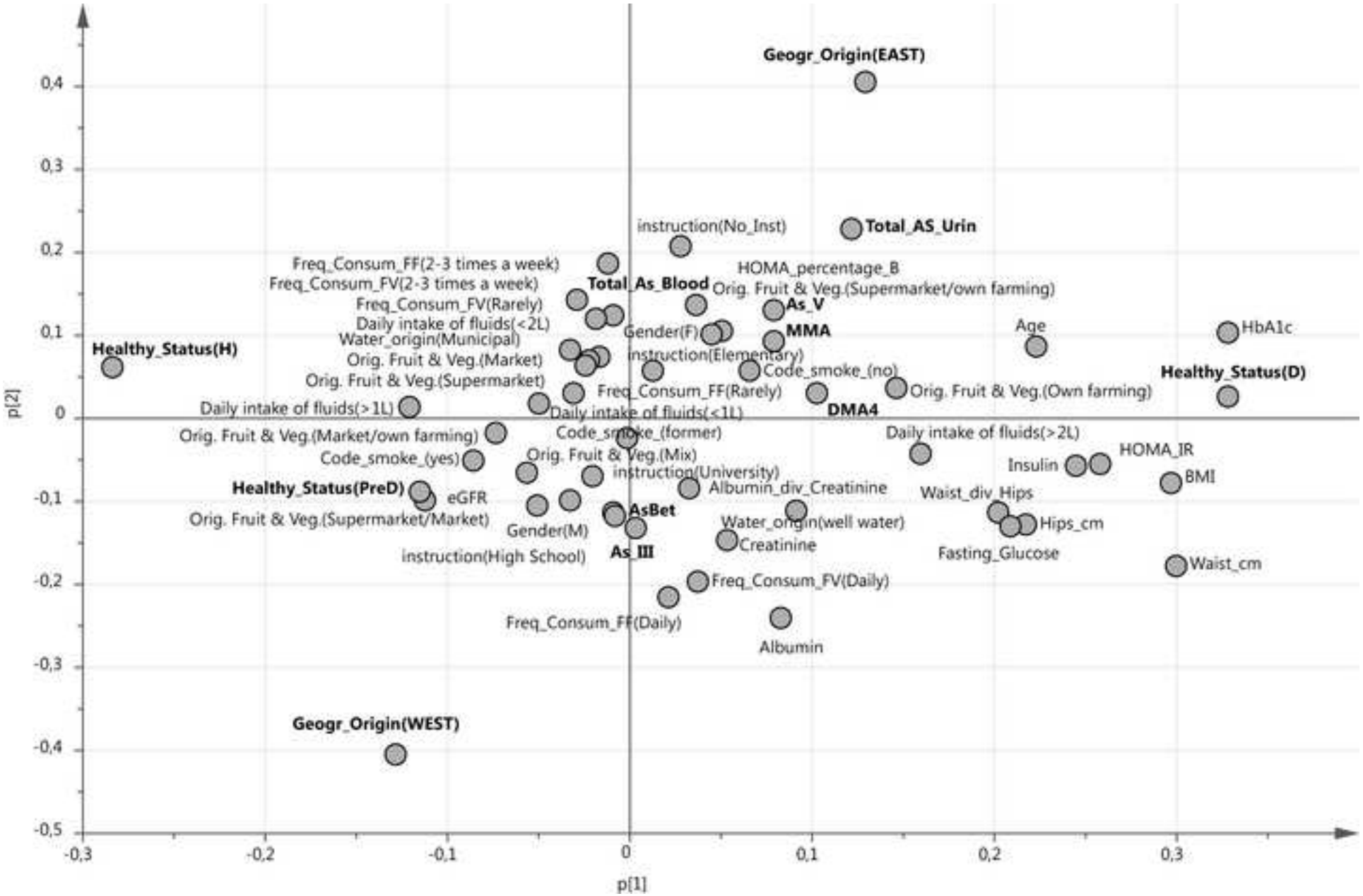
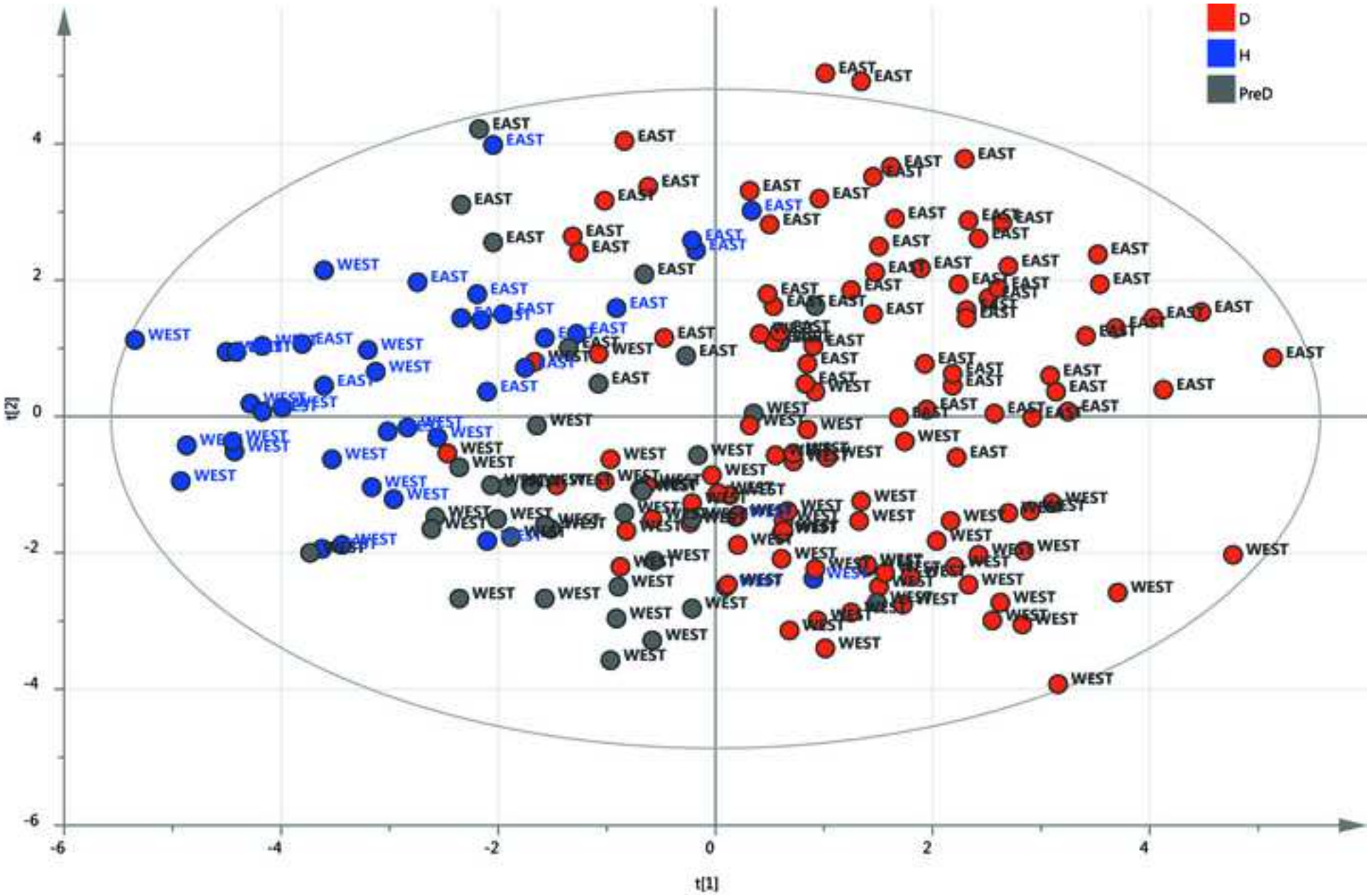


Figure 2

Click here to download high resolution image



Supplementary material for on-line publication only

Click here to download Supplementary material for on-line publication only: Supporting Information CroDiAs_REVISED.docx

***conflict of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT author statement:

Marianna Lucio: Data curation, Methodology, Visualization, Validation, Writing - Original Draft; Writing – review & editing. **Rinea Barbir**: Investigation, Formal analysis; Writing - Original Draft; Writing – review & editing. **Marijana Vučić Lovrenčić**: Conceptualization, Methodology, Investigation, Funding acquisition, Writing - Review & Editing. **Silvija Canecki Varžić**: Investigation, Formal analysis. **Spomenka Ljubić**: Investigation, Supervision, Resources. **Lea Smirčić Duvnjak:** Investigation, Supervision; **Vatroslav Šerić**: Methodology, Resources, Funding acquisition. **Mirta Milić**: Formal analysis; Investigation; Writing – review & editing. **Blanka Tariba Lovaković**: Formal analysis; Visualization; Writing - Original Draft; Writing – review & editing. **Adela Krivohlavek**: Resources, Writing – review & editing. **Ivana Vinković Vrček**: Conceptualization, Methodology, Supervision, Writing - Original Draft, Writing - Review & Editing, Project administration. **Bernhard Michalke**: Conceptualization, Methodology, Formal analysis; Supervision, Writing - Original Draft, Writing - Review & Editing, Funding acquisition, Project administration.