

# Subclinical Hypothyroidism in Families Due to the Chronic Consumption of Nitrate-contaminated Water in Rural Areas of Durango, Mexico.

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## Research

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# Abstract

**Background:** Nitrate is a water pollutant widely disseminated and has been linked to health disorders, including thyroid disorders as subclinical hypothyroidism.

**Methods:** A familial, observational, descriptive, and cross-sectional study was conducted on individuals in rural areas of Durango, Mexico. The sample comprised 102 subjects forming part of 26 families' resident in the abovementioned areas. All those fulfilling the inclusion criteria were tested for exposure biomarkers (plasma and urinary nitrite), an effect biomarker (percentage of methemoglobin), and thyroid function (thyroid profile). The genotypes corresponding to the single nucleotide polymorphisms (SNPs) rs965513 and rs1867277 of the *FOXE1* gene were also determined. The presence of anti-thyroid peroxidase antibody (TPO-Ab) was determined in those subjects who were found to have subclinical hypothyroidism. The variables of interest comprised the subject's age, sex, body mass index (BMI), and thyroid profile, as well as the exposure and effect biomarkers, and the levels of nitrate exposure via drinking water. Pearson's correlation, principal component analysis (PCA), heatmap and cluster analysis as well as post hoc Kruskal-Wallis and chi-squared were performed.

**Results:** 102 individuals were analyzed, 45% presented subclinical hypothyroidism, a negative correlation was observed between methemoglobin and total T3 ( $r = -0.43$ ,  $r^2 = 18.49$ ,  $p = 0.001$ ) and free T3 levels ( $r = -0.34$ ,  $r^2 = 11.56$ ,  $p = 0.001$ ); also between plasma nitrite and free T4 ( $r = -0.20$ ,  $r^2 = 4$ ,  $p = 0.045$ ) and total T3 ( $r = 0.23$ ,  $r^2 = 5.29$ ,  $p = 0.022$ ). Only 15.7% had positive antithyroid ab-TPO, while the polymorphic genotype (AA) represent only 3% (rs965513) and 4% (rs1867277) among subclinical hypothyroidism.

**Conclusions:** The subclinical hypothyroidism in the population studied is mainly due to nitrate contamination in drinking water.

## Background

The main source of potable water, groundwater is a stable and reliable resource that meets the demands of human consumption (Carrard et al. 2019) and a wide range of processes involved in the development and industrialization of urban and rural areas (Velis et al. 2017). However, in recent years, the contamination of these groundwater reserves has increased due to the increased levels of nitrogenated compounds, specifically nitrate (Shukla & Saxena 2018), resulting from their lixiviation, which is mainly caused by anthropogenic activities and soil use, such as the following: intensive agriculture (Serio et al. 2018); intensive livestock farming (Sahoo et al. 2016); the use of nitrogenated chemical fertilizers (Yu et al. 2020); the use of animal manure as an organic fertilizer (Galindo et al. 2019); the production and disposal of urban wastewater (Shalev et al. 2015); and, the disposal of septic waste (Wang et al. 2017). Said contamination is also affected by the following specific characteristics of the area: geological characteristics (Wongsanit et al. 2015); pluvial fluctuations (Kawagoshi et al. 2019); interaction with surface water (Lasagna et al. 2016a); changes in phreatic levels (Rhymes et al. 2016); and, the presence of biological processes such as denitrification (Lasagna et al. 2016b). The foregoing result in levels

exceeding the permissible limits in water for human consumption, as established by the World Health Organization (WHO), of 50 mg/l nitrate ion, the equivalent of 11.3 mg/l N-NO<sub>3</sub><sup>-</sup> (nitrate nitrogen) (WHO 2017).

Nitrate is a nitrogenated compound with a wide natural distribution (Takai 2019) via plants, soil, air, and water (Gassara et al. 2016). Notable among its characteristics are that it is colorless, odorless, tasteless, and highly soluble, making it difficult to detect in water used for human consumption (Almasi et al. 2016). As a result of the action of nitrifying algae and bacteria, nitrite and ammonia are easily converted via oxidation into nitrate, which is the most abundant stable nitrogenated form (Elisante & Muzuka 2016). Nitrogen is a component of many biomolecules, for which reason it can also be obtained via an endogenous pathway that metabolizes nitric oxide via L-arginine-nitric oxide synthase. In the exogenous pathway, nitrogen is obtained via the diet (Lundberg & Weitzberg 2018), from the consumption of vegetables, green leafy vegetables, processed foods containing nitrogen as an additive, and water contaminated with high nitrate concentrations. Once ingested, nitrogen is absorbed via the gastrointestinal mucosa and the proximal duodenum, reaching a bioavailability of over 90% (Harper et al. 2017). It is then disseminated around the entire body via systemic circulation, with approximately 25% absorbed via salivary gland uptake and then transported to the stomach by means of the enterosalivary circulation. Finally, 65% of the nitrate ingested is eliminated in urine in the form of nitrate, ammonium, or urea (Lundberg and Weitzberg 2017).

The exogenous nitrate-nitrite-NO pathway plays an important role due to its precursor action in the formation of nitrite and, in turn, of the genotoxic N-nitroso compounds present in endogenous nitrosation (Schullehner et al. 2018). Moreover, the ingestion of high nitrate concentrations in water used for human consumption has been found to be related to the following: the appearance of methemoglobinemia (Johnson 2019) with alterations in biochemical parameters (Bahadoran et al. 2015) and metabolic functions (Piccoli 2015); the development of cancers, such as bladder (Espejo et al. 2016), colorectal (Jones et al. 2016), gastric (Song et al. 2015), and thyroid (Ward et al. 2010) cancer; and, the development of thyroid function disorders (Gatseva & Argirova 2008), such as goiter (Gatseva & Argirova 2008 b), thyroid hypertrophy (Van Maanen 1994; Tajtakova 2006), and subclinical hypothyroidism (Aschebrook-Kilfoy 2012).

Further to its well-established carcinogenic action (Cantwell & Elliott 2017), nitrate has been suggested as a thyroid disruptor (Poulsen et al. 2017), as it is one of the major inhibitors of the sodium-iodide symporter channel. Acting competitively in the uptake of iodine by the thyroid glands, nitrate alters the hypothalamic-pituitary-thyroid axis, thus conditioning the appearance of hypothyroidism (Yilmaz et al. 2019). The presence of this compound in the thyroid follicle has been associated with changes in the gene expression of sodium-iodide symporter, thyroid peroxidase, thyroglobulin, and the *FOXO1* transcription factor (Montesinos et al. 2016), which are key elements in the biosynthesis of thyroid hormones.

Subclinical hypothyroidism (SCH) is characterized by thyroid stimulating hormone (TSH) levels that exceed the normal parameters for thyroid hormone levels in both total and free T4 tests (Hennessey & Espailat 2015). On a global level, the main cause of SCH is a low level of iodine ingestion (Zimmermann 2019), while auto-immune disorders are the second most common cause (Ragusa 2019), followed by genetic factors (Persani 2018) and endocrine disruptors (Oliveira et al. 2019). The global prevalence of SCH is 10% (Biondi et al. 2019), while in Mexico this is 8% (Bruneel et al. 2016).

Some rural areas of the state of Durango are characterized by intensive agricultural practices (Cerutti 2008), comprising one of the main producing regions of dairy and beef cattle and one of the largest milk basins in Mexico (González et al. 2018). These practices cause serious deterioration and contamination of the region's groundwater reserves due to the high nitrate levels, which are mainly attributed to agriculture and livestock farming. Across different rural locations in the municipality of Lerdo, Durango, studies have been conducted to ascertain the extent of the levels of nitrate in water used for human consumption. A high nitrate level in potable water is considered to be 11 mg/l nitrate nitrogen ( $\text{N-NO}_3^-$ ), as established in the 2019 amendment to the corresponding Official Mexican Standard, NOM-127-SSA-1994 (SSA 2019). Moreover, impacts on the health of the region's inhabitants have been reported, such as high levels of methemoglobin (MetHb) (Calleros et al. 2012), effects on sperm quality (Calleros et al. 2012b), and the presence of Heinz bodies (Calleros et al. 2018).

A study undertaken on women living in this area found alterations to their metabolic parameters and thyroid hormone profiles, as well as genotoxic damage and an increased incidence of SCH (Gandarilla et al., in review). Furthermore, inhabitants report more than one member of their family experiencing thyroid hormone alterations, as detected in the region's primary care health centers. In light of the foregoing, the present study sought to analyze the cases identified in the abovementioned study and broaden the research to the majority of the family members of the index case in order to identify the presence of more cases in the family. Moreover, the study aimed to identify the environmental, genetic, and immunological factors that are present in these populations and may explain the increased incidence of SCH.

## Methods

### 2.1. Study population

A familial, observational, descriptive, and cross-sectional study was conducted on individuals previously identified with thyroid dysfunction and residing in rural areas of the municipality of Lerdo, Durango. The study population comprised families resident in properties that form the *villa* (village) Juan E. García, the *ejido* (communally-held indigenous land) San Jacinto, the *ejido 21 de marzo*, the *villa* La Loma, and the *villa* Nazareno. The sample comprised 102 subjects forming part of 26 families resident in the abovementioned communities. The inclusion criteria were the following: voluntary participation; signed informed consent; any sex; any age; more than a year's residence in the locations of interest; consumption of water from the municipal network; and, not receiving medication with either anti-thyroid effects or compounds containing nitrate salts. All those fulfilling the inclusion criteria were tested for exposure

biomarkers (plasma and urinary nitrite), an effect biomarker (percentage of methemoglobin), and thyroid function (thyroid profile). The genotypes corresponding to the single nucleotide polymorphisms (SNPs) rs965513 and rs1867277 of the *FOXE1* gene were also determined. The presence of anti-thyroid peroxidase antibody (TPO-Ab) was determined in those subjects who were found to have SCH. The variables of interest comprised the subject's age, sex, body mass index (BMI), and thyroid profile, as well as the exposure and effect biomarkers, and the levels of nitrate exposure via drinking water.

## 2.2. Biological sample collection

Study participants were asked to fast for eight hours prior to the collection of a 10 ml peripheral blood sample and a 10 ml sample of the first urine void in the morning, with both samples collected in sterile containers. The blood sample collection was conducted using BD Vacutainer® tubes containing 6 ml coagulation activator for the recovery of the serum to be used for establishing both, the thyroid profile and TPO-Ab levels. Tubes containing 4 ml ethylenediamine tetraacetic acid (EDTA) were also used to measure plasma nitrite and methemoglobin levels and for the purification of genomic DNA. Once obtained, the samples were kept at -70°C until processing.

## 2.3. Nitrate exposure via drinking water

Once the nitrate levels in the water for human consumption had been established, the study locations were then stratified according to their exposure level. A low level was classified as  $4.7 \pm 3.3$  mg/l (*ejido 21 de marzo*), while a medium level was classified as  $32.1 \pm 3.7$  mg/l (*ejido San Jacinto and Juan E. García*) and a high level as  $56.9 \pm 14.7$  mg/l (*La Loma and Nazareno*).

## 2.4. Measurement of methemoglobin percentage

The percentage of MetHb was established via the spectrophotometry method reported by Sakata et al. (1982), using 100 µl of the blood collected in the EDTA tubes and following the manufacturer's instructions (FAR Diagnostics Verona, Italy) for measurements conducted via four readings at 630 nm (HACH-DR5000). The first reading (D1) is a hemolyzed preparation and the second reading (D2) is conducted via the addition of sodium azide, while the third (D3) corresponds to a hemolyzed preparation to which potassium ferricyanide has been added, to which, in turn, sodium azide is finally added for the fourth reading (D4). The percentage of methemoglobin is calculated via the following equation: % MetHb =  $[(D1-D2) / (D3-D4)] \times 100$  with values of over 1.5% total methemoglobin considered to be above the reference values. The reagents used complied with high purity standards (Merck KGaA, Darmstadt, Germany and/or its affiliates).

## 2.5. Nitrite measurement

The nitrite concentration was determined, as a stable metabolite, for the plasma and urine samples, which were then deproteinized using absolute ethanol at a 1:7 ratio and centrifuged at 3500 rpm for 15 minutes. The nitrite levels in the plasma and urine were measured by subjecting 100 µl of supernatant to the Griess colorimetric method (Merck KGaA, Darmstadt, Germany and/or its affiliates), as reported by

Miranda et al. 2001, using spectrophotometric quantification (HACH-DR5000) at 540 nm. A standard curve was generated using sodium nitrite for the quantitative determination.

## 2.6. Measurement of thyroid function

Thyroid function (TSH, total and free T3, and total and free T4) was measured via chemiluminescent immunoassay (IMMULITE®1000 Siemens™, Gwynedd, United Kingdom). The sensitivity of the TSH assay was 0.004 µIU/mL, with an upper limit of 75 µIU/mL, while the cut-off points for thyroid function were TSH µIU/ml 0.400–4.00, T3T ng/dl 82.0–179, T3L pg/ml 1.00–40.0, and T4L ng/dL 0.300 – 6.00.

## 2.7. Measurement of TPO-Ab concentration

For those individuals presenting TSH levels above the normal range, 100µl blood serum was used to conduct a sandwich-type enzyme-linked immunosorbent assay (ELISA), using a commercial TPO-Ab detection kit in accordance with the manufacturer's instructions (Merck KGaA, Darmstadt, Germany and/or its affiliates). The levels of TPO-Ab + were determined once values of > 35 UI/ml were observed.

## 2.8. DNA extraction and genotypification of FOXE1

Genomic DNA extraction was conducted using whole blood following the protocol described by Lahiri and Nurnberger (1991), while DNA concentration and purity was established using the NanoDrop 2000 (Thermo Fisher Scientific Inc., Germering, Germany). The SNPs rs965513 and rs1867277 of *FOXE1* were genotyped in the StepOne Real-Time PCR (polymerase chain reaction) (Applied Biosystems, Foster City, California, USA), using hybridisation probes (Integrated DNA Technologies rhAmp®, ID Hs.GT.rs965513. G.1 y Hs.GT.rs1867277. G.1) in accordance with the manufacturer's instructions.

## 2.9. Statistical analysis

Measures of central tendency were analyzed for the description of the data and Pearson's correlation for quantitative variables with normal distribution. The information for 16 variables was compiled for 102 individuals, with the database including numerical and categorical variables. The variables were standardized by subtracting the average corresponding to the variable from the value of each data point and then dividing by the standard deviation. The multivariate analysis comprised a correlation analysis applied solely for the 11 numerical variables, given that principal component analysis (PCA) requires that the variables used present some level of correlation. For this reason, only those variables with a level of significance greater or equal to 0.90 were considered. Once the variables presenting a correlation had been identified, a Mahalanobis distance analysis was undertaken in order to discard the data considered atypical for this set of variables. The PCA was carried out to show, firstly, the distribution of the individuals based on their subclinical condition and, secondly, on their nitrate exposure according to the levels detected in the communities in which they live. A heatmap analysis was carried out in order to group the individuals according to the 11 numerical variables and, at the same time, show the levels of correlation of each of the variables for each individual. The cluster analysis was undertaken using the Euclidean distance index and the Ward algorithm in order to construct the groupings, while genotype, subclinical condition, sex, and the  $\text{N-NO}_3^-$  level to which they were exposed were mapped for each

individual in the heatmap. Moreover, in order to reassign those individuals who had been classified with a non-hypothyroid subclinical condition prior to the analysis, a discriminant function analysis was conducted to establish the percentage of correct classifications, which was then statistically corroborated via the Wilkins test. Finally, post hoc and chi-squared Kruskal-Wallis tests were performed to determine the differences between the different exposure scenarios, where applicable. All statistical and multivariate analysis was undertaken using the R 6.6.1 software, a p value of  $< 0.05$  was considered as statistically significant.

### **3. Ethical considerations**

This study has been approved by the Ethics Committee of the Faculty of Chemical Sciences at the Juarez University of the State of Durango, with a unique assigned registration number of R-2017-123301538X0201-026. The present study followed the guidelines, as pertaining to health research, stipulated in articles 13, 14, 17, 21, and 22 of the General Health Law in Mexico.

### **Results**

The study population initially comprised 26 female subjects, who had been previously diagnosed with SCH and who described other cases in their families. The analysis was conducted on the highest number of the subject's family members possible, in order to understand the potential causes behind the increased incidence of SCH in these rural populations, which had been exposed to a possible endocrine disruptor.

The total sample comprised 102 people with an average age of 22 years, 69% of whom were women, 15% had no schooling and 36% had been educated up to lower secondary level, while 58% were overweight or presented some degree of obesity and the majority worked in the home or were students. With regard to water consumption, 85.5 % consumed water drawn from the public water system or from private wells, while only 14.5 % occasionally consumed water from water purification machines.

Table 1  
General characteristics of the total study population.

<b>Variables n = 102</b>		<b>n (%)</b>
Sex	Women	70 (69)
	Man	32 (31)
Age		
Median (mín.-máx.)	22 (2–72)	
Education	No schooling	15 (15)
	Primary education	37 (36)
	Middle school	36 (35)
	High school	10 (10)
	University	4 (4)
	Postgraduate	0
BMI (kg/m <sup>2</sup> )	Underweight	4 (4)
	Normal weight	39 (38)
	Overweight	28 (27)
	Class I obesity	20 (20)
	Class II obesity	5 (5)
	Class III obesity	6 (6)
Occupation	Homemaker	56 (55)
	Seasonal agriculture	8 (8)
	Student	32 (31)
	Other	6 (6)
Drinking water consumption	Public water system	63 (62)
	Well	24 (23.5)
	Purified	15 (14.5)
Consumption of water to prepare food	Public water system	70 (69)
	Well	26 (25)
BMI: body mass index		

Variables n = 102		n (%)
	Purified	6 (6)
Boiled water for consumption	Yes	2 (2)
	No	100 (98)
BMI: body mass index		

The measurement of MetHb found 79% of subjects to fall outside the reference limit, while the average levels of nitrite in plasma and nitrite in urine were 24.62  $\mu\text{mol/mL}$  and 3.46  $\mu\text{mol/mL}$ , respectively. In terms of the thyroid function parameters, 45% of subjects presented TSH levels over the reference limits (SCH), 49% presented reduced total T3 levels, and 19.6% presented a reduced total T4 levels. The detection of TPO-Ab found that 15.7% of the SCH cases were positive for the presence of antibodies.

Table 2  
Determination of biomarkers of interest in the study population.

Parameters	n	Median (mín-máx)	Reference rank	Out of rank n (%)
Methemoglobin (%)	102	2.44 (0.26–12.35)	> 1.5	81 (79)
Nitrite $\mu\text{mol/ml}$ blood	102	24.62(3.19–45.65)	>7	97 (95)
Nitrite $\mu\text{mol/ml}$ urine	102	3.46 (0.04–25.50)	> 1	87 (85)
TSH $\mu\text{UI/ml}$	102	3.72 (0.49–95.49)	0.04-4.00	46 (45) †
T3T ng/dl	102	89.7 (0.27–210)	82.0-179	51(49) ‡
T3F pg/ml	102	3.3 (1-10.2)	1.00–40.0	0(0)
T4T $\mu\text{g/dl}$	102	6.38 (1.3–23.1)	4.50–12.5	20 (19.6) ‡
T4F ng/dl	102	1.17 (0.36–3.21)	0.300-6.00	0 (0)
Ab anti-TPO	38	1 (1-1268.7)	> 35 UI/ml	6 (15.7) §
§ The determination of Ab Anti-TPO was only carried out in 38 of the 46 samples with a TSH level above the normal range. † indicates increase, ‡ indicates decrease.				

Table 3 presents the genotypic and allelic distribution of the SNPs rs965513 and rs1867277 for each of the levels established by the present study for nitrate exposure in water used for human consumption. The SNP rs1867277 was not found in Hardy-Weinberg equilibrium ( $J_i^2 = 0.012$ ) and its genotypic distribution across the exposure scenarios was found to be different ( $J_i^2 = 0.019$ ).

Table 3  
Genotypic and allelic frequencies of polymorphisms in the *FOXE1* gene, observed in the study population.

	<b>Total</b> <b>n = 102</b>	<b>Baja</b> <b>n = 25</b>	<b>Media</b> <b>n = 46</b>	<b>Alta</b> <b>n = 31</b>	<b>P-value</b>
<i>FOXE1</i> rs965513					
<i>Genotype</i>					
GG	47 (46 %)	12 (48 %)	25 (54 %)	10 (32 %)	
GA	49 (48 %)	12 (48 %)	20 (43 %)	17 (55 %)	0.177
AA	6 (6 %)	1 (4 %)	1 (2 %)	4 (13 %)	
<i>Allele</i>					
G	143 (70 %)	36 (72 %)	70 (76 %)	37 (60 %)	
A	61 (30 %)	14 (28 %)	22 (24 %)	25 (40 %)	
HWE					0.080
<i>FOXE1</i> rs1867277					
<i>Genotype</i>					
GG	34(33 %)	4 (16 %)	19 (41 %)	11 (35 %)	
GA	61 (60 %)	16 (64 %)	26 (57 %)	19 (61 %)	0.019
AA	7 (7 %)	5 (20 %)	1 (2 %)	1 (3 %)	
<i>Allele</i>					
G	129 (63 %)	24 (48 %)	64 (70 %)	41 (66 %)	
A	75 (37 %)	26 (52 %)	28 (30 %)	21 (34 %)	
HWE					0.012
P- value corresponds to Chi squared test					
HWE = Hardy Weinberg Equilibrium					

Table 4 presents a summary of the alterations observed in the following variables, in light of the different level of chronic exposure to nitrates in the water used for human consumption: exposure and effect biomarkers for nitrates (via the measurement of nitrites); thyroid dysfunction (SCH); the genotypic frequency of the SNPs; and, the presence of TPO as an autoimmune factor. Differences were observed in the levels of both nitrates and the exposure (nitrite in urine  $p = 0.001$ ) and effect (methemoglobin  $p =$

0.042) biomarkers, while the development of SCH was observed for all levels of chronic exposure ( $Ji^2 = 0.817$ ). The SNP rs1867277 only presented in the low exposure scenario, corresponding to 4% ( $Ji^2 = 0.043$ ), and, finally, the TPO-Ab percentage was highest in the medium exposure scenario, with 14.2%.

Table 4  
Summary of events by scenario of exposure to nitrates through drinking water.

Parameters	Exposure scenarios to NO <sub>3</sub> <sup>-</sup> mg/l			P-value
	Low (4.7 ± 3.3)	Medium (32.1 ± 3.7)	High (56.9 ± 14.7)	
Families	5	12	9	
All individuals	25	46	31	
MetHb > 1.5 % n (%)	21 (84)	32 (69.5) <sup>b</sup>	28 (90.3)	0.042 <sup>†</sup>
NO <sub>2</sub> <sup>-</sup> Blood > 7µmol/ml n (%)	22 (88)	45 (97.8)	30 (96.7)	0.097 <sup>†</sup>
NO <sub>2</sub> <sup>-</sup> Urine > 1µmol/ml n (%)	21 (84) <sup>a</sup>	43(93.5) <sup>b</sup>	28 (90.3)	0.001 <sup>†</sup>
Subclinical Hypothyroidism Cases n (%)	10 (40)	21 (45.6)	15 (48.4)	0.817 <sup>‡</sup>
<i>FOXE1</i> Risk genotype AA + HtS				
rs965513 n(%)	0 (0)	0 (0)	1 (3)	0.314 <sup>‡</sup>
rs1867277 n(%)	2 (4)	0 (0)	0 (0)	0.043 <sup>‡</sup>
Ab Anti- TPO + > 35 UI/ml n (%)	1 (10)	3 (14.2)	2 (13.3)	0.899 <sup>‡</sup>
Note: The percentage of the parameters is given based on the total number of people per exposure scenario.				
‡ Chi squared test; † Kruskal Wallis test, with post-hoc test (p < 0.05), showed with superscript letter: exposure <sup>a</sup> low-high, <sup>b</sup> médium-high.				

The statistical analysis compiled the information pertaining to 16 variables for the 102 individuals in the study, while the Pearson correlation analysis solely included the continuous quantitative variables. A positive correlation between the variables of age and BMI was found, while a dispersal denoting a moderate positive curvilinear relationship is also observed ( $r = 0.62$ ,  $r^2 = 38.44$ ,  $p = < 0.001$ ). The correlation between age and thyroid profile shows a moderate positive linear relationship between age and TSH ( $r = 0.062$ ,  $r^2 = 0.38$ ,  $p = 0.537$ ), and a weak negative correlation between age and T4T ( $r = -0.20$ ,  $r^2 = 4$ ,  $p = 0.049$ ), age and T4F ( $r = -0.19$ ,  $r^2 = 3.61$ ,  $p = 0.055$ ), age and T3T ( $r = -0.16$ ,  $r^2 = 2.56$ ,  $p = 0.109$ ),

and age and T3F ( $r = -0.43$ ,  $r^2 = 18.49$ ,  $p = 0.001$ ). Analysis of the correlation between the effect and exposure biomarkers revealed no statistically significant correlation, while a weak negative correlation was observed between the effect biomarkers (MetHb) and thyroid profile. However, a statistically significant correlation was found between the MetHb percentage and the levels of T3T and T3F ( $r = -0.43$ ,  $r^2 = 18.49$ ,  $p = 0.001$ , and  $r = -0.34$ ,  $r^2 = 11.56$ ,  $p = 0.001$ , respectively). A statistical significance was observed for the level of nitrite in the blood and its correlation with the thyroid hormones, but only for T4F and T3T ( $r = -0.20$ ,  $r^2 = 4$ , and  $p = 0.045$ , and  $r = 0.23$ ,  $r^2 = 5.29$ , and  $p = 0.022$ , respectively). In terms of the correlation between BMI and thyroid hormone levels, a negative relationship was found across almost the entire thyroid profile with the exception of TSH, while only a weak negative, but statistically significant, correlation was also found between BMI and T3F ( $r = -0.31$ ,  $r^2 = 0.096$ ,  $p = 0.01$ ).

The PCA revealed that the three first components represent 63.3% of the variability, wherein the variables that most contributed to the analysis of the first component were T3T, T3F, and T4T, while the main variables in the second component were age and BMI [Figure 2A *near here*]. The majority of the individuals presenting a SCH condition were identified by increases in the  $\text{NO}_2^-$  levels in the blood, BMI, age, TSH level, and MetHb levels, while a greater relationship was found between those individuals with a non-SCH clinical condition and increases in the variables T3T, T4T, T3F, and, mainly, T4F. When the nitrate exposure scenarios were classified, the PCA analysis did not identify a clear condition, but did establish that high levels of MetHb are more related to those study locations presenting high levels of nitrate in potable water.

Additionally, both multivariate conglomerate and heatmap analysis was undertaken to group subjects according to the numerical variables and, at the same time, present the levels of correlation for each of the variables for each individual. The conglomerate analysis was carried out using the using the Euclidean distance index and the Ward algorithm to construct the groupings, while the heatmap analysis mapped the subject's genotype and subclinical condition and the level of  $\text{N-NO}_3^-$  to which they had been exposed.

Finally, the discriminant function analysis revealed 81% accuracy in the classification of the subjects. This contrasts with the *a priori* group, wherein discriminant function analysis showed that four of the 95 subjects (numbers 70, 73, 74, and 76) from this group should be classified as likely to develop SCH if the levels for all the numerical variables do not decrease. The discriminant function analysis was statistically corroborated via the Wilkins test.

## Discussion

The present study is the first to seek evidence as to the possible direct relationship between chronic nitrate exposure via the consumption of contaminated water and alterations in thyroid function in rural areas of Durango.

As widely described in detail in the literature, human exposure to nitrates and nitrites occurs endogenously due to the metabolism of NO (Lundberg, Weitzberg & Gladwin 2008). It also occurs exogenously in the diet via different concentrations in drinking water and green leafy vegetables (Jonvik et al. 2016), food preservatives or flavorings in processed foods (Mortensen et al. 2017), and food supplements such as potassium nitrate and sodium nitrate (McDonagh et al. 2018). It is also present in medications that are mainly used to treat cardiovascular diseases (Lee & Gerriets 2019). The fact that the endogenous metabolism produces a much lower amount of nitrates and nitrites than that produced via the diet (Moretti et al. 2019) and the subjects' null ingestion of food supplements should both be noted. In light of this, the main source of nitrate exposure in the study population is attributable to the consumption of water contaminated with these compounds.

While few studies have established base levels of nitrates in the organism, Mikiwa et al. (2002) found nitrate and nitrite levels of  $36.6 \pm 18.5 \mu\text{mol/l}$  and  $6.4 \pm 2.1 \mu\text{mol/l}$ , respectively, in the plasma of a sample of ten healthy subjects of Japanese origin. Pimková et al. (2014) found nitrite and nitrate levels of  $1149 \pm 86 \text{ nmol/l}$  and  $32.78 \pm 10.33 \mu\text{mol/l}$ , respectively, in the plasma of a sample of 23 healthy subjects in the Czech Republic, while Lundberg & Weitzberg (2017) reported normal plasma levels of nitrates and nitrites ranging from 20–40  $\mu\text{mol/l}$  and 50–300  $\text{nmol/l}$ , respectively. In contrast, the present study obtained blood and urine nitrite levels of  $23.95 \pm 9 \mu\text{mol/ml}$  and  $4.90 \pm 4.90 \mu\text{mol/ml}$ , respectively, which may be related to the degree of exposure to nitrate via contaminated drinking water.

The physiological level of MetHb ranges from 0%-1% (Queirós et al. 2017) to 2% (Gómez et al. 2017). The lactating population is more prone to developing this condition as a result of the low level of methemoglobin reductase activity, while the ease with which fetal hemoglobin is oxidized and its more acidic gastric pH enable the intestinal microbiota to reduce the amount of nitrate ingested into nitrite. However, a notable correlation is not observed between the levels of nitrites in the blood and urine, while the high MetHb percentages observed is notable, wherein 79% of individuals analyzed presented levels over 1.5%, with an average of  $2.80 \pm 1.88$ .

Methemoglobinemia is a condition characterized by an abnormally high level (> 40%) of MetHb, which is mainly produced by acute exposure to oxidizing compounds, pharmaceuticals, and most chemical agents (Alanazi 2017). However, in the present study, the highest MetHb percentage, of 12.35%, was obtained from a study population which indicated, via the questionnaire, to having had no prior contact with any medication or chemical agent that could explain these high MetHb levels.

Another health aspect of interest to the present study is the state of thyroid function in the presence of chronic nitrate exposure. Nitrate, together with other sodium-iodide symporter inhibitors, is able to alter thyroid function (Horton et al. 2015), leading to it being suggested as a possible thyroid disruptor in humans (Bahadoran et al. 2015; Poulsen et al. 2018). In contrast to the findings obtained by Van Maanen et al. (1994), Tájtková et al. (2006), Gatseva & Argirova (2008, 2008b), and Ward et al. (2010), our results do not provide data on abnormal growth, thyroid nodules, or the presence of any type of thyroid carcinoma. Moreover, while do not they concur with the reduced TSH levels and increased thyroid

hormone levels found by the foregoing studies, they do show altered thyroid function after chronic nitrate exposure via potable water, ranging from  $4.7 \pm 3.3$  mg/l to  $56.9 \pm 14.7$  mg/l. Said alteration found in the present study corresponds to SCH and occurs due to the increased levels of TSH (up to 45%) and a reduction in T4T and T3T (up to 49% and 19.6%, respectively), a finding concurring with that reported by Aschebrook-Kilfoy et al. (2012). Another significant finding of the present study is the 85% urinary nitrite detected, which indicates that the study population exceeds the baseline (1  $\mu$ mol/ml) and, thus, that high levels of these nitrogenated compounds are renally excreted in the presence of high nitrate concentrations in drinking water. Van Maanen et al. (1994) observed an increase in urinary nitrate which corresponded to the increased nitrate concentration in the drinking water of the study population, thus showing a dose-response relationship.

While previous studies have indicated the relationship between high levels of nitrates in potable water and altered thyroid function, others have shown that said relationship is not completely established and have not found structural or functional changes in the thyroid gland (Hunault et al. 2007). In contrast, the present study identified nitrate concentrations in potable water of up to  $56.9 \pm 14.7$  mg/l, which is considered to be chronic exposure ( $\geq 1$  year), further to identifying the presence of other conditioning factors for the development of SCH.

The prevalence of SCH found by the present study was 45%, which exceeds the national and global prevalence of 8% and 10%, respectively (Bruneel et al. 2016; Duntas 2019), and remains high even under the scenarios of low (40%), medium (45.6%), and high (48.4%) exposure. Hashimoto's thyroiditis (a form of chronic lymphocytic thyroiditis or chronic autoimmune thyroiditis) is characterized by the autoimmune destruction of the thyroid gland, which leads to epithelial cell apoptosis and diffuse lymphocytic infiltration due to the action of specific B and T cells. Moreover, it also causes follicular destruction (Liontiris & Mazokopakis 2017) that leads to reduced levels of thyroid hormone synthesis. This chronic autoimmune thyroiditis is the main cause of both clinical and subclinical primary hypothyroidisms in areas with sufficient or excessive iodine content (Duntas 2019; Zimmermann & Boelaert 2015). This is accompanied by the presence of anti-thyroid antibodies, mainly TPO and anti-thyroglobulin antibodies, which are considered biomarkers of thyroid gland damage (Radetti 2014). In contrast with the 60% established by Bromińska et al. (2017) and Malathi et al. (2013) and the 50% established by Jayashankar et al. (2015), the present study found an SCH level of 15.7% in the presence of TPO-Ab ( $> 35$  UI/ml). Therefore, autoimmune disorders would not be the main cause of SCH in the families of the study population. Although the exact etiology of the development of Hashimoto's thyroiditis remains to be established, the interaction among genetic susceptibility factors, nutritional factors, and environmental triggers may be involved (Hu & Rayman 2017).

Alterations in the synthesis and secretion of thyroid hormones may be a consequence of a perturbation in the expression of the genes encoding thyroid transcription factors or the presence of genetic variations therein (Fernández et al. 2015). The gene *FOXE1* plays an important role in the growth and development of the thyroid glands and the proliferation and differentiation of follicular thyroid cells and acts as a regulator of cell function, growth, and differentiation (Chen & Zhang 2018). Two of its polymorphisms,

rs965513[A] and rs1867277[A], contribute independently to establishing a predisposition for the development of papillary thyroid cancer (Nikitski et al. 2017). Similarly, the presence of the allele of rs965513 polymorphism impacts on thyroid function, reducing the levels of TSH and T4F, increases T3F levels (Gudmundsson et al. 2009), and is associated with the development of hypothyroidism and goiter (Denny et al. 2011). Furthermore, the presence of the allele of rs965513 polymorphism participates in the recruitment of the USF1/2 factors by the FOXE1 promoter, resulting in an alteration in the state of *FOXE1* gene expression (Landa et al. 2009). Furthermore, it has been found that genetic variations in *FOXE1*, including rs965513 and rs1867277, are risk factors associated with increased susceptibility to differentiated thyroid cancer (Chen & Zhang 2018; Geng et al. 2015). In terms of the results of the present study, the frequency of the occurrence of the polymorphic allele in the study population was 30% for rs965513 [A] and 37% for rs1867277 [A], which is in line with global (rs965513 [A] = 20% and rs1867277 [A] = 31%) and national (rs965513 [A] = 26% and rs1867277 [A] = 29%) levels. Considering that only the AA genotype of both polymorphisms could be the cause of thyroid function alterations, the present study found the presence of the genetic variants rs965513 and rs2200733 in only 3 and 4%, respectively, of the SCH cases found, thus reducing the impact of genetic factors in the study population.

Increased body weight is one correlation plausibly connected to the presence of SCH, in that hypothyroidism and SCH provide the conditions for reduced metabolic function, alterations in thermogenesis, and processes involving the metabolism of both glucose and lipids. This translates into weight gain over the long-term, once the level of thyroid hormone synthesis begins to decrease (Sanyal & Raychaudhuri 2016). Given the foregoing, it is possible that the correlation between age and BMI found in the present study may reveal that the older a subject, the greater their exposure to nitrates and, consequently, the greater the alteration in metabolic processes, thus resulting in weight gain. However, we also consider that said correlation would be affected by other factors such as nutritional variation, sedentarism, and, even, the inherent relationship with growth, without underplaying the importance of said correlation in itself.

It should be noted that some studies indicate a relationship between increased BMI and increased thyroid hormone (T3F) levels (Taylor et al. 2016; Xu et al. 2019), while others maintain that changes in serum thyroid hormone (F4T) may cause increases in BMI (Abdi et al. 2017). The foregoing is the opposite of the negative correlation found in the present study between BMI and T3L levels ( $r = 0.031$ ,  $r^2 = 0.096$ ,  $p = 0.001$ ), for which reason, we consider that high nitrate exposure would play an important role in the thyroid profile and merits attention in the study population.

In terms of the relationship observed between age and thyroid profile, the results of the present study provide evidence of a negative and statistically significant correlation. However, the production of hormones regulated by the endocrine system, including the thyroid hormones, decreases due to aging, which causes general morphological and physiological changes (Barbesino 2019). Nevertheless, the importance of the chronic presence of nitrate as an inhibitor of iodine uptake in the thyroid gland cannot be understated.

It is of the utmost importance to highlight that the following environmental characteristics of the rural region in which the present study was carried out are a problem with real impacts on human health: intermittent rainfall; soil/aquifer contamination via inorganic nitrate and other compounds; over-fertilization; over-use of land for forage crops; and, intensive livestock and agricultural practices. Not only are said problems present in rural zones of Durango, Mexico, but are also, today, without a doubt, a public health and environmental problem in large parts of the world (Shukla & Saxena, 2019).

As discussed above, the development of SCH may have a multifactorial origin (Ibañez, 2017). However, Table 4 clearly shows that SCH presents in each of the exposure scenarios, thus suggesting that, further to the high nitrate levels, it is chronic nitrate exposure that provides the conditions for alterations in thyroid function.

## Conclusions

The results of the present study suggest that environmental factors have a direct and significant influence, via chronic exposure to nitrate in potable water, on thyroid dysfunction in general and specifically the disruption of the synthesis and secretion of thyroid hormones. Therefore, the high frequency of SCH in the study population is mainly due to the nitrate contamination of potable water and not as a response to an additive effect and other causal factors.

The present study draws attention to the nitrate concentrations that may be found in potable water and suggests that exposing individuals to such concentrations during critical periods of their development may have significant long-term consequences. There is an evident need to ascertain the role played by nitrate as an endocrine disruptor via potable water. Our findings contribute to the implementation of public health measures aiming to ensure access to water resources of adequate quality, further to raising awareness in the rural population of Durango as to the dangers of consuming water with high nitrate content.

## Declarations

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**Availability of data and material:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Ethics approval:** This study has been approved by the Ethics Committee of the Faculty of Chemical Sciences at the Juarez University of the State of Durango, with a unique assigned registration number of R-2017-123301538X0201-026. The present study followed the guidelines, as pertaining to health research, stipulated in articles 13, 14, 17, 21, and 22 of the General Health Law in Mexico.

**Consent to participate:** Informed consent was obtained from all individual participants included in the study.

**Consent for publication:** Patients signed informed consent regarding publishing their data.

**Authors' contributions:** EGT performed experimental work, sampling and wrote the manuscript; AGZ performed the PCA, cluster and heatmap models and statistical analyses; EYCR participated in the design of the study, sampling and writing. RPM participated in the design of the study, implementation of techniques and revision of the manuscript, all the authors participated in the approval of the final version.

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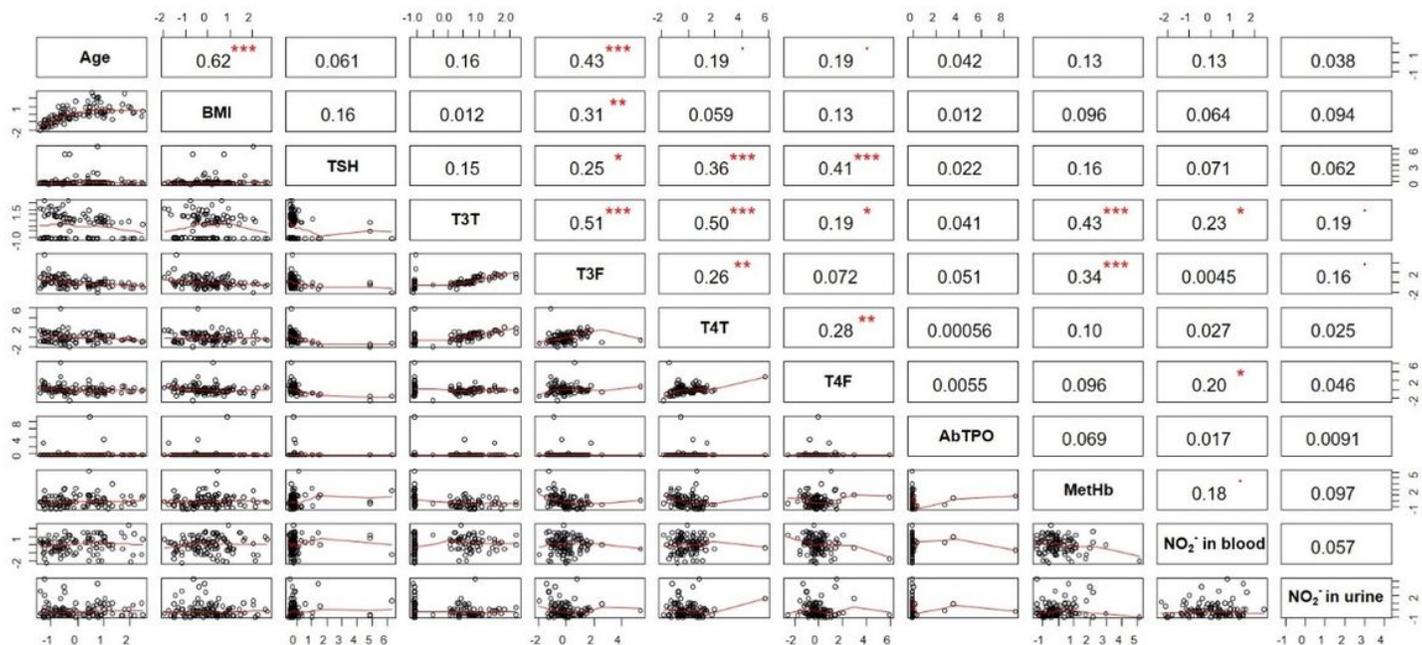
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## Figures



**Figure 1**

Pearson correlation analysis (Significance level, \*\*\* = 0.99; \*\* = 0.95; \* = 0.90). BMI, Body mass index; TSH, Thyroid stimulant hormone; T3T, Total T3; T3F, Free T3; T4T, Total T4; T4F, Free T4; AbTPO, Thyroid peroxidase antibody; MetHb, Methemoglobin; NO<sub>2</sub><sup>-</sup>, Nitrite.

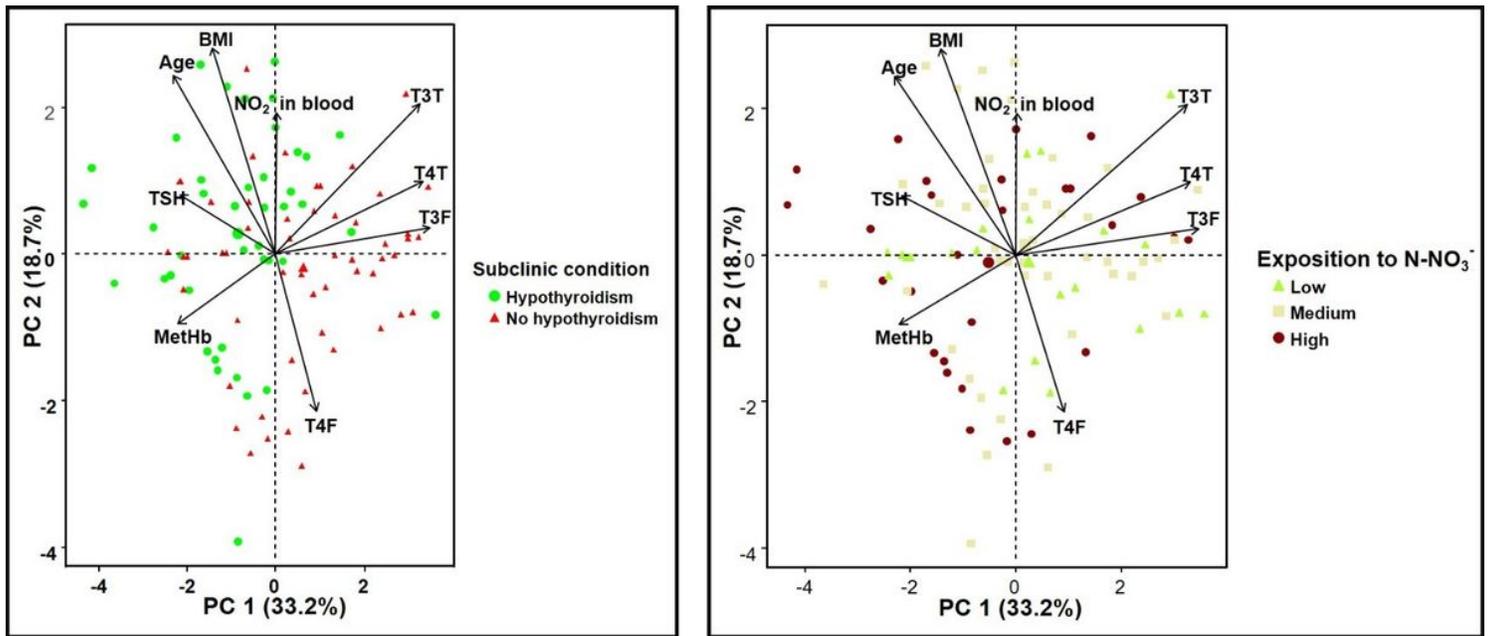


Figure 2

Principal component analysis (PCA). A) PCA analysis classifying the condition of subclinical hypothyroidism and B) PCA analysis classifying nitrate exposure scenarios through drinking water.

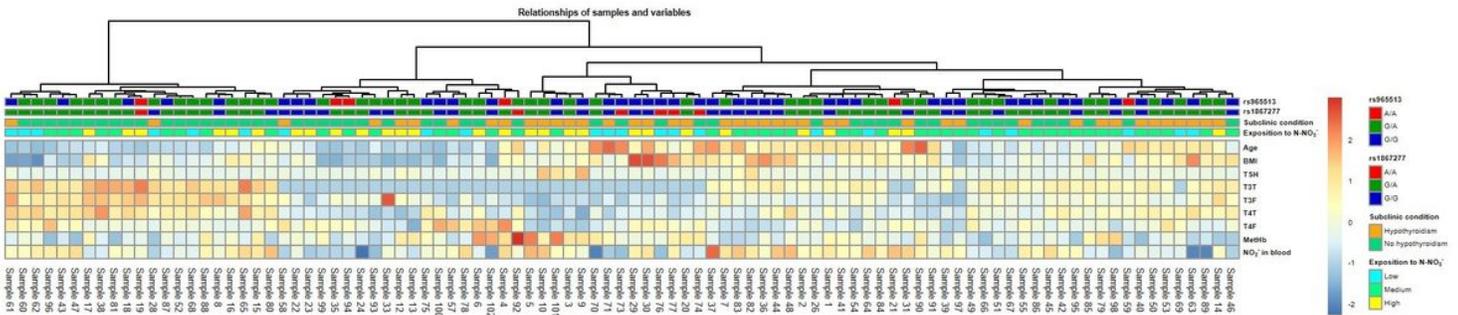


Figure 3

Multivariate cluster and Heatmap analysis. BMI, Body mass index; TSH, Thyroid stimulant hormone; T3T, Total T3; T3F, Free T3; T4T, Total T4; T4F, Free T4; AbTPO, Thyroid peroxidase antibody; MetHb, Methaemoglobin; NO<sub>2</sub><sup>-</sup>, Nitrite. The cluster analysis was performed using the Euclidean distance index and using Ward's algorithm to construct the clusters; in the Heatmap analysis, the genotype presented by each individual, the subclinical condition, and the level of N-NO<sub>3</sub><sup>-</sup> to which they are exposed were mapped.