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Serum copper profile in patients with type 1 diabetes in comparison to other metals

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ABSTRACT

Background: Type 1 diabetes (T1D) is a chronic condition in which the pancreas loses the ability to produce insulin due to an autoimmune destruction of the insulin producing beta cells in the pancreatic islets of Langerhans. Pathophysiological complications related to diabetes include micro and macrovascular disease, nephropathy, and neuropathy that can also be affected by environmental factors such as lifestyle and diet.
Objectives: The current study aimed to evaluate the serum levels of total copper, the copper-carrying protein, ceruloplasmin and nonceruloplasmin bound copper (nonceruloplasmin-Cu) and other essential and environmental metals and metalloids in subjects with T1D compared with healthy controls.
Methods: A cohort of 63 subjects with T1D attending Diabetes Clinics at the University of Miami and 65 healthy control subjects was studied. Metals and metalloids were measured by inductively coupled plasma mass spectrometry.
Results: A main finding of this study was that total copper and ceruloplasmin levels were higher in persons with T1D compared to healthy controls. In comparison to other metals and clinical variables, elevated copper was the strongest factor associated with T1D resulting in a15-fold increased odds of having the disease per standard deviation increase.
Conclusion: Our results suggest a metal and metalloid perturbation in T1D with a significant involvement of Copper dysfunction in the disease pathology, possibly linked to inflammatory processes.

1. Introduction

Diabetes represents a cluster of chronic metabolic conditions associated with defective insulin action and/or secretion causing high blood sugar levels [1] and an increased risk of macro- and micro-vascular complications, such as coronary artery disease [2,3], retinopathy [4], neuropathy [5–7]. In 2014 it was estimated that over 422 million people throughout the world had diabetes [8]. Insulin resistance is the predominant condition in type 2 diabetes (T2D) wherein more insulin than usual is needed for glucose to enter cells. Approximately 10% of people with diabetes have type 1 that typically appears in childhood or early adulthood. People with type 1 diabetes (T1D) lose control of blood glucose levels due to a near-total or complete autoimmune destruction of beta cells in the pancreatic islets of Langerhans. As a complex disease, diabetes onset is influenced by multifactorial effects, such as auto-antigens, viruses, diet, trace elements, including essential and environmental metals (Manganese, Copper, Iron, Zinc, Aluminum, Chromium, Cobalt, Nickel, Lead, Cadmium, Beryllium, Molybdenum and Thallium) and metalloids (Selenium and Arsenic).

Most of the evidence linking Copper abnormalities and diabetes concerns T2D, and the association with insulin resistance [9]. However, in both T1D and T2D, Copper is associated with advanced glycosylation end products (AGEs) formation through non-enzymatic protein glycation [10]. The products are Schiff bases, produced by Maillard reactions, that when rearranged, generate Amadori compounds resulting in formation of protein-bound compounds, the AGEs. AGEs formation is

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accelerated by redox active metals such as Copper that can also promote protein glycoxidation processes [11]. AGEs accumulate in the extracellular matrix (ECM) of damaged arterial wall in diabetes contributing to the pathogenesis of diabetic complications [12] including vascular disease and neuropathy. The role of catalytically active Copper in causing oxidative stress and tissue damage in diabetes has been shown to be suppressed by selective Copper chelation [13], suggesting a direct role for biologically active Copper in these processes.

Copper is an essential trace metal that is required for the physiological activity of many proteins and some vital enzymes in metabolism. These include ubiquitously expressed proteins (e.g., cytochrome c oxidase or superoxide dismutase 1) as well as tissue specific proteins such as lysyl oxidase secreted by fibroblasts, tyrosinase in skin melanocytes, the plasma protein ceruloplasmin and the intestinal haephestin; all proteins that need copper for the stabilization for the protein structure or for enzymatic activities based on its reduction-oxidative (redox) properties [14]. Enzymes and Copper proteins are involved in various metabolic pathways, ranging from oxidative phosphorylation, antioxidant defense, collagen or pigment synthesis, Iron homeostasis, neurotransmitter synthesis and modulation [15].

The effects of a breakdown in Copper homeostasis is evident in the rare genetic disorders Wilson disease and Menkes disease, caused by mutations in the Copper transporters ATPase7B and ATPase7A, respectively. ATPase7A and ATPase7B are expressed in the endoplasmic reticulum and *trans*-Golgi network, where they function to load Copper into cuproproteins (e.g. ceruloplasmin, dopamine β-monooxygenase, peptidylglycine α-amidating monooxygenase). ATPase7A and ATPase7B can move to the cell membrane and eliminate excess cellular Copper. ATPase7A generally traffics toward the basolateral membrane of polarized cells in the gastro-entorhinal track and in the choroid plexus in the brain, while ATPase7B traffics to the apical side mainly in hepatocytes and in the brain [16]. In the liver, ATP7aseB facilitates Copper incorporation into ceruloplasmin, the most abundant Copper protein in serum (accounting for 70–95% of the serum Copper) [17]. It also controls Copper entry into lysosomes and Copper excretion into the bile, accounting for the pleiotropic effects of *ATP7B* gene mutations and polymorphisms on Copper homeostasis [15,18]. Loss of function mutations in the ATPase7B copper pump can cause failure of Copper excretion into the bile, as well as a decrease in plasma ceruloplasmin along with a spillover of Copper into the blood in the form of non-ceruloplasmin-Copper (-Cu) (also known as 'free' Copper) [15]. Non-ceruloplasmin-Cu, when exceeding the normal reference range (0.1–1.6 μmol/L [19]), is an intrinsically toxic form of Copper since it participates in the redox cycling between the Cu(II) and Cu(I) oxidation states, facilitating oxidative stress via Haber Weiss and Fenton like reactions.

Chronic complex diseases have been reported to be influenced by alteration of trace essential metal homeostasis. A number of studies reported increases in the levels of total Copper in TID and T2D, recently confirmed in a meta-analysis study [20]. The metal, with particular emphasis on the toxic form of nonceruloplasmin-Cu, has been evaluated and shown to be increased in several diverse age related neurodegenerative disorders that share a complex disease etiology, such as Alzheimer's disease (AD), Parkinson's disease, and Frontotemporal lobar degeneration [21–23], but reports in persons with diabetes are still scant. Alteration of Copper homeostasis by means of a panel of Copper markers including Copper, ceruloplasmin and nonceruloplasmin-Cu has been evaluated in TD1 patients and results are discussed herein in comparisons to other elements.

2. Methods

2.1. Research subjects

Subject characteristics and recruitment of persons with T1D and controls were previously described [24]. Briefly, subjects were recruited at the Diabetes Clinic at the Diabetes Research Institute/University of Miami Miller School of Medicine. Exclusion criteria included age less than18 years, pregnancy, the presence of chronic kidney disease, a recent cardiovascular event or other systemic disease, or therapy with fibrates or niacin. In the current study a subset of samples (63 T1D and 65 control subject) that had available serum samples not previously thawed were evaluated. All subjects with T1D were on insulin therapy, 28% were taking stains and 30% were taking antihypertension medicines. In contrast, only 5% and 8% of control subjects were taking statins or antihypertension medicines, respectively. The study was approved by the Human Subjects Research Office of the University of Miami, and written consent was obtained from all individuals.

2.2. Clinical and biochemical measures

The collection of clinical variables and biochemical assays used has been described in detail [24]. In addition to previous data, serum ceruloplasmin was measured by immunoassay on a Roche Cobas 6000 analyzer using manufacturer's reagents and procedures (Roche Diagnostics, Indianapolis, IN). The method had intra and inter assay CVs of $< 2.0\%$.

2.3. Metals and metalloids measures

All metals and metalloids were measured by inductively coupled plasma mass spectrometry (ICP-MS) at IGEA Research Lab (Miami, FL). Deionized 18.2 MΩ water was prepared by Millipore Simplicity UV (MilliporeSigma™ SIMS00001) and used for sample dilution and standard preparation. SPEX CertiPrep ICP–MS multi-element standard solutions were used for external calibration. Yttrium (SPEX CertiPrep.) was used as the internal standard throughout all measurements. Nitric acid (Fisher TraceMetal Grade) was diluted to 2% and added to the samples and standards. Freeze-dried reference pooled healthy male's serum (Innovative Research, USA) was used for validation and as a standard for the serum sample measurements. Thermo Fisher Scientific iCAP Q ICP- MS (Thermo Fisher Scientific, USA) was used in all experiments. The plasma source was 99.998% argon (Airgas, USA). For the preparation of standards and samples only polyvinyl chloride (PVC) tubes were employed. All operating parameters were under computer control, which allowed simple and fast optimization routines for different matrices. For Manganese (55; isotope detected), Iron (57), Zinc (66), Selenium (77), Beryllium (9), Aluminum (27), Chromium (52), Cobalt (59), Nickel (60), Arsenic (75), Molybdenum (95), Lead (208), Cadmium (111) and Thallium (205) measurements, 100 μL of serum was mixed with 400 μL of water and 1000 μL of 2% nitric acid containing 0.1 μg/mL of Yttrium (as internal standard). ICP-MS was calibrated for all metals for the range of 1–100 μg/L (0.0157–1.57 μmol/L).

For nonceruloplasmin-Cu measurements, serum samples were filtered through an Amicon Ultra centrifugal filter unit according to previous published methods [25,26]. 300 μL of filtrate was mixed with 900 μL of 2% nitric acid containing 100 μg/L of Yttrium (as internal standard) and analyzed by ICMPS. For total Copper measurements, 30 μL of serum was mixed with 270 μL of water and 900 μL of 2% nitric acid containing 0.1 μg/mL of Yttrium (as internal standard). ICP-MS was calibrated for Copper (Copper 65) for the range of 1–100 μg/L (0.0157–1.57 μmol/L).

2.4. Statistical analysis

The distribution of each variable was examined. First, one outlier was removed for iron and two outliers were removed for lead. Non-normal distributions were log-transformed. All metal variables were log-transformed.

Means and standard deviations (SD) were calculated for all demographic and clinical characteristics by group (control vs. T1D). Means

Table 1
Demographic and clinical characteristics of the sample.

	Control *n* = *65*	Diabetes Type 1 (T1D) *n* = *63*	Statistical analysis	*p-value*
Age - years	37.3 ± 9.6	40.4 ± 15.3	F(1,123) = 1.8	0.18
Age of onset*		18.69 (95%CI 14.87, 22.51)	N/A	
HbA_{1C}*		7.67 (95%CI 7.30, 8.03)	N/A	
C-peptide*		0.22 (95%CI 0.08, 0.36)	N/A	
Duration T1D*		24.52 (95%CI 20.62, 28.43)	N/A	
Sex - Female (%)	34 (52.3%)	44 (49.2%)	$\chi^2 = 0.123^{(1)}$	0.726 (*n.s.*)
Body Mass index (BMI)	24.5 ± 3.3	26.4 ± 4.5		0.014 (*n.s.*)
Cholesterol	189.6 ± 34	191.3 ± 35.5	t = −0.30	0.765
Triglycerides	108 ± 67	108 ± 60	t = −0.20	0.842
HDL-Cholesterol	52 ± 14	60 ± 17	t = −3.18	0.0019
LDL-Cholesterol	116 ± 31	110 ± 29	t = 1.13	0.259
Adiponectin	10.7 ± 4.0	13.3 ± 6.3	t = −2.79	0.0060 (*n.s.*)
apoA-I	149.6 ± 24.7	168.4 ± 35.2	t = −3.87	0.0002
S-creatinine	0.8 ± 0.2	0.8 ± 0.2	t = 0.73	0.465 (*n.s.*)
C-reactive protein (mg/L)	1.46 ± 1.49	2.93 ± 2.73	t = −3.81	0.0002

Data are expressed as mean ± standard deviation adjusted for age and sex or as * median (95% confidence interval, CI). Bonferroni correction applied to all mean comparisons. The p-value needed for significance at the 0.05 level was 0.0045.
N/A = not applicable.
F: F-statistic of ANOVA test.
(1) χ^2: chi-squared statistic test.
(*n.s*) = non-significant mean comparison.

were adjusted for age and sex. F- and t-tests were used to examine differences between groups for continuous variables. The chi-square test of independence was used to test group differences for categorical variables. Due to the exploratory nature of the study, Bonferroni correction was applied to all mean comparisons. The alpha level threshold for significance at .05 was .0005.

Next, a multiple logistic regression model was used to compare standardized elements between healthy controls and patients with T1D. The outcome was the disease variable (T1D or no T1D/healthy control). HDL, adiponectin, apoA-I, CRP, Copper, nonceruloplasmin-Cu, Zinc, Manganese, and Selenium were included in the model. Z-scores were used for each variable in order to interpret the results in terms of standard deviation units. Odds ratios and 95% confidence intervals (95% CI) are reported per standard deviation unit.

3. Results

Persons with T1D and control subjects did not differ in age or sex (Table 1). Since most of the biological variables were influenced by sex, as previously reported for this cohort [24], the statistical analyses carried out in the current study were adjusted for age and sex. T1D patients had significantly higher levels of BMI, HDL-cholesterol, apoA-I, C-reactive protein (CRP), and adiponectin compared to controls (Table 1).

None of the essential metals and metalloids measured correlated with age, except for Manganese, which was inversely correlated ($r = -0.24$, $p = 0.011$). Data on the Copper profile and other measured essential trace elements in T1D and controls are summarized in Table 2. Total Copper levels and the copper-carrying protein, ceruloplasmin, were both significantly elevated in T1D subjects (Table 2). Manganese, Zinc and Selenium were significantly lower in TD1 patients than in healthy controls, while Iron did not differ between the two groups. Copper:Zinc (Cu:Zn) ratio and Copper:Selenium (Cu:Se) ratio were higher in T1D than controls (Table 2). The correlation between the essential metals and metalloids and the clinical variables revealed that Copper, ceruloplasmin and nonceruloplasmin-Cu were all positively correlated with CRP ($r = 0.546$, $p < 0.0001$; $r = 0.633$, $p < 0.000$; $r = 0.365$, $p < 0.0001$, respectively) and total cholesterol ($r = 0.254$, $p = 0.005$; $r = 0.271$, $p = 0.002$; $r = 0.257$, $p = 0.004$, respectively) after adjusting for age and sex. In contrast Zinc was negatively associated with CRP ($r = -0.266$, $p = 0.003$).

Table 2
Serum essential metal profile of the sample.

	Control *n* = *65*	Type 1 Diabetes *n* = *63*	t test	*P value*
Copper (μmol/L)	14.1 ± 3.8	17.9 ± 4.8	6.54	< 0.0001
Ceruloplasmin (mg/dL)	24.4 ± 6.4	30.1 ± 9.5	5.51	< 0.0001
Nonceruloplasmin-Cu (μmol/L)	1.3 ± 0.4	1.5 ± 0.4	2.59	0.011 (*n.s.*)
Cu:Zn	0.9 ± 0.3	1.3 ± 0.5	7.25	< 0.0001
Cu:Se	4.4 ± 1.3	6.4 ± 1.8	9.44	< 0.0001
Manganese (μg/L)	3.7 ± 1.8	2.6 ± 1.9	2.98	< 0.0035
Iron (μg/L)*	1447 ± 563	1729 ± 3080	1.37	0.1707
Zinc (μg/L)	1113 ± 259	966 ± 386	3.63	0.0004
Selenium (μg/L)	209 ± 31	185 ± 50	3.84	0.0002

Data are expressed as the mean ± Standard Deviation and were adjusted for sex and age.
All variables were log-transformed prior to comparison of adjusted means. Bonferroni correction was applied to all mean comparisons. The p-value needed for significance at the 0.05 level was 0.005.
(*n.s*) = non-significant mean comparison.
* 1 outlier removed.

Among the environmental metals Beryllium was lower and the level of Molybdenum was higher in T1D subjects compared to controls. There were no differences in the other environmental variables (Table 3), while Cadmium and Thallium were not detectable in any of the study subjects.

In order to assess the associations of the biological variables under study on the probability of developing T1D, a multiple logistic regression model was performed with T1D versus control as the dependent variable, and HDL, adiponectin, apoA-I, CRP, Copper, nonceruloplasmin-Cu, Zinc, Manganese, Selenium as the independent variables (Table 4). The results revealed that total Copper and Selenium were associated with the risk of having T1D (Table 4). More specifically, controlling for age, sex, and BMI (95% CI: 3.65–65.21) the association of Copper was stronger than that of Selenium, considering that for a standard deviation unit, there was a 15-fold higher odds of copper and a 0.21 lower odds of Selenium in adults with T1D compared to adults without T1D.

Table 3
Serum environmental metal profile of the sample.

	Control *n* = 65	Type 1 Diabetes *n* = 63	t test	*p-value*
Berillium (μg/L)	0.117 ± 0.1	0.053 ± 0.08	3.13	0.002
Aluminum (μg/L)	32.6 ± 16	36.2 ± 52	−0.03	0.9784 (*n.s.*)
Chromium (μg/L)	23.6 ± 17.3	18.5 ± 19.6	1.78	0.078 (*n.s.*)
Cobalt (μg/L)	0.31 ± 0.32	0.23 ± 0.17	0.83	0.407 (*n.s.*)
Nichel (μg/L)	12.03 ± 13.9	4.2 ± 8.2	2.49	0.0151 (*n.s.*)
Arsenic (μg/L)	1.02 ± 0.9	1.1 ± 1.9	1.41	0.1606 (*n.s.*)
Molybdenum (μg/L)	3.2 ± 1.2	4 ± 1.8	−3.19	0.002
Lead (μg/L)*	0.02 ± 0.06	0.2 ± 1.3	−0.28	0.78 (*n.s.*)

Data are expressed as the mean ± Standard Deviation adjusted for sex and age. All variables were log-transformed prior to comparison of means adjusted for sex and age. Bonferroni correction applied to all mean comparisons. The p-value needed for significance at the 0.05 level was 0.005.
(*n.s*) = non-significant mean comparison.
* 2 outliers removed.

Table 4
Results of multiple logistic regression modeling comparing standardized metals in healthy controls (n = 65) and in patients with T1D (n = 653), including age sex BMI as covariates.

Biological variable	Odds Ratio	95% CI
HDL-Cholesterol	2.79	0.76, 10.06
Adiponectin	2.58	1.20, 5.58
apoAI	0.49	0.13, 1.795
C-reactive protein	1.15	0.37, 3.48
Copper*	15.42	3.65, 65.21
nonceruloplasmin-Cu	1.24	0.38, 4.02
Zinc	1.23	0.65, 2.31
Manganese	0.46	0.22, 0.93
Selenium*	0.21	0.10, 0.45

Estimates are adjusted for age, sex, and body mass index. Z-scores used for all variables. Maximum likelihood estimation was used to handle missing data.
* $p < 0.001$.

4. Discussion

A main finding of this study is that total Copper and ceruloplasmin levels were higher in persons with T1D compared to healthy controls and, in comparison to other metals and clinical variables, it was the strongest factor associated with T1D, explaining a 15-fold increased odds of having the disease per standard deviation unit. Abnormalities in other essential elements and metals were also associated with T1D, such as lower levels of Zinc, Manganese, Selenium and Beryllium and higher levels of the environmental metal Molybdenum. However, the fact that total Copper and ceruloplasmin levels were significantly elevated in T1D patients, suggests an alteration of Copper homeostasis may be an important hallmark of the disease. In a previous study, higher levels of serum Copper and ceruloplasmin have also been reported in subjects with T1D [20]. Along with increased levels of glucose that trigger free radical reactions, Copper dysregulation also strongly facilitates oxidative stress in disease states. Streptozotocin (STZ) induced models of T1D show that this beta-cell toxin also induced disruption of Copper homeostasis, with increased levels of the metal in many tissues and organs after the first week of the diabetes onset (reviewed in [27]). A rise in urinary Copper excretion, indicative of an increase in the non-ceruloplasmin-Cu fraction in the blood, has been reported in the STZ diabetic rat 14 days after diabetes onset [28]. The increase in in urinary Copper was reduced after insulin treatment, suggesting that hormonal status directly influenced Copper excretion [28]. In line with the STZ diabetic rat evidence, we observed increased levels of serum non-ceruloplasmin-Cu in TD1, though not reaching the statistical threshold, relative to healthy controls. Studies in larger cohorts may unveil the significance in the trend of raised nonceruloplasmin-Cu levels but also measures of urinary Copper excretion should be considered as another approach to measuring levels of free copper. However, in a previous study of ours, employing a different method to measure nonceruloplasmin-Cu detection, we observed no difference in non-ceruloplasmin-Cu in T1D patients compared to controls, but did observe significantly higher levels in participants with T2D [29].

Some authors showed that insulin can facilitate ATPase7B activity reducing the Copper content in hepatic cells, by the retrograde trafficking of ATPase7B from the canalicular membranes [27,30]. On this bases they proposed that diabetes mellitus can have an inhibitory effect on ATPase7B that in turn can result in increased levels of non-ceruloplasmin-Cu [27,30]. Such as perturbation of the ATPase7B pathway at liver level has been fully described in Wilson disease and appears to also be triggered in AD [31], a chronic complex disease typified by extensive oxidative stress and Copper homeostasis abnormalities. In both diseases, even though they have a different etiology, *ATP7B* genetic variants are modulators of non-ceruloplasmin-Cu levels and Copper dysfunction [15,32,33]. However, in our T1D patients, this pathway appears to play a secondary role, given that nonceruloplasmin-Cu was not different from controls in the current study: the elevation observed is below the level of the normal reference values in most of the patients (60%) and lower than the values observed in Wilson disease and AD [34]. Furthermore, in our T1D patients, relative to controls, the ceruloplasmin concentration was significantly higher. On this basis, the inflammatory machinery triggered by the autoimmune condition that is often present in T1D appears to be a likely factor for the higher levels of Copper and ceruloplasmin in T1D patients. Our data show a strong association between CRP, an established marker of systemic inflammation, and ceruloplasmin, an acute phase inflammatory reactant. We also observed a difference in the Cu:Zn and Cu:Se ratios that may be associated with decreased blood antioxidant capacity and increased inflammatory response and could be a marker of inflammation status and oxidative stress.

The lower levels of Selenium found in T1D patients compared with referents is consistent with previous observations [35] but not with other studies, which found comparable or even slightly higher selenium values in patients [36,37]. In the present study, the lower Selenium levels in patients are reflected by a low odds ratio for the disease associated with an indicator of exposure to this metalloid, thus indicating either an involvement of a low Selenium intake in disease etiology or a derangement of selenium status following disease onset and progression. Unfortunately, these two differential hypotheses cannot be adequately tested in the present investigation, since they can be addressed only using a longitudinal study design. However, given the ability of Selenium to increase the risk of T2D, as detected in both observational and intervention studies [38] possibly due to the deleterious effects of selenoprotein P and other selenoproteins on glucose metabolism and homeostasis [39–41], a reverse causation effect seems to be more likely, thus suggesting that diabetes *per se* might lower Selenium status.

Additionally, Zinc and Manganese levels were also lower in T1D patients with respect to healthy controls. Both Zinc and Manganese insufficiency and increased Copper concentrations may influence the

equilibrium of the antioxidant system, as they are important for the Cu/Zn-superoxide dismutase (SOD) and Mn-SOD activities [42,43]. Furthermore, glycosylation of the Cu/Zn-SOD results in fragmentation and inactivation of the enzyme [44] further decreasing the antioxidant capacity in subjects with T1D. Our results are in line with some recent observations in T2D showing that Zinc supplementation (50 mg/day) had a beneficial effect on increasing gene expression and enzyme activity of Cu/Zn SOD and insulin, accompanied by a reduction of fasting blood glucose, HbA_{1c}, triglycerides and total cholesterol [45]. The associations of all markers of Copper panel with cholesterol suggest a direct link of Copper dysfunction with membrane metabolism through lipid peroxidation. In fact, administration of Zinc, that decreases the absorption of Copper reinforcing the mucosal block through metallothioneins induced expression, has some effects in reducing cholesterol abnormalities as shown in T2D [45].

The current study has a number of limitations including the small size of the sample and the lack of a clinical follow-up of the T1D patients to provide information about Copper and other metals involvement in disease onset and progression. Even though future studies in larger and longitudinal cohorts are needed in order to address this hypothesis, our results suggest a metal and metalloid perturbation in T1D and suggest that Copper dysfunction may contribute to disease pathologies associated with known diabetes-associated comorbidities, potentially linked to pro-oxidative and inflammatory processes.

Declaration of Competing Interest

RS is Chief Scientific Officer of IGEA Pharma N.V.; she has some shares in IGEA Pharma N.V., but does not receive monetary compensation. CR is a member of The Scientific Advisory Board of IGEA; he has some shares in IGEA Pharma N.V., but does not receive monetary compensation. VN is employed at IGEA Pharma N.V. Other authors declare no commercial or noncommercial conflicts of interest relating to this work.

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