

Diabetes and Alzheimer's Disease: Can Elevated Free Copper Predict the Risk of the Disease?

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Accepted 30 November 2016

Abstract.

Background: Defective copper regulation, primarily referred to as chelatable redox active Cu(II), has been involved in the etiology of diabetes, and Alzheimer's disease (AD).

Objectives: However, no study has determined levels of labile copper non-bound to ceruloplasmin (non-Cp Cu, also known as 'free' copper) in the blood of subjects with diabetes compared with that of AD patients.

Methods: To this aim, values of non-Cp Cu were measured in 25 Type 1 (T1D) and 31 Type 2 (T2D) subjects and in 28 healthy controls, along with measurements of C-reactive protein, glycated hemoglobin A_{1c}, cholesterol, and triglycerides. Non-Cp Cu levels were compared with those of an AD group previously studied.

Results: T2D subjects had significantly higher non-Cp Cu levels than Controls and T1D subjects (both $p < 0.001$ after adjusting for age, sex, and body mass index). A multinomial logistic model revealed that a one unit standard deviation increase of non-Cp Cu increased the relative risk of having T2D by 9.64 with respect to Controls (95% CI: 2.86–32.47). The comparison of non-Cp Cu levels in T2D with those of an AD population previously studied shows rising blood non-Cp Cu copper levels from Controls to T2D and AD.

Conclusion: These results suggest the involvement of catalytically-active Cu(II) and glucose dysregulation in oxidative stress reactions leading to tissue damage in both diseases.

Keywords: Alzheimer's disease, copper, diabetes, free copper, Type 1, Type 2

INTRODUCTION

Diabetes represents a cluster of chronic metabolic conditions associated with defective insulin action and/or secretion, causing high blood sugar levels and an increased risk of microvascular complications, such as diabetic retinopathy, neuropathy, and

nephropathy. In 2013 it was estimated that over 382 million people throughout the world had diabetes. In type 1 diabetes (T1D), insulin production becomes inadequate for the control of blood glucose levels due to a near-total or complete destruction of beta cells in the pancreas. Approximately 10% of all diabetes cases are type 1. Typically, the disease first appears in childhood or early adulthood, but it can occur at any age.

In type 2 diabetes (T2D), insulin resistance is the predominant condition and more insulin than usual is needed for glucose to enter cells. Insulin resistance

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in the liver results in more glucose production, while resistance in peripheral tissues means that glucose uptake is impaired. The impairment stimulates the pancreas to make more insulin, but eventually the pancreas is unable to make enough to prevent blood sugar levels from rising too high.

The number of adults diagnosed with diabetes in the US has risen significantly in the past 30 years; it rose almost 5 fold from 5.5 million cases in 1980 to 29.1 million in 2014 [1]. Type 2 is the most common form of diabetes, accounting for over 90% of all diabetes cases.

Diabetes is a complex disease, in which polygenic and multifactorial effects, including auto-antigens, viruses, diet, and exercise can contribute to disease pathogenesis.

Since late 1970s, attention has been devoted to essential metal abnormalities in the etiology of this chronic metabolic disorder. Among the metals, copper, in particular, drew attention since hyperglycemia has been linked to tissue damage through impaired copper homeostasis. Non-clinical models of diabetes demonstrated that it causes defective copper regulation [2], primarily involving advanced-glycation end products (AGEs), that can modify amino acid residues producing pathological copper-binding sites within extracellular matrix components of the blood-vessel walls.

Alzheimer's disease (AD) is another common age-associated disorder with a complex etiology in which metal abnormalities have been implicated. In the brain, AD is characterized by the presence of several kinds of amyloid plaques and neurofibrillary tangles, which consist mostly of the amyloid- β ($A\beta$) protein and hyperphosphorylated tau. Extensive experiments conducted by numerous groups using animal and cell models, as well as *in vitro* assays, have reached consensus regarding the involvement of metals and particularly copper in the toxic gain of function of the $A\beta$, which has been indicated to be central in the disease, a dogma which has been challenged in recent years. These studies demonstrated that the hypermetallation of the $A\beta$ peptide is at the basis of the reduction-oxidation (redox) cycles of oxidative stress, which produce H_2O_2 as a by-product and are catalyzed by transition metals (Fenton's and Haber Weiss chemistry) [3–5]. Evidence is also increasing about dietary AGEs as important risk factors for AD [6].

A number of studies have been performed investigating copper levels in T1D and T2D, most of them demonstrating elevated levels of copper in the serum

or plasma of T2D patients. Studies of urinary copper excretion indirectly demonstrated excess of systemic chelatable free copper in diabetes patients [2, 7]. These findings were confirmed in clinical as well as nonclinical models of the disease [7–10]. Free copper, or more precisely copper not bound to ceruloplasmin (non-Cp Cu), is a pool of circulating redox-active copper loosely bound to albumin and micronutrients in the blood, available to meet tissue needs in the body. If the non-Cp Cu pool becomes expanded, this copper becomes toxic, as exemplified by Wilson's disease, the paradigmatic disease of non-Cp Cu copper intoxication and accumulation [11–13]. More recently, elevations of non-Cp Cu have been identified in AD [14]. Even though a number of studies have investigated copper abnormalities in T1D and T2D, including those testing copper chelating agents in diabetic cardiovascular disease [2, 7, 8, 15], no study has been carried out so far testing the presence of labile non-Cp Cu copper in the blood of diabetic patients. The current study was exactly designed to explore the hypothesis of the existence of an expanded pool of chelatable non-Cp Cu in general circulation of persons with T2D.

METHODS

Subjects

Control, T1D, and T2D subjects were recruited from the University of Miami affiliated clinics. All patients provided informed consent; recruitment and all procedures were approved by the University of Miami Human Subjects Research Office. All procedures involving experiments on human subjects have been done in accordance with the ethical standards of the Committee on Human Experimentation of our institution and in accordance with the Helsinki Declaration of 1975 (revised 2008). Blind procedures for data collection and analyses were applied. Serum samples were obtained from fasting blood, and aliquots were stored frozen at -80°C until analysis.

Biochemical analyses

The direct fluorescent non-Cp Cu test is an *in vitro* method [16] carried out at IGEA Research Corporation Laboratories in Miami (FL, US). The method is based on two steps, as follows: i) a size exclusion solid-phase extraction (SPE) flow system separates the portion of low molecular weight non-Cp Cu

present in serum from the protein-bound copper; ii) it is a direct fluorescent assay based on a coumarin fluorescent probe; finally, it is a microplate device for the reading of the fluorescence signal. The fluorescent coumarin probe is specific for Cu(II) and is added to the soluble fraction eluted from the previous steps for the fluorescence emission signal detection. When the probe captures the labile non-Cp Cu — as a Cu(II) — from small peptides and amino acids, there is a decay in its fluorescence emission, proportional to non-Cp Cu concentration. During step (i), serum is loaded to a SPE chromatography (Ultra-high molecular weight polyethylene; micro-column with reservoir-3 ml capacity). The stationary phase is compressed with 6 mL physiological solution (0.9% NaCl) using a peristaltic pump (ISMATEC IPC, Cole-Parmer GmbH, Germany) at a flow rate of 500 $\mu\text{L}/\text{min}$. Serum (200 μL) was loaded on the column and eluted at a flow rate of 200 $\mu\text{L}/\text{min}$. The fraction eluted, containing non-Cp Cu, is collected into polypropylene tubes. During step (ii), a 50 μL volume of the fraction eluted (in duplicate) is co-incubated with the fluorescent probe at a final concentration of 10^{-5} M into the well of a 96/wells micro-plate reader, and the complex is read in a Victor X2 fluorimeter (Perkin Elmer) at excitation and emission wavelength of 380 nm and 500 nm, respectively. The interaction of the fluorescent probe with Cu(II) present in the eluate of step (i) switches-off the fluorescent probe. The intensity of the decay of the fluorescent emission is proportional to the quantity of non-Cp Cu present in the original specimen.

Body mass index (BMI) was calculated as body weight (kg) divided by height squared (m^2). C reactive protein (CRP), glycated hemoglobin A_{1c} (HbA_{1c}), cholesterol, and triglycerides were measured on a Cobas 6000 analyzer and using reagents from Roche Diagnostics (Indianapolis, IN), following all manufacturer instructions and procedures.

Statistics

Statistical significance of the differences between groups in the functional assays were made by one-way ANOVA, followed by Tukey's multiple comparison test. A one-way analysis of covariance (ANCOVA) was conducted to determine if there were significant differences between groups on non-Cp Cu after adjusting for significant variables. Multiple comparisons tests were conducted to evaluate pairwise differences among the adjusted means using Bonferroni's correction. Correlation analyses were

evaluated by the Pearson correlation coefficient r . A multinomial logistic model was applied to predict the probability of developing one of the diagnoses (dependent variable), on the basis of the non-Cp Cu levels, BMI, age, and sex (independent variables). Control group was chosen as the referent group. Results were presented in term of relative risk ratios (RRR) and the corresponding 95% confidence interval (95% CI). Statistical analysis was made in Graphad Prism 7.0a (Graphad Software, La Jolla, CA, USA) or SPSS version 23 (IBM, Armonk, NY, USA).

RESULTS

Non-Cp Cu levels in healthy controls, T1D, and T2D patients

Table 1 shows the demographic and selected clinical variables of the study population. In general, the majority of subjects were Hispanic (62% of the entire cohort), 28% were non-Hispanic Caucasian, and 10% were African-American, representative of the Miami population. The T2D group was significantly older and had a higher BMI than the Control and T1D subjects. CRP levels, as a measure of systemic inflammation, were not statistically different between any of the groups. HbA_{1c} was similar for T1D and T2D subjects. Lastly T1D and T2D subjects had significantly lower cholesterol levels than controls, likely accounted for by the increased use of statins in the diabetic subjects.

Table 1
Characteristics of study subjects

	Control	T1D	T2D
n	28	25	31
Sex (M/F)	14/14	12/13	10/21
Ethnicity (NHW/ HIS/BLA)	8/19/1	11/10/4	5/23/3
Age (y)	42.0 \pm 11.0	45.7 \pm 13.0	57.1 \pm 6.9*
Statin Use	11%	36%	100%
BMI (kg/m^2)	25.7 \pm 4.0	26.8 \pm 4.4	31.3 \pm 5.7*
HbA _{1c} (%)	—	8.2 \pm 1.5	7.5 \pm 1.3
CRP (mg/L)	1.8 \pm 1.6	3.0 \pm 2.6	3.0 \pm 2.4
Cholesterol (mg/dL)	206.8 \pm 37.5	166.1 \pm 25.6***	183.9 \pm 36.6***
Triglyceride (mg/dL)	131.5 \pm 88.8	64.8 \pm 24.6**	156.1 \pm 78.0

Data are expressed as the mean \pm SD. CRP and Triglycerides log transformed for statistical analysis. *T2D significantly different from Control and T1D, $p < 0.002$ by ANOVA. **T1D significantly different from Controls ($p < 0.001$) and T2D ($p < 0.05$) by ANOVA. ***T1D and T2D significantly different from Controls ($p < 0.05$) by ANOVA. In bold are reported the significant results.

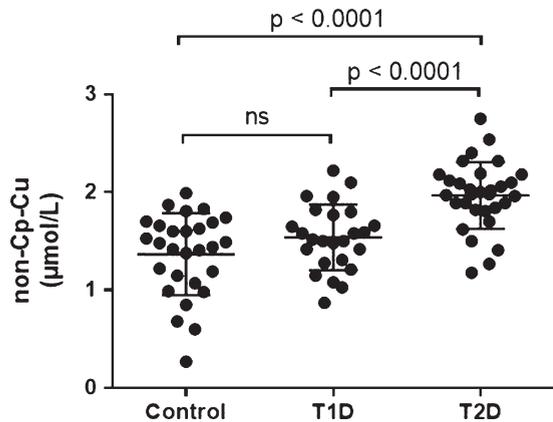


Fig. 1. Non-Cp Cu levels in Control, T1D, and T2D subjects. Scatter plots show the mean and SD of each group. p values determined by ANOVA.

Figure 1 shows non-Cp Cu copper levels measured in the serum samples of all subjects. T2D subjects had significantly higher non-Cp Cu levels ($1.97 \pm 0.31 \mu\text{M}$, mean \pm Standard Deviation, SD) than Controls and T1D subjects (1.37 ± 0.42 and 1.54 ± 0.33 , respectively, both $p < 0.001$ compared to T2D). There was no significant difference between the Control and T1D group.

The relationship between age and non-Cp Cu levels was evaluated, and no significant correlations were observed in any of the study cohorts (Pearson r all $p > 0.1$; Fig. 2). However, since age was significantly higher in the T2D subjects compared to Control and T1D subjects (Table 1), it was used as a covariate in the ANCOVA. There was a significant group effect on non-Cp Cu after controlling for age [$F(1, 80) = 16.2$, $p < 0.001$]. There were significant differences in the age-adjusted non-Cp Cu values between Control and T2D (1.35 ± 0.08 versus 1.98 ± 0.07 , $p < 0.001$) and T1D versus T2D (1.53 ± 0.08 versus 1.98 ± 0.07 , $p < 0.001$). Thus, the differences in non-Cp Cu in T1D were not due to the increased age of this group, relative to the Controls or T1D subjects.

The relationship between BMI and non-Cp Cu levels was evaluated and no significant correlations were observed (Pearson r all $p > 0.1$) in any of the study cohorts (Fig. 3). BMI was significantly higher for T2D compared to Control and T1D subjects (Table 1) and was used as a covariate in the ANCOVA (as above) to evaluate differences between groups (Control, T1D, T2D) that might be accounted for by the increased BMI. There remained a significant group effect on non-Cp Cu after controlling for BMI

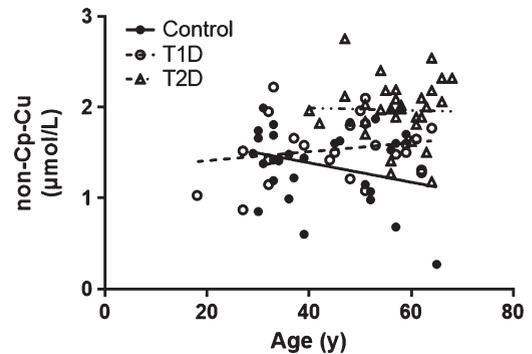


Fig. 2. (Lack of) Correlation between non-Cp Cu and age. The Pearson correlation (r) were -0.280 , 0.191 , and -0.026 for Control, T1D and T2D subjects, respectively (all $p > 0.1$).

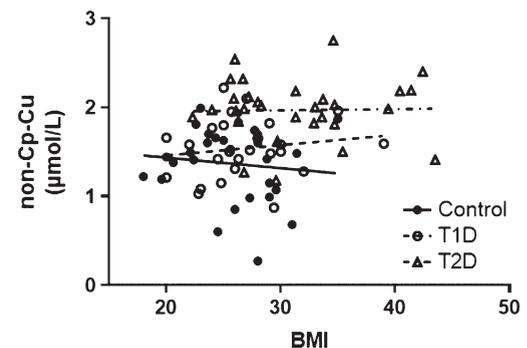


Fig. 3. (Lack of) Correlation between non-Cp Cu and BMI. The Pearson correlation (r) were -0.112 , 0.150 , and -0.022 for Control, T1D, and T2D subjects, respectively (all $p > 0.1$).

[$F(1, 80) = 16.1$, $p < 0.001$]. Significant differences persisted in the adjusted means between Control and T2D (1.37 ± 0.07 and 1.97 ± 0.07 , $p < 0.001$) and between T1D and T2D (1.54 ± 0.08 and 1.97 ± 0.07 , respectively, $p < 0.001$).

There were no significant correlations between non-Cp Cu and HbA_{1c} in the diabetic subjects or CRP in any of the study groups (data not shown).

Multinomial logistic model

A multinomial logistic model has been applied to investigate the relationship between the diagnosis groups (T1D and T2D), assuming as the dependent variable, and the biological and demographical variables, as follows: non-Cp Cu, BMI, age, and sex (Table 2). The model revealed that none of the considered variables had an effect on the risk of having T1D. Conversely, the effect of non-Cp Cu and age on the relative risk (RRR) of having T2D was significant

(Table 2). More specifically, a one unit standard deviation increase of non-Cp Cu increased the relative risk of having T2D by 9.64 with respect to Controls (95% CI: 2.86–32.47, holding constant the effect of the other variables in the model). In other words, we can say that if two subjects have identical age, BMI, and sex, but diverse non-Cp Cu, the subject with the higher standardized non-Cp Cu has a 10-times greater relative risk of having T2D than the subject with the lower value.

Even age had an effect on the risk of T2D but was less significant. In this model, a one-year increase of age raised the relative risk for T2D by about 17% (RRR = 1.17; 95% CI: 1.06–1.29, holding constant the effect of the other variables in the model).

Comparison of non-Cp Cu levels in Controls and T2D and AD

Figure 4 shows the comparison of non-Cp Cu levels in Controls and T2D of the current study with those of an AD population previously studied [17]. Controls had significantly lower non-Cp Cu levels ($p < 0.0005$) than T2D and AD subjects. Levels in T2D ($2.0 \pm 0.3 \mu\text{M}$; mean \pm SD) approached those of the AD cohort ($2.1 \pm 0.6 \mu\text{M}$) with no significant difference between these two groups. It should be noted that the AD subjects were older (81 ± 7 versus 67 ± 7 years), had a greater proportion of females (68 versus 50%), and were mostly European Caucasians compared to mostly Hispanic ethnicity relative to the T2D group in the current study. In spite of these demographic differences, these results demonstrate that elevated serum non-Cp Cu in T2D are comparable to levels observed in AD subjects [17].

Table 2
Multinomial logistic regression results

	RRR	95% CI	<i>p</i>
T1D			
Sex, F versus M	0.76	0.24–2.47	0.653
Standardized non-Cp Cu, standard deviation unit	1.67	0.89–3.14	0.109
BMI, kg/m ²	1.04	0.89–1.21	0.593
Age, years	1.03	0.87–1.08	0.317
T2D			
Sex, F versus M	0.66	0.13–3.41	0.618
Standardized non-Cp Cu, standard deviation unit	9.64	2.86–32.47	<0.001
BMI, kg/m ²	1.18	0.98–1.41	0.076
Age, years	1.17	1.06–1.29	0.002

CONTROL BASE CATEGORY
In bold are reported the significant results.

Comparison of non-Cp Cu levels as revealed through the direct and the indirect method

Figure 5 shows the comparison of two methods of evaluation of non-Cp Cu levels [18]: the direct fluorescent method and the indirect method known as Walshe’s index [13] based on the subtraction of the quantity of the copper bound to ceruloplasmin from the total concentration of copper in the blood serum are compared in samples of 147 healthy subjects ((77 women, 52.7%), mean age 49 ± 12.7 years (25–84), Mini-Mental State Examination (MMSE) [19] 29 ± 1) and 89 AD patients ((52 women, 67.5%),

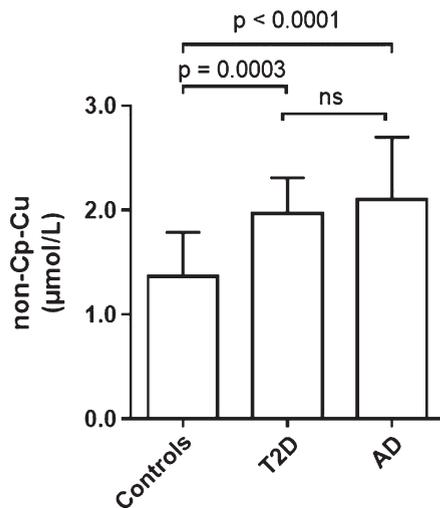


Fig. 4. Comparison of non-Cp Cu levels in Control, and T2D subjects to AD subjects. Scatter plots show the mean and SD of each group. *p* values determined by ANOVA.

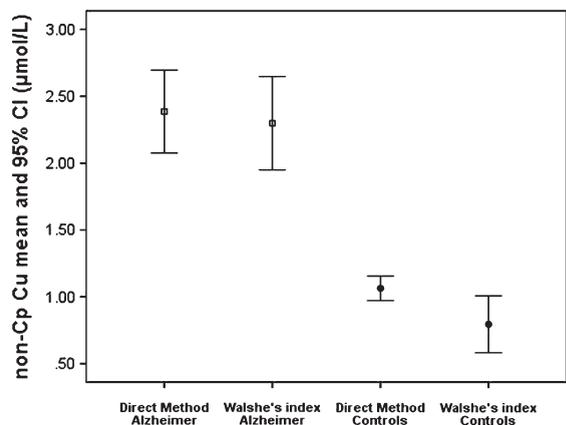


Fig. 5. Comparison of non-Cp Cu levels as revealed through the direct method and the indirect non-Cp Cu Walshe’s index in samples from 147 healthy subjects and 89 AD patients.

mean age 73 ± 8.5 (50–86), MMSE 19 ± 4.7], studied in-depth elsewhere [18]. In healthy controls, the mean value of the direct measurement of non-Cp Cu with the fluorescent method was $1.07 (\pm 0.6)$, and that calculated with the Walshe's index was $0.79 (\pm 1.3)$. In AD patients, the mean value of the direct measurement of non-Cp Cu with the fluorescent method was $2.3 (\pm 1.5)$ and that calculated with the Walshe's index $2.1 (\pm 1.7)$.

DISCUSSION

The main result of the current study is that T2D patients have increased levels of labile non-Cp Cu in the peripheral circulation, which corresponds to an increase in the relative risk of having T2D that is approximately ten times higher than in Controls. This is not true for T1D. T1D, if anything, can be considered more severe than T2D, because of the total or near total immune destruction of insulin producing cells. Copper seems to play no role in T1D. Conversely, an increase in the risk of having T2D corresponding to an expanded pool of non-Cp Cu resembles a chronic dysfunction possibly associated with progressive amyloidosis in the pancreatic islets or other target tissue, theoretically comparable to what happens in the AD brain. A direct link between Cu(II) and AGEs as interacting risk factors increasing the probability of having AD [3, 20] can be suggested. Longitudinal studies are in progress to deepen the connection of copper and glucose altered metabolism and their effects on the cognitive state of diabetes patients.

The results presented in this study confirm previous indirect evidence of elevated systemic chelatable copper, as revealed by increased levels of basal urinary copper excretion in T2D [2, 7–10]. Moreover, they are in line with previous literature of systemic copper excess in T2D patients. More precisely, since the late 1970s, eleven studies demonstrated that copper in serum was increased in patients affected by T2D [21–31], four studies reported no change [32–35], and only two studies reported decreased level of copper in T2D, in comparison with healthy controls [36, 37]. Furthermore, our data can explain results of elevated copper balance in T2D, demonstrated by the fact that oral administration of trientine, a copper chelating agent, increased the urinary copper excretion in T2D patients in comparison with controls, as well as intravenous infusion of the drug dose in Zucker diabetic rats [38, 39].

T1D subjects showed no sign of copper abnormalities in terms of non-Cp Cu elevation, in agreement with previous evidence [31], even though literature regarding serum copper level in patients with T1D is very limited.

Another result of the current investigation is that abnormalities in non-Cp Cu levels revealed in T2D subjects approach those described in an AD population recently studied using the same methodology [17]. The mean value of non-Cp Cu in the AD population used in this comparison is $2.1 \mu\text{M}$, but in previous studies, we reported higher values of non-Cp Cu in AD, ranging from 2.5 – $2.6 \mu\text{M}$ [40, 41] to 2.72 [42]. In the latter cited studies, the non-Cp Cu was evaluated by means of an indirect method, consisting of subtracting the quantity of the copper bound to ceruloplasmin from the total amount of the serum copper, with a calculation known as the Walshe's index [13]. As discussed extensively in previous reports [14], this copper index has limitations. It assumes that ceruloplasmin is saturated, that it is in the correct stoichiometry with the moles of copper, and the most important limit is that the ceruloplasmin is overestimated by the immunologic assay, since it measures either the holo-form of ceruloplasmin or apo-forms of the protein (fragments of the protein not containing copper), thus providing an underestimation of the levels of non-Cp Cu in the calculation. This underestimation has as a result that the values of the Walshe's index can be lower than the corresponding values measured with the direct fluorescent method (the Walshe's formula can provide even negative values). This is evident in Fig. 5 that shows the comparison between the non-Cp Cu evaluated through the Walshe's index and that obtained through the fluorescent direct method, which was applied in this current study and elsewhere [17, 43]. The comparison of the results shows that the two methods highly agree, albeit they do not correlate perfectly. However, the comparison cannot provide any information about the contribution of each copper component (albumin, transcuprein, small copper carriers [44]) present in the blood plasma to the non-Cp Cu pool. The variation in the average of the non-Cp Cu in AD estimated so far can be explained in terms of the method used, the size of the sample, and the demographic characteristics, but mostly by the percentage of the AD patients exhibiting abnormal non-Cp Cu levels present in the subject sample, since it is known that copper homeostasis abnormality is not a general condition of AD, but pertains to a subpopulation of patients, as recently demonstrated [17, 45].

As a whole, these findings strongly sustain a disease cascade pathway connecting high blood glucose levels with copper imbalance and tissue damage, affecting primarily vascular and cardiac tissues in T2D, through AGEs and oxidative stress, as proposed by some authors [2, 46]. From experimental models of the T2D, it derives that the amount of chelatable copper in the plasma of diabetic rats is approximately twice that in normal rat plasma [46]. Furthermore, there is substantial evidence that Cu(II) reacts with collagen and other extracellular matrix components, producing AGE-modification of proteins in blood-vessel walls. This appears as a widespread phenomenon [46–51], also confirmed in studies on humans [8, 39], closely linking copper alterations to the pathogenesis of vasculopathy and organ damage. More specifically, the mechanism appears to involve glycated proteins, which bind transition metals such as copper, and that the complexes Cu(II)-AGEs accumulate within the vasculature [46]. It has been demonstrated that AGEs can bind and localize the oxidative form of Cu(II), which is catalytically-active, thus prone to trigger oxidative stress reactions [8, 52]. The direct fluorescent method we employed to measure non-Cp Cu is specific for Cu(II) oxidative state, thus sustaining a precise involvement of this catalytically active Cu(II) in diabetes oxidative stress leading to tissue damage. Non-clinical models of the disease provided extensive evidence that once the Cu(II) mediated oxidative stress process becomes activated, it results in the progressive enhancement of further protein-modification and consequent copper-trapping potential [50], in a vicious circle of oxidative stress.

AD is another common age-associated disorder with a complex etiology in which metal abnormalities have been implicated. Elevated levels of chelatable non-Cp Cu in brain tissues [53] and body fluids [14] have been reported in humans and extensively in experimental models of AD as well: redox reactions promoting A β interaction with copper in AD have been proposed to cause processes of auto-oxidation promoting A β self-aggregation, eventually leading to oxidative stress and cell death (reviewed in [54]). Studies at the basis of the model of the toxic gain of function of A β mediated by transition metals [55, 56] demonstrated that the amyloid- β protein precursor (A β PP) binds and reduces copper from Cu(II) to Cu(I), modulating copper-induced toxicity in cell cultures and oxidative stress through the production of H₂O₂ [57], which appears to temporally precede A β plaques formation [58]. This results in

A β and metals packed together in plaques [59, 60]. A β plaques disturb brain physiology, entrapping metals within the plaques, while cell-associated copper appears decreased [55].

Even though A β plaques remain major lesions in AD brain, the notion that A β PP mutations, through a gain of function process and A β deposits, are *per se* neurotoxic has repeatedly been called into question [58, 61]. Alternative hypotheses of A β 's loss of function positing a protective role of A β against toxic levels of metals are now substantiated by a critical mass of evidence [58, 62]. A β is proposed to chelate toxic metals and protect neurons from oxidative insult which appears to precede A β pathology [58, 62]. The loss of this protecting function cannot counteract the increase in the metal-oxidative stress insult, prompting the disease cascade [58, 62].

Even though the exact mechanism is still elusive, strong support of the breakdown of copper homeostasis as a risk factor pertinent to AD comes from a myriad of experimental, as well as clinical, genetic, and large population studies [63–67]. In this line, the most representative are the studies of meta-analyses, which show copper imbalance in AD, consisting of copper decreases in the brain [68], along with increases of total copper and non-Cp chelatable copper in the serum of AD patients [14, 69]. This condition is a typical sign of copper breakdown homeostasis, as shown by Wilson's disease, the paradigmatic disorder of non-Cp Cu toxicosis and accumulation [11, 12].

Our results, therefore, would indicate that non-Cp Cu is specifically linked to T2D pathophysiology [2], suggesting a role similar to that already proposed in AD [70]. In past years, considerable evidence indicated that AD and diabetes share common pathways [71] and that altered copper and glucose metabolisms can contribute to the disease onset of these complex diseases. Enzymes and second messenger abnormalities in terms of mitochondrial dysfunctions and glucose and copper abnormalities have been described in both diseases. A receptor for AGEs (RAGE), a multi-ligand receptor, has been described to play a role in several inflammatory pathways shared among diabetes, obesity, and AD, transporting the circulating A β across the brain-blood barrier, producing oxidative stress and cerebral blood flow abnormalities [6, 72].

Current results strengthen previous evidence [2], which demonstrated that diabetes-evoked copper dysregulation has the potential to be a target for therapeutic and preventive interventions aimed at

preserving organ function in diabetes, heart failure, and some neurological disorders connected with aging, in line with recent suggestions to the public [73].

Limits of our study are the small size of the sample, possible sampling bias relative to the subjects studied and most of all the lack of a cognitive follow-up of the T2D patients. Moreover, our estimation of the RRR has a large IC due to the non-Cp Cu variability in the T2D group, which has then to be repeated in a new T2D population. Confirmation of the present findings in larger longitudinal cohorts to explore the connection between copper, alterations of glucose metabolism, risk of developing T2D, and their relationships with cognitive impairment and AD are therefore necessary.

CONCLUSIONS

The comparison of non-Cp Cu levels in T2D with those of an AD population previously studied shows rising blood non-Cp Cu copper levels from Controls to T2D and AD, demonstrating the involvement of catalytically-active Cu(II) and glucose dysregulation in oxidative stress reactions leading to tissue damage in both diseases. We suggest that the increased risk of AD in patients with T2D, as reported by epidemiological studies, may be mediated by abnormal copper metabolism, which even alone affects the risk on the disease.

ACKNOWLEDGMENTS

Authors thanks Dr. Valentine Negrouk for technical support.

This work was supported by National Research Council [Aging Program 2012–2014, ‘A low-copper diet as a preventive strategy for cognitive disability in Aging’]; Italian Ministry of Health [5XMille project ‘Un metodo sensibile, diretto e preciso per misurare il rame Non-legato alla Ceruloplasmina nel siero per applicazione in ambiente clinico’ 02/09/2013 al 31/08/2015]; Italian Ministry of Health [‘Tolerability and efficacy of Zinc therapy in Mild Cognitive Impairment for treatment and prevention of Alzheimer’s disease: a prospective, randomized, double blind, parallel, placebo controlled Phase II clinical trial’ (Project Code: CO-2013-02358488)]; Canox4drug SpA [2013–2016 ‘Non-Ceruloplasmin copper in Alzheimer’s disease’ (Prot. 30/2013)], Italian Ministry of Health, Ricerca Corrente.

Authors’ disclosures available online (<http://j-alz.com/manuscript-disclosures/16-1033r2>).

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