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Copper imbalance in Alzheimer's disease: meta-analyses of serum, plasma, brain studies, and a replication study accounting for ATP7B gene variants

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Corresponding Author:	Rosanna Squitti IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy Rome,, Rome ITALY
Order of Authors:	Rosanna Squitti
	Mariacarla Ventriglia
	Ilaria Simonelli
	Cristian Bonvicini
	Alfredo Costa
	Giulia Perini
	Giuliano Binetti
	Luisa Benussi
	Roberta Ghidoni
	Giacomo Koch
	Barbara Borroni
	Alberto Albanese
	Stefano Sensi
	Mauro Rongioletti
Abstract:	Background: Previous evidence indicates that Alzheimer's disease (AD) patients exhibit signs of copper dyshomeostasis. However, a meta-analysis of all eligible copper studies on brain and serum/plasma, aimed at establishing whether copper dyshomeostasis can be considered as an AD susceptibility factor, has never been performed. Methods: We performed several meta-analyses of 51 studies of levels of copper markers in serum/plasma (pooled total of 2592 AD and 3335 controls), and in brain specimens (pooled total of 155 AD and 135 controls). We also performed a replication study of serum biomarkers of copper status (copper, ceruloplasmin, ceruloplasmin specific activity, non-ceruloplasmin copper, and copper to ceruloplasmin ratio) in 97 AD patients and 70 controls screened for rs732774 and rs1061472 of ATP7B gene (encoding for the copper transporter ATPase7B). Results: Our meta-analyses show that brain copper is decreased ($p<0.01$) while serum/plasma copper ($p<0.001$) and non-ceruloplasmin copper are increased ($p<0.001$) in AD patients compared with healthy controls, while ceruloplasmin levels don't change. The excess of serum/plasma copper is associated with a 3 to 4-fold increase of the risk of contracting AD. Our replication study confirmed the meta-analyses results and the increased frequency of the mutated alleles ATP7B rs732774 and rs1061472 in AD (pcorrect=0.012). Conclusions: Overall, the results show that AD patients fail to maintain a correct metabolic balance of copper that appears to be linked to the ATP7B gene. This suggests an urgent need for precision medicine approaches that, by fully contemplating the heterogeneity of AD, may target specific biological subsets rather than broad clinical entities.

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Suggested Reviewers:	Matthew Schrag Department of Neurology, Yale University, New Haven, Connecticut matthew.schrag@yale.edu
	Marco Vinceti University of Modena and Reggio Emilia, marco.vinceti@unimore.it
	Ashley I Bush The Florey Institute of Neuroscience and Mental Health, Kenneth Myer Building, The University of Melbourne, Parkville, VIC, Australia ashley.bush@florey.edu.au
	George Brewer University of Michigan Medical School brewergj@umich.edu
	Anthony R White University of Melbourne arwhite@unimelb.edu.au
	Stephen Kaler National Institutes of Health KalerS@mail.nih.gov
Opposed Reviewers:	

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Biological Psychiatry
Editor-in-Chief
Prof. John H. Krystal, MD
Yale University, School of Medicine
and VA Connecticut Healthcare System,
West Haven, Connecticut, United States

Dear Prof. Krystal,

In the attachment, please find the manuscript '**Copper imbalance in Alzheimer's disease: meta-analyses of serum, plasma, brain studies, and a replication study accounting for *ATP7B* gene variants**' that we are submitting to *Biological Psychiatry* for consideration as an *Archival Report*.

Since your Journal covers a broad range of topics related to thepathophysiology and treatment of major neuropsychiatric disorders we hope thatour clinical neuroscience contribution that also addresses genetic and environmentalrisk factor, as copper exposure, and Alzheimer's disease might be ofinterest. Current meta-analysis updates data of copper in the brain presented in a 2011 (Schrag et al Prog Neurobiol2011) paper and integrate them by extending meta-analyses of copper in the blood/serum, showing the global picture of copper misbalance in Alzheimer's disease. The main findings of the study, a rigorous systematic analysis that we think can make a significant contribution to thefield, support the notion that copper dyshomeostasis is a contributing feature of Alzheimer's disease brain. Our data also provide the rationale for a phase II clinical trial, funded by the Alzheimer's Association (number PTC-19-602325;EudraCT No.: 2019-000604-15), and in which we are evaluating the tolerability and efficacy of a zinc-based approach to block the cognitive decline of a subset of subjects suffering from Mild Cognitive Impairment who also show signsof copper dysmetabolism. The choice of employing a zinc-based approach moves from the analogous therapy that is used to treat the copper dysregulation that is driving the onset and the progression of Wilson disease.

The content of the paper has not been published or submitted for publication elsewhere. The manuscript conforms to Ethical Adherence, grants availability of data and all authors have contributed significantly and are in agreement with the content of the manuscript. Specifically, Rosanna Squitti, Mariacarla Ventriglia, Stefano Sensi and Mauro Rongioletti performed conceptualization; Rosanna Squitti, Mariacarla Ventriglia, Cristian Bonvicini Alfredo Costa, Giulia Perini and Ilaria Simonellli also contributed to data curation and formal analyses; Rosanna Squitti, Giuliano Binetti, Luisa Benussi, Roberta Ghidoni, Giacomo Koch, Barbara Borroni, Alberto Albanese, Stefano Sensi significantly contributed in writing the original draft, review and editing.

We hope you will find the study suitable for publication in *Biological Psychiatry*.

Warm regards,

Rosanna Squitti and Stefano Sensi

Molecular Markers Laboratory
IRCCS Istituto Centro San Giovanni di Dio- Fatebenefratelli
Via Pilastroni, 4

25125 Brescia, Italy
phone 0039-030-3501725
fax 0039-030-3501592

We feel that the following scientists have the expertise to review the study.

Matthew Schrag, MD,PhD
Department of Neurology, Yale University, New Haven, Connecticut.
email address: matthew.schrag@yale.edu

Marco Vinceti, MD
University of Modena and Reggio Emilia, Research Center in Environmental, Nutritional and Genetic Epidemiology (CREAGEN), Department of Biomedical, Metabolic and Neural Sciences, Via Campi 287, Modena, Italy, 41125.
email address: marco.vinceti@unimore.it

Ashley I Bush, MD PhD
The Florey Institute of Neuroscience and Mental Health, Kenneth Myer Building, The University of Melbourne, Parkville, VIC, Australia.
Abstract Australia
email address: ashley.bush@florey.edu.au

George Brewer, MD
Departments of Human Genetics and Internal Medicine
University of Michigan Medical School
5022 Kresge Bldg. II, Ann Arbor, MI 48109-0534
Phone: (734) 764-5499, Fax: (734) 615-2048
email address: brewergj@umich.edu

Tjaard Hoogenraad, MD
Department of Neurology
University Hospital
Utrecht, The Netherlands
email address: tu.hoogenraad@planet.nl

Assoc. Prof. Anthony R. White
Head, Biometals in Brain Health and Disease
Department of Pathology
University of Melbourne
Victoria, 3010
Australia
Tel: +61 3 8344 1805
email address: arwhite@unimelb.edu.au

Kaler, Stephen G. (NIH/NICHD) [E]
Stephen G. Kaler, MD
Senior Investigator and Head,
Section on Translational Neuroscience;

Molecular Medicine Branch, NICHD
Porter Neuroscience Research Center II
Building 35, Room 2D-971
35A Convent Drive, MSC 3754
National Institutes of Health
Bethesda, MD 20892-3754
email address: KalerS@mail.nih.gov>,

Frank LaFerla, PhD
University of California, Irvine 3212 Biological Sciences III MailCode: 4545. Irvine, CA 92697.
email address: laferla@uci.edu

Patrick Sullivan, PhD
University of Kentucky College of Medicine
https://neuroscience.med.uky.edu/users/patsull
email address: patsullivan@uky.edu

Massimo Tabaton, MD
Università di Genova, NEUROLOGIA.
Phone (+39) 010 353 7064.
Email address: mtabaton@unige.it

Prof. Amos D. Korczyn, MD
Sieratzki Professor of Neurology at Tel-Aviv University.
Email address: amoskor@tau.ac.il

Copper imbalance in Alzheimer's disease: meta-analyses of serum, plasma, brain studies, and a replication study accounting for *ATP7B* gene variants

Rosanna Squitti,[a] Mariacarla Ventriglia,[b] Ilaria Simonelli,[b] Cristian Bonvicini,[a] Alfredo Costa[c,d], Giulia Perini[c,d], Giuliano Binetti[e], Luisa Benussi[a], Roberta Ghidoni[a], Giacomo Koch[f,g], Barbara Borroni[h], Alberto Albanese[i], Stefano L. Sensi[j,k,l] and Mauro Rongioletti[m]

[a]Molecular Markers Laboratory, IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy

[b]Fatebenefratelli Foundation for Health Research and Education, AFaR Division, San Giovanni Calibita Fatebenefratelli Hospital, Isola Tiberina, Rome, Italy

[c]Unit of Behavioral Neurology, IRCCS Mondino Foundation, Pavia, Italy

[d]Dept. Of Brain and Behavior, University of Pavia, Italy

[e]MAC Memory Clinic and Molecular Markers Laboratory, IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy

[f]Section of Human Physiology, University of Ferrara, Ferrara, Italy

[g]Department of Clinical and Behavioural Neurology, Santa Lucia Foundation IRCCS, Rome, Italy

[h]Centre for Neurodegenerative Disorders, Department of Clinical and Experimental Sciences, University of Brescia, Italy

[i]Department of Neurology, IRCCS, Istituto Clinico Humanitas, Rozzano, Milan, Italy

[j]Department of Neuroscience, Imaging and Clinical Science, "G. D'Annunzio" University of Chieti-Pescara

[k]Institute for Mind Impairments and Neurological Disorders - iMIND, University of California - Irvine, Irvine, USA

[l]Molecular Neurology Units, Center for Advanced Studies and Technology (CAST), University G. D'Annunzio of Chieti-Pescara, Italy

[m]Department of Laboratory Medicine, Research and Development Division, San Giovanni Calibita Fatebenefratelli Hospital, Isola Tiberina, Rome, Italy

Correspondence: *Rosanna Squitti*, Molecular Markers Laboratory, IRCCS Istituto Centro San Giovanni di Dio- Fatebenefratelli, 25125 Brescia, Italy telephone 0039-030-3501725; fax 0039-030-3501592; email: rosanna.squitti@afar.it

Stefano Sensi, Molecular Neurology Units, Center for Advanced Studies and Technology (CAST), University G. D'Annunzio of Chieti-Pescara, Italy; *Tel.: 0039- 0871-541542;* email: ssensi@unich.it

Running title: Cu imbalance in AD

Key words: Alzheimer's disease, copper, ceruloplasmin, meta-analysis, brain, serum, ATP7B, Wilson disease

Abstract

Background: Previous evidence indicates that Alzheimer's disease (AD) patients exhibit signs of copper dyshomeostasis. However, a meta-analysis of all eligible copper studies on brain and serum/plasma, aimed at establishing whether copper dyshomeostasis can be considered as an AD susceptibility factor, has never been performed.

Methods: We performed several meta-analyses of 51 studies of levels of copper markers in serum/plasma (pooled total of 2592 AD and 3335 controls), and in brain specimens (pooled total of 155 AD and 135 controls). We also performed a replication study of serum biomarkers of copper status (copper, ceruloplasmin, ceruloplasmin specific activity, non-ceruloplasmin copper, and copper to ceruloplasmin ratio) in 97 AD patients and 70 controls screened for rs732774 and rs1061472 of *ATP7B* gene (encoding for the copper transporter ATPase7B).

Results: Our meta-analyses show that brain copper is decreased ($p<0.01$) while serum/plasma copper ($p<0.001$) and non-ceruloplasmin copper are increased ($p<0.001$) in AD patients compared with healthy controls, while ceruloplasmin levels don't change. The excess of serum/plasma copper is associated with a 3 to 4-fold increase of the risk of contracting AD. Our replication study confirmed the meta-analyses results and the increased frequency of the mutated alleles *ATP7B* rs732774 and rs1061472 in AD ($p_{correct}=0.012$).

Conclusions: Overall, the results show that AD patients fail to maintain a correct metabolic balance of copper that appears to be linked to the *ATP7B* gene. This suggests an urgent need for precision medicine approaches that, by fully contemplating the heterogeneity of AD, may target specific biological subsets rather than broad clinical entities.

INTRODUCTION

Alzheimer's disease (AD), the most common neurodegenerative disorder, is a multi-factorial condition for which an effective therapy is still missing. Hallmarks of AD are extracellular deposits of the beta-amyloid protein (Aβ), intraneuronal aggregates of protein Tau and reactive gliosis. Many modifiable risk factors, including diabetes, midlife hypertension, midlife obesity, physical inactivity, depression, smoking and even low educational and personal realization levels (1) concur in shaping the disease's onset and progression. Several seminal epidemiological studies have been published but they have neglected the investigation of a possible role of dietary factors and metal imbalance in modulating AD, since data of their prevalence were missing in the literature (1). This omission has likely contributed to the fact that metals have never been considered as modifiable risk factors of AD. Almost a decade ago, however, a novel metal hypothesis (2) and several meta-analyses (3-6) have triggered a new interest in the interaction between some metals (mainly iron, copper and zinc) and AD-related proteins (primarily the APP/Aβ system).

Copper (Cu) is an essential micronutrient, catalyst or component of many metallo-proteins as well as enzymes involved in systems that span from connectivity to brain function and help to control physiological cellular life and energy production. Non-ceruloplasmin Cu is the fraction of Cu in serum/plasma that does not bind to ceruloplasmin, the protein that carries Cu in the blood [review in (7)]. The expansion of non-ceruloplasmin Cu in plasma is toxic since it can cross the blood brain barrier (BBB) and increase the labile pool of the metal in the brain. In fact, this is what happens in Wilson disease, a rare autosomal recessive disorder caused by mutations of the *ATP7B* gene. ATPase7B (encoded by *ATP7B*) is a Cu pump located in the hepatocyte and the endothelial cells at BBB. The pump loads Cu onto nascent ceruloplasmin in the hepatocyte and onto the glycophosphatidyl inositol ceruloplasmin expressed in the astrocyte [review in (8)]. Defects in this process are associated to an increased release of non-ceruloplasmin Cu in the blood [review in (8)].

Here we report on new meta-analyses specifically focused on Cu metabolism in the brain and serum/plasma of AD patients and healthy controls. Moreover, we describe a replication study of

serum Cu and non-ceruloplasmin Cu, ceruloplasmin concentration, ceruloplasmin activity, ceruloplasmin specific activity (iCp/eCp), and Cu to ceruloplasmin ratio (Cu:Cp), along with genotyping of the 2 functional single nucleotide polymorphisms (SNPs) rs1061472 and rs732774 of *ATP7B* (9), in a sample of 97 AD patients and 70 healthy controls.

METHODSAND MATERIALS

Meta-analyses

Search strategy

To identify appropriate studies for meta-analysis, we followed the procedural steps indicated by Cochrane (http://www.cochrane-handbook.org). We entered the keywords "Alzheimer's disease", "copper", "serum", "plasma", "brain", "metals" and their combinations in PubMed and selected studies from 1984 to 2019. We also identified other studies by using "Scopus" and "ISI Web of Knowledge" databases. Besides, reviewing the contents of this first selection, we also evaluated their reference lists to search for additional studies via Google Scholar. We considered only papers showing comparative analyses of AD and healthy populations, presenting original results and published in peer-reviewed journals. In most studies, the severity of the AD patients' cognitive decline had been assessed by a Mini-Mental State Examination (MMSE, (10)). The target of our meta-analysis was the comparison, between AD patients and controls, of the Cu weighted means reported in the selected papers.

Our first selection consisted of 54 studies. At this point, we further required that manuscripts explicitly present Cu mean and standard deviation values for both patient and control groups. From the first selection, one study that did not report standard deviation values (11) has been excluded and one that did not report the exact value of Cu levels (12) was dropped. We excluded the study by Alimonti et al. (13) because it was conducted partially on the same sample as the study by Bocca and colleagues (14). We also included data from 721 subjects investigated in our previous studies

(15) excluding the studies from our laboratory in which the patient samples partially were overlapped.

At this point, our selection had been reduced to 51 studies: 10 on Cu in brain (**Table 1**), 37 on Cu in serum and 4 in plasma (**Table 1 and Table 2 Supplementary material**); 18 studies of the latter section reported data on non-ceruloplasmin Cu in serum and on ceruloplasmin (**Table 2)**]. We performed separate meta-analyses of: Cu in brain, Cu in serum and plasma pooled together, non-ceruloplasmin Cu and ceruloplasmin in serum plasma pooled together.

Of these 10 studies in brain, we also performed a meta-analysis stratified on the basis of the functions of different areas of brain: hippocampus, amygdala, frontal cortex, cerebral cortex, cerebellum.

Data extraction for Meta-analysis

All the steps for studies selection, data extraction and statistical procedures applied in the meta-analysis are reported in the **Supplementary Materials**.

Replication study

Human Subjects

The study was approved by the Local Ethics Committees (approval number 53/2016), and all subjects signed an informed consent (approval number 2/1992; 26/2014). Ninety-seven AD patients (16, 17) [with a Mini-Mental State Examination (MMSE) score of 25 or less(10)] and 70 cognitively normal individuals were included in the replication study. Procedures applied for fasting blood sample collections, biochemical and genetic investigation, as well as the statistical analyses applied in the replication study, are described in **Supplementary Materials**.

RESULTS

Demographic and Cu data from the studies selected for our meta-analyses of brain Cu are listed in **Table 1**. Demographic data from the studies selected for our meta-analyses of Cu, non-ceruloplasmin Cu, and ceruloplasmin in serum/plasma are reported in **Table 1 Supplementary material**. Biochemical indices of the studies included in the meta-analyses of Cu in serum/plasma are reported in **Table 2 Supplementary material**, while those evaluated in the meta-analyses of non-ceruloplasmin Cu and ceruloplasmin status are shown in **Table 2**.

Meta-analyses of Cu in brain tissues

Sixteen studies were identified in the primary screening; 6 were excluded (refer to **Table 1),** after the application of the exclusion criteria. Clinical data and demographic information limited to age, sex, and cerebral area of the metal measurements are reported in **Table 1**. The pooled sample was made of 155 AD samples and 135 control samples. **Figure 1** shows Cu concentrations stratified for the hippocampus, amygdala, an undefined 'cerebral cortex' area, and the frontal cortex. Only one study analyzed the cerebellum (18). The standardized mean difference (SMD) was statistically significant and equals to -0.77 (95% CI -1.16 to -0.38; $p<0.001$), thereby indicating that Cu levels were depleted in the AD group in most regions (**Figure 1**).

We also analysed data from 4 studies focused on the hippocampus (18-21) with a pooled sample made of 42 AD patients and 41 controls. All these studies reported lower Cu levels in AD patients when compared to healthy controls but statistical significance was achieved only in 2 studies (18, 21) (SMD=-0.89, 95% CI from -1.54 to -0.24; $p=0.007$). The heterogeneity was not significant ($I^2=45.8\%$, $p=0.136$).

The amygdala was considered only in 2 studies (18, 19) for a total of 28 patients and 27 controls. One study reported significantly lower Cu concentrations in AD patients compared to controls(18); the pooled SMD in this group was statistically significant (SMD=-0.94, 95% CI from -1.82 to -0.07; $p=0.035$). The heterogeneity was moderate and not significant ($I^2=55\%$, $p=0.136$).

Three studies reported data collected from the frontal cortex (22-24), for a pooled of 34 AD and 28 controls. While Magaki and colleagues (22) reported significantly lower levels of Cu in the AD brain, Loeffler and colleagues indicated Cu increases (24). In this subset, the pooled SMD was not significant (SMD= -0.80, 95% CI from -2.96 to 1.36; p=0.468) and an elevate heterogeneity was observed (I^2=91.2%, p<0.001).

Data on the cerebral cortex were obtained from 4 studies (18, 25-27). All these studies reported lower levels of Cu in AD patients than in healthy controls, even though the difference was statistically significant only in 2 studies (25, 27), where the pooled SMD was -0.78 (95% CI from -1.14 to -0.41; p<0.001). The heterogeneity was not significant (I^2=0%; p=0.619).
Only one study reported data about Cu concentration in the cerebellum (18).

In general, the high heterogeneity emerging from the analysis was due to differences in the magnitude of the effect size and the methodology used to assess Cu in each study. There was no significant difference among the different brain areas, (Test for subgroups χ^2 (4)=0.57, p=0.97).

Meta-analyses of Cu in serum/plasma

Data regarding the 37 studies on serum Cu and the 4 studies investigating plasma are shown in **Figure 2** and **Table 1 and Table 2 Supplementary material**. Data were from 2592 AD patients and 3335 healthy controls. Patient samples ranged from 5 (28) to 385(15) individuals. The mean age of the participants was>70 years, except for a few studies (29-32) that investigated younger subjects. The percentage of women in the AD group ranged from 20% (28) to 100% (33). The analyzed population was Caucasian in 20 studies, Asian in 17, and Argentinean in one study (34). **Figure 2** shows increased Cu levels in serum/plasma in the AD group (SMD = 0.64, 95% CI 0.33-0.96, p < 0.0001). Heterogeneity was high (I^2= 96.3%; p<0.001).

The funnel plot (**Figure 1 Supplementary material**) appears asymmetrical, but asymmetry is not statistically significant (Egger test: beta=0.77, SE=2.10; p=0.716). Meta-regression revealed that the difference in mean age between AD patients and healthy control had no effect on the

analysis (beta=-0.05, SE=0.32; p=0.300). The difference of the SMD for Cu in serum/plasma was 0.64 so that the formula

$$SMD = \sqrt{lnOR}$$

delivers OR=3.19. This indicates that a µmol/L unit increase of serum/plasma Cu generates 4-fold higher odds in AD patients as compared to healthy controls.

Meta-analyses of non-ceruloplasmin Cu

Data regarding the 18 studies included in the meta-analysis are reported in **Figure 3** and **Table 2.** Data were from 1595 AD subjects and 2399 healthy controls. The patient sample size ranged from 28 (35, 36) to 385(15) individuals and the control sample size from 20 (37) to 716 (38).

Results, shown in **Figure 3**, indicate that AD subjects had higher levels of non-ceruloplasmin Cu than healthy controls (SMD = 0.32, 95% CI 0.06–0.57, p =0.014). There was high heterogeneity among the included studies (I^2 = 91.5%; p<0.001). The heterogeneity was likely due to the difference in the methodology applied to quantify the levels of Cu. In order to check for possible publication bias, we performed a funnel plot (**Figure 2 Supplementary material**) that revealed no evidence of asymmetry, as confirmed by the egger test (b=-1.98, SE=2.19; p=0.380).

We ran a sensitivity analysis taking into account the Cu:Cp ratio as a Cu index for internal quality control verification of ceruloplasmin calibration that provides information about the actual stochiometry between Cu and ceruloplasmin in the specimens. As we previously discussed in detail (6), 6-8 range obtained in healthy controls for Cu:Cp allows to obtain a more reliable non-ceruloplasmin Cu value when applying Walshe's formula (6, 39) (see Methods Section). On this basis, 7 studies with a Cu:Cp ratio lower than 6 and higher than 8 in healthy controls were excluded from the meta-analysis (29, 35, 36, 38, 40, 41) (**Figure 4**). By excluding these 7 studies, the pooled AD sample was made of 1082 and the pooled healthy control sample of 1289 subjects. The result was SMD = 0.59 (95% CI 0.38–0.81) and remained significant (p < 0.001) (I^2=79.7%, p<0.001).

Meta-regression revealed that the difference in mean age between AD patients and healthy controls had no effect on the analysis (beta=0.34, SE=0.19; p=0.095). The difference of the SMD for non-ceruloplasmin Cu in serum/plasma was 0.32 which delivers OR=1.79 when applying the formula

$$SMD = \sqrt{lnOR}$$

indicating that for a µmol/L unit increase of non-ceruloplasmin Cu there was an a ca. 2-fold increase in odds of having AD compared to healthy controls. Considering the results of the sensitivity analysis, SMD=0.59 gives OR=2.91, indicating that for a µmol/L unit increase of non-ceruloplasmin Cu there was a 3-fold increase in odds of having AD compared to healthy controls.

Meta-analysis of ceruloplasmin in serum/plasma

Data regarding the 17 studies included in the meta-analysis are reported in **Figure 3 Supplementary material** and **Table 2**. Data were from 1551 AD patients and 2371 healthy controls. Results reported in **Figure 3 Supplementary material** show no difference in ceruloplasmin levels between AD patients and healthy controls [SMD=0.04, 95% CI -0.11 to 0.19 (p=0.589)]. There was a high heterogeneity among the included studies (I^2=75.3%, p<0.001).

No significant publication bias was observed (beta=0.48, SE=1.34; p=0.727; **Figure 4 Supplementary material**). The meta-regression did not show a significant effect of the difference in mean age between AD patients and healthy controls (beta=0.088, SE=0.12; p=0.487).

Replication study

A total of 167 participants were recruited for this study (97 patients and 70 healthy controls). The demographic and serum variables of Cu are shown in **Table 3**. The two groups were different for age and sex (**Table 3**). While ceruloplasmin did not correlate significantly with age, a slightly positive association between age and specific activity of ceruloplasmin (r=0.244; p=0.002), non-ceruloplasmin Cu (r=0.311; p<0.001) and Cu:Cp ratio (r=0.28; p<0.001) was observed.

The two study groups were no different for sex distribution, even though approaching the significance threshold [healthy control men: 22.9% (16/70) vs. AD men: 37.1% (36/97)]; Chi square =3.85, df (1) p = 0.050), and Cu, ceruloplasmin levels and activity were found to be different between men and women. To account for this potential bias, all the analyses were corrected for sex and age. In healthy controls, the correlation between ceruloplasmin concentrations and serum Cu levels was 0.86 ($p<0.001$), the correlation between ceruloplasmin concentrations and ceruloplasmin activity values was 0.75 ($p<0.001$), the correlation between ceruloplasmin activity and serum Cu levels was 0.77 ($p<0.001$) and between non-ceruloplasmin Cu and the Cu:Cp ratio was 0.97 ($p<0.001$). Cu:Cp ratio in healthy controls was 6.6 (0.81), thereby demonstrating good agreement between Cu and ceruloplasmin, allowing the application of Walshe's formula(39). Among the variables under study, total serum Cu, non-ceruloplasmin Cu and the Cu:Cp ratio were higher in AD patients (**Table 3**).

Allele, genotype and haplotype distribution of *ATP7B* rs732774 and rs1061472 along with main demographic and clinical characteristics of the 91 AD patients and 69 healthy controls (genotype data from 6 AD patients and 1 healthy control were not available, see method section in Supplementary material) participating in the genetic study are reported in **Table 4**.

A difference between patients and controls was found when analyzing genotype frequencies for rs732774 ($p_{correct}$=0.032). As for allele carrier frequencies (AA+AG), carriers of at least one A allele were more frequent in the AD group compared to controls (92% vs.78%; $p_{correct}$=0.011, OR=3.33 95%CI: 1.28-8.70; **Table 4**).

As for rs1061472, no differences emerged between the two study groups ($p_{correct}$=0.097), while carriers of at least one G allele (GG+GA) were more frequent in the patient group (89% vs.75%; $p_{correct}$=0.031, OR=2.65 95%CI: 1.13-6.21).

Finally, we built the haplotypes of the two polymorphisms and observed that cluster genotype frequencies were significantly different between AD and controls ($p_{correct}$=0.022); in

particular, AG haplotype carriers were more frequent in the AD group (89% vs. 75%; $p_{correct}$=0.012, OR=3.30 95%CI: 1.26-8.69).

To evaluate the effect of biochemical variables on the probability of being susceptible to AD, an univariable binary logistic regression model was applied. The model also took into consideration carriers and non-carriers of the AG haplotype, age, and sex (**Table 5**). A multivariable analysis was performed that included the model biochemical variables with a p value<0.10 at the univariable analysis. The model revealed a significant effect of non-ceruloplasmin Cu on the probability of having AD with an increased risk of 1.32 times (95% CI 1.06-1.64; p=0.012) for each µmol/L unit increase in non-ceruloplasmin Cu when keeping all other independent variables constant. In the multivariable model, the effect of the AG haplotype on the probability of having AD was borderline significant, with a probability of having AD about 2.49 times higher in individuals carrying the AG haplotype compared to GA/GA haplotype carriers (95% CI 0.99-6.28; p=0.053; only 27 individuals were GA/GA carriers; **Table 5**).

DISCUSSION

The results of our meta-analyses and the replication study show that decreased Cu levels in brain regions as the hippocampus, amygdala, and cerebral cortex characterize the AD group. The AD cohort also exhibited increased levels of Cu and non-ceruloplasmin Cu in serum/plasma, despite no changes in ceruloplasmin levels. Distinction between brain Cu deficiency and excess non-ceruloplasmin Cu breaks down in the notion of a systemic AD Cu dyshomeostasis that features both aspects directly, being two facets of the same biological process (6, 8).

The meta-analyses investigating differences of Cu and non-ceruloplasmin Cu levels in the serum/plasma fractions revealed high heterogeneity. The phenomenon is possibly explained by either methodological heterogeneity or the presence of a skewed percentage of AD subjects with increased levels of non-ceruloplasmin Cu in the study cohorts. As a matter of fact, previous studies have shown that the use of non-ceruloplasmin Cu as a biomarker to identify AD patients resulted in

a 95% specificity but only a 40-50% sensitivity. Therefore and non-ceruloplasmin Cu is not a suitable AD diagnostic biomarker. Nevertheless, as previously indicated (42-46), non-ceruloplasmin Cu can well serve as a susceptibility/risk and stratification biomarker for a subset of AD patients susceptible to Cu exposure.

Given the obtained SMD, the probability of having AD increases 4 times for each µmol/L unit increment of Cu and 3 times for each µmol/L unit increase in non-ceruloplasmin Cu in serum/plasma. These findings are in line with previous retrospective (47) and prospective studies (46, 48).

Results of our replication study confirm the findings of the meta-analyses, indicating increased non-ceruloplasmin Cu levels in an independent cohort of AD patients who were compared with healthy control individuals. Moreover, we found that the functional SNPs rs732774 and G rs1061472 as well as the derived AG haplotype of *ATP7B* (9) were more frequent in AD patients. The multivariate model indicated that non-ceruloplasmin Cu may explain a 1.32-fold increased risk of AD for each µmol/L unit increase. The effect of *ATP7B* AG haplotype on the risk of having AD, even though significant in the univarable model, was borderline when considering a multivariate model **(Table 3)**. The last analysis suffered from the small size of the subjects sample even though the genetic study was not underpowered.

Which are the biological processes underlying these intriguing results? Non-ceruloplasmin Cu is a low molecular weight pool of Cu in the bloodstream that easily crosses the BBB and that is toxic above a certain cut-off [higher than 1.6 µmol/L (39, 49)]. Increased labile Cu within the brain can potentially underlie a number of the pathological abnormalities common to AD and other neurodegenerative disorders. Labile Cu can help drive aggregation and accumulation of Aβ peptide and plaque formation, as well as drive intracellular accumulation of phosphorylated tau due to the presence of Cu-binding sites on these proteins. Furthermore, Cu imbalance can facilitate bioenergetic abnormalities in mitochondria, impaired glucose utilization, oxidative stress, inflammation, calcium dyshomeostasis, and heterogeneously disturbed iron, Cu, and zinc levels of

the brain [review in (8)]. An important role for abnormal Cu balance is also major effects on key proteins implied in Cu buffering and functions at the synapses during glutamatergic transmission. This has been reported for Aβ, prion protein, α-synuclein, cytochrome C oxidase, superoxide dismutase 1, ceruloplasmin, ATPase7B, metallothioneins and dopamine beta-hydroxylase and recently reviewed in (8), further sustaining the *Metal Hypothesis* of AD (2) postulated some years ago. The authors proposed that the AD pathogenesis is driven by the interaction of Aβ with metals at glutamatergic synapses that facilitates the aggregation of the oligomers in insoluble plaques, short-circuiting the entire neuronal network to which those synapses belong. Overall, Cu homeostatic machinery in the brain is a complex pathway and it seems that when the metal balance in some brain areas is sufficiently disrupted (12) it can eventually facilitate oxidative stress, mitochondrial and autophagic dysfunctions and neurodegeneration [review in (8)].

The systemic Cu imbalance in AD may occur with mechanisms that share some similarities with the ones described in Wilson disease, as shown for serum and urine indicators (15). Wilson disease is typified by excess non-ceruloplasmin Cu and in early stages by normal or even low level of Cu-bound proteins in the brain, as reported in studies on the toxic milk mouse (50) and the Long Evans Cinnamon rat, 2 preclinical models of Wilson disease (51). In line with the analogy between the 2 diseases, functional SNPs of *ATP7B* (9) (also known as the Wilson disease gene) have been associated with increased susceptibility to AD in a subset of patients (44, 52-55), as confirmed by current results on the distribution of the *ATP7B* AG haplotype. It should be stressed, though, that Cu dyshomeostasis in AD patients has a different aetiology, is less severe than in Wilson disease and pertains to a subset of individuals (56). However, given the multiple neurotoxic activities exerted by unregulated Cu in the brain, the metal is a contributing element in the multi-factor pathogenesis of AD increasing the global susceptibility to the disease in those susceptible to Cu exposure.

The current study has several limitations. The major ones concern the lack of *APOE* genotypes. Another limit concerns the potential sampling bias of the investigated subjects as most of the studies evaluated in the meta-analyses adopted the NINCDS-ADRDA criteria (16, 57) for patient selection. These are less stringent than the currently employed IGW criteria (17). Notwithstanding its limitations, this study modifies the general view in the AD community proposing that Cu dyshomeostasis may be an AD susceptibility factor. Furthermore, our results indicate that the issue of Cu dyshomeostasis deserves attention and can be a valid therapeutic avenue to explore by precision medicine approaches that contemplates the heterogeneity of disease to target specific biological subsets rather than broad clinical entities. To this aim, the present team is currently involved in a phase II clinical trial funded by the Alzheimer's Association (number PTC-19-602325; EudraCT No.: 2019-000604-15), set at investigating the role of a zinc-based therapy (a measure employed in analogy with Wilson disease therapy) as a way to delay the cognitive decline in mild cognitive impairment due to AD subjects who exhibit non-ceruloplasmin Cu higher than normal values (0.1-1.6 µmol/L (49)).

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DISCLOSURES

RS is Chief Scientific Officer and has some shares in IGEA Pharma N.V.; other authors declare no commercial or noncommercial conflicts of interest relating to this work.

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FIGURE LEGENDS

Figure 1. **Standardized mean difference (SMD) computed from the studies on Cu brain levels (µg/g) in Alzheimer's disease patients and healthy controls.** SMDs between patients and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the centre representing the point estimate and the width the associated 95% CI.

The forest plot of the meta-analysis of brain Cu in AD revealed no variation (2 studies for the hippocampus area, 1 for the amygdala, 1 for frontal cortex, 2 for cerebral cortex and 1 for cerebellum), decreased values (2 for the hippocampus area, 1 for the amygdala, 1 for frontal cortex, 2 for cerebral cortex) as well as increased values (a study in the frontal cortex) of Cu in the brain that as a whole results in a Cu depletion in the brain as indicated by the overall diamond at the left of the zero line.

Figure 2. Standardized mean difference (SMD) computed from the studies on Cu serum/plasma levels (µmol/L) in Alzheimer's disease patients and healthy controls.

SMDs between patients and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the centre representing the point estimate and the width the associated 95% CI.

Most of the studies carried out in serum/plasma demonstrated increased values (26 studies) or no variation (13 studies) while only 2 studies reported decreased values of Cu in serum/plasma of AD patients in comparison with healthy controls. As a whole, the forest plot of the meta-analysis reveals increased values of Cu in serum/plasma of AD patients in comparison with healthy controls as indicated by the diamond at the right of the zero line.

Figure 3. Standardized mean difference (SMD) computed from the studies on non-ceruloplasmin Cu (µmol/L) in Alzheimer's disease patients and healthy controls.

SMDs between AD subjects and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the centre representing the point estimate and the width the associated 95% CI.

Most of the non-ceruloplasmin Cu studies carried out in serum/plasma demonstrated increased values (10 studies) of non-ceruloplasmin Cu in serum/plasma while 6 studies reported no variation, and 2 studies decreased values of non-ceruloplasmin Cu in AD patients in comparison with healthy controls. As a whole, the forest plot of the meta-analysis reveals increased values of non-ceruloplasmin Cu in serum/plasma of AD patients in comparison with healthy controls as indicated by the diamond at the right of the zero line.

Figure 4. Standardized mean difference (SMD) computed from the studies on non-ceruloplasmin Cu (µmol/L) in Alzheimer's disease patients and healthy controls, considering studies with a Cu:Cp ratio lower than 6 and higher than 8.

SMDs between AD subjects and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the centre representing the point estimate and the width the associated 95% CI.

Most of the non-ceruloplasmin Cu studies carried out in serum/plasma demonstrated increased values (9 studies) of non-ceruloplasmin Cu in serum/plasma while 2 studies reported no variation values of Cu in serum/plasma of AD patients in comparison with healthy controls. As a whole, the forest plot of the meta-analysis reveals increased values of non-ceruloplasmin Cu in serum/plasma of AD patients in comparison with healthy controls as indicated by the diamond at the right of the zero line.

The sensitivity analysis suggests the relevance of the Cu:Cp as a quality control of Cu status measurements (58). Two studies reported increased levels of non-ceruloplasmin Cu in AD and MCI subjects using direct methods of non-ceruloplasmin Cu assessment (45, 59).

Table 1. Effect of Alzheimer's disease on brain Cu levels

				Alzheimer's patients				Healthy controls			
Study (Authors, year)	**Country**	**Brain region**	**Meta-analysis classification**	**N°**	**Sex (F)**	**Mean age (DS)**	**Cu µg/g (DS)**	**N°**	**Sex (F)**	**Mean age (SD)**	**Cu µg/g (DS)**
Plantin et al, 1987 (21)	Sweden	temporal lobe	hippocampus	5			1.8 (0.7)	6			3.1 (0.6)
Corrigan et al, 1993 (20)	UK	hippocampal tissue	hippocampus	12	10	79.5 (9.2)	4.5 (2.2)	12	4	78.5 (9.0)	5.1 (1.1)
Loeffler et al, 1996 (24)	USA	frontal cortex	frontal cortex	12		79.4 (1.7)	7.8 (0.6)	7		75.7 (2.8)	6.8 (1.0)
		amygdala	amygdala	10	3	80.4	2.7 (0.9)	11	8	81.7	4.1 (1.0)
		hippocampus	hippocampus				2.3 (0.6)				3.2 (0.7)
Deibel et al, 1996 (18)	USA	superior and middle temporal	cerebral cortex				3.2 (0.6)				4.0 (0.6)
		inferior parietal	cerebral cortex				3.8 (0.9)				4.1 (0.6)
		cerebellum	cerebellum				5.1 (0.1)				5.7 (1.0)
Squitti et al, 2007 (27)	Italian	cortical tissue	cerebral cortex	9		84.6	5.1 (1.6)	10		80.2 (6.8)	6.8 (1.5)
Religa et al, 2006 (26)	Australia	neocortical tissue	cerebral cortex	10	7	81.6 (11)	2.3 (1.0)	14	12	82.8 (11.2)	2.8 (0.9)
Magaki et al, 2007 (22)	USA	frontal cortex	frontal cortex	8	6	78 (12)	3.9 (0.3)	6	2	72 (11.0)	6.9 (1.2)
Akatsu et al, 2012(19)	Japan	hippocampus	hippocampus	15	9	87.5 (7.6)	3.78 (8.9)	12	6	84 (6.7)	7.4 (2.1)
		amygdala	amygdala	18	11		4.3 (9.1)	16	10	85.9 (7.3)	8.3 (3.9)
Graham et al, 2014 (25)	UK	Brodman area 7	cerebral cortex	42	21	83 (7.2)	19.3 (2.9)	26	13	81.7 (6.2)	22.5 (4.3)
Szabo et al, 2016 (23)	USA	frontal cortex	frontal cortex	14		78	2.1 (0.7)	15		88	2.4 (0.7)

Sixteen studies were identified in the primary screening; 6 were excluded (60-65) after the application of the exclusion criteria.

Table 2. Indices of serum Cu status in all the 18 studies analyzed in the non-ceruloplasmin Cu (Non-Cp Cu) and ceruloplasmin (Cp) meta-analyses

	Alzhimer's disease							Health controls							
Study (Authors, year)	**N°**	**Female (%)**	**Mean age (SD)**	**MMSE (SD)**	**Cu µmol/L (SD)**	**Cp mg/dl (SD)**	**Non-Cp Cu µmol/L (SD)**	**N°**	**Female (%)**	**Mean age (SD)**	**MMSE (SD)**	**Cu µmol/L (SD)**	**Cp mg/dl (SD)**	**Non-Cp Cu µmol/L (SD)**	**Cu:Cp ratio**
Rozzini et al, 2018(59)	44	61%	77.1 (7.6)	18.1 (5.6)	18.3 (3.5)	-	0.57 (0.34)	28	43%	76.1 (8.4)	29.3 (1.0)	16 (3.3)	-	0.3 (0.11)	n.a.
Squitti et al, 2018 (15)	385	73%	76 (2.5)	≤25	15.2 (13-17)*	27.4 (5.2)	2.2 (2.1)	336	60%	65 (3.5)		13.2 (11.3-15)*	27.1 (5.0)	0.4 (2.1)	6.5
Shere et al, 2018 (32)	44	54%	68.3 (9.7)	13.7 (6.6)	16.1 (3.7)	22.6 (4.9)	5.4 (1.8)	52	48%	65.7 (7.6)	29.5 (1.1)	18.2 (4.4)	23.3 (4.7)	7.2 (2.3)	n.a.
Squitti et al, 2018 (45)	89	67.5%	73 (8.5)	16 (4.7)	15.9 (3.59)	28.8 (5.59)	2.31 (1.64)	147	52.7%	49 (12.7)	29 (1)	16.04 (6.39)	30.48 (10.38)	1.68 (2.46)	6.8
Talwar et al, 2017 (31)	108	39%	68.8 (9.2)	13.5 (5.7)	22.1 (9.6)	36.1 (9.3)	5.0 (5.2)	159	48%	63.2 (7.5)	28.2 (1.5)	18.7 (7.0)	36.6 (10.8)	1.4 (1.9)	6.7
	284	46%	73.4 (6.0)	19.8 (3.3)	16.9 (6.4)	22 (52)	6.5 (22.1)								
Pu et al, 2017 (36) **	42	45%	75.8 (6.7)	13.5 (3.0)	20.3 (6.7)	17 (48)	12.1 (20.1)	40	47%	74.2 (6.5)	24.2 (3.3)	16.3 (6.5)	24 (54)	27.6	8.96
	55	45%	77.8 (6.5)	5.62 (1.1)	21.3 (6.9)	15 (41)	13.5 (17.0)								
Siotto et al, 2016 (66)	84	69%	77 (8.7)	≤25	15.5 (3.3)	28.2 (4.3)	2.1 (1.8)	58	47%	64.5 (18)		14 (2.3)	26.3 (3.7)	1.5 (1.5)	7
Park et al, 2014 (40)	64	50%	75.0 (5.4)	17.9 (5.1)	28.4 (4.7)	23.3 (4.1)	7.0 (1.9)	67	55%	73.4 (5.3)	24.2 (3.8)	26.7 (5.0)	21.7 (4.9)	6.7 (2.2)	10.3
Lopez et al, 2013 (67)	36	55%	77.7 (5.3)	20.7 (4.4)	15.8 (2.9)	25.9 (4.1)	3.6 (2.1)	33	64%	74.0 (5.0)	28.9 (1.3)	13.8 (3.7)	25.8 (4.3)	1.6 (3.0)	7.06
Rembach et al, 2013 (38)	152	59%	77 (7.9)	18.9 (5.3)	13.9 (2.7)	31.6 (7.9)	-0.6 (2.7)	716	58%	69.0 (6.8)	28.9 (1.2)	14.6 (2.9)	31.7 (7.9)	-0.6 (3.1)	5.97
Brewer et al, 2010 (35)	28	46%	76.2	24 (3.9)	17 (2.4)	24.4 (3.0)	5.5 (1.8)	29	69%	68.6	29.8 (0.7)	18.4 (3.1)	25.7 (3.7)	6.3 (2.5)	9.45
Arnal et al, 2010 (34)	110	56%	70 (5.6)	22.2 (3.1)	15.6 (3.1)	25.2 (10.7)	3.8 (4.5)	79	48%	77.8 (3.7)	27.8 (1.1)	12.7 (0.2)	26.5 (7.0)	0.2 (2.8)	6.34
Agarwal et al, 2008 (29)	50	38%	60.0 (11.6)	14.1 (7.6)	24.6 (4.8)	41.6 (9.6)	4.9 (3.7)	50	34%	55.3 (10.9)		21.2 (5.0)	28.8 (8.0)	7.5 (4.1)	9.68
Zappasodi et al, 2008 (37)	54	81%	73.7 (8.7)	19.5 (3.8)	15.1 (3.4)	27.6 (5.9)	2.1 (2.5)	20	65%	71.5 (9.2)	28	12.9 (3.0)	27 (5.3)	0.2 (2.5)	6.31
Sedighi et al, 2006 (41)	50	48%	76.4	14.3 (4.6)	21.7 (3.1)	27.7 (9.6)	8.6 (3.3)	50	50%	67.8	25.8	20.8 (2.5)	31.1 (5.4)	6.2 (2.2)	8.85
Snaedal et al, 1998 (68)	44	73%	74.3 (53-89)	15.9 (6-28)	19.1 (3.9)	38.2 (6.8)	1.1 (2.9)	44	matched	matched		19.4 (2.2)	38.3 (8.1)	1.3 (3.0)	6.69
Molaschi et al, 1996 (33)	31	100%	77.2 (2.4)		18.9 (3.4)	38.8 (7.1)	0.5 (2.7)	421	100%	77.6 (2.3)		19.3 (3.8)	40.6 (9.1)	0.1 (3.6)	6.27
Present study	97	64%	70.4 (7.0)	17.9 (5.8)	16.3 (4.6)	29.4 (4.7)	2.39 (3.4)	73	77%	67.1 (9.2)	28.3	14.8 (3.2)	29.5 (4.3)	0.86 (1.8)	6.6

*25-75th percentiles. **Data were pooled for the analyses.

Table 3: Demographic characteristics of participants to the replication study and comparison of serum Cu biological variables between AD and healthy controls.

		Alzheimer's disease N=97	Healthy controls N=70	Statistics	P value
Sex, M	% (n/subjects)	37.1% (36/97)	22.9% (16/70)		0.05
Age	mean (SD)	71.1 (7.18)	65.9 (7.53)		0.01
MMSE	median (25-75th percentile)	19 (15-23)	29 (27.3-30)		<0.001*
Cu (µmol/L)	mean (SD)	15.8 (3.76)	14.6 (2.95)	F(1, 163)=6.69#	0.011
	median(25-75th percentile)	14.9 (13 - 18.3)	14.7 (12.58-16.22)		
Ceruloplasmin& (mg/dL)	mean (SD)	29.3 (4.89)	29.2 (4.08)	F(1, 163)=1.13#	0.289
	median(25-75th percentile)	28.6 (25.7 - 32.25)	28.8 (26.47 - 32.17)		
Non-ceruloplasmin Cu(µmol/L)**	mean (SD)	2 (2.29)	0.9 (1.59)	F(1, 163)=11.23#	0.001
	median(25-75th percentile)	1.4 (0.6 - 2.8)	0.75 (-0.16 - 1.71)		
Ceruloplasmin activity (IU)	mean (SD)	117.9 (26.13)	110.4 (18.06)	F(1, 163)=10.21#	0.008
	median(25-75th percentile)	112.6 (101 - 129)	110.5 (96.8 - 123.03)		
eCp:iCp ratio	mean (SD)	4.0 (0.51)	3.8 (0.44)	F(1, 163)=7.02#	0.009
	median(25-75th percentile)	3.9 (3.69 - 4.31)	3.8 (3.50 - 4.06)		
Cu:Cp ratio	mean (SD)	7.1 (0.74)	6.6 (0.93)	F(1, 163)=10.21#	0.002
	median(25-75th percentile)	6.8 (6.44 - 7.09)	6.6 (6.45 - 6.9)		

#All analyses were adjusted for sex and age; *non-parametric Mann-Whitney test; &logarithmic transformation was applied.

Table 4. genetic characteristics of the patients under study

	Healthy controls		AD		Chi-square				Odd Ratio		
rs732774	**n**	**Freq**	**n**	**Freq**	**Value**	**df**	**p**	**$p_{correct}$**	**Value**	**95% CI**	
Alleles											
A	78	0.57	113	0.62							
G	60	0.43	69	0.38	1.011	1	0.315	0.409			
Total	138	1.00	182	1.00							
Genotypes											
AA	24	0.35	29	0.32							
AG	30	0.43	55	0.60	7.857	2	0.020	**0.032**			
GG	15	0.22	7	0.08							
Total	69	1.00	91	1.00							
Carriers											
Allele A	54	0.78	84	0.92	6.529	1	0.011	**0.011**	**3.33**	**1.28**	**8.70**
Allele G	45	0.65	62	0.68	0.151	1	0.698	0.799	1.14	0.59	2.21

	Healthy controls		AD		Chi-square				Odd Ratio		
rs1061472	**N**	**freq**	**N**	**freq**	**Value**	**df**	**p**	**$p_{correct}$**	**Value**	**95% CI**	
Alleles											
A	64	0.46	72	0.40							
G	74	0.54	110	0.60	1.492	1	0.222	0.315			
Total	138	1.00	182	1.00							
Genotypes											
AA	17	0.25	10	0.11							
AG	30	0.43	52	0.57	5.762	2	0.056	0.097			
GG	22	0.32	29	0.32							
Total	69	1.00	92	1.00							
Carriers											
Allele A	47	0.68	62	0.68	$4.6*10^{-6}$	1	0.998	1.000	1.00	0.51	1.96
Allele G	52	0.75	81	0.89	5.212	1	0.022	**0.031**	**2.65**	**1.13**	**6.21**

n subjects in the analysis= 160; AD= 91, healthy controls= 69

Table 5. Results of a uni- and a multivariable binary logistic model developed to evaluate the effect of molecular variables under study on the probability of having AD

	Univariate analysis			Multivariate analysis*		
	OR	**95% CI**	**p value**	**OR**	**95% CI**	**p value**
Sex (M vs F)	1.99	0.99-3.98	0.051	2.51	1.13-5.56	0.023
Age	1.06	1.01-1.1	**0.008**	1.03	0.98-1.08	0.193
Cu	1.11	1.01-1.22	**0.037**			
iCp	1.01	0.94-1.08	0.845			
eCp	1.02	1.0-1.032	0.045			
non-ceruloplasmin Cu	1.4	1.14-1.70	**0.001**	1.32	1.06-1.64	**0.012**
eCp:iCp ratio	2.86	1.36-6.0	**0.006**	2.17	0.96-4.91	0.062
Cu:Cp ratio	1.17	0.46-3.01	0.74			
Haplotypes*						
AG vs GA/GA	2.7	1.13-6.23	**0.026**	2.49	0.99-6.28	0.053

Table notes:
Biochemical variables with a p value<0.10 at the univariable analysis (bold values in the table) were included into the multivariable analysis.
Cu and eCp were not included to avoid the multicollinearity with non-ceruloplasmin Cu and eCp:iCp ratio respectively.
*The analysis was carried out on 160 individuals; OR: odds ratio; CI: confidential Interval

Figure 1

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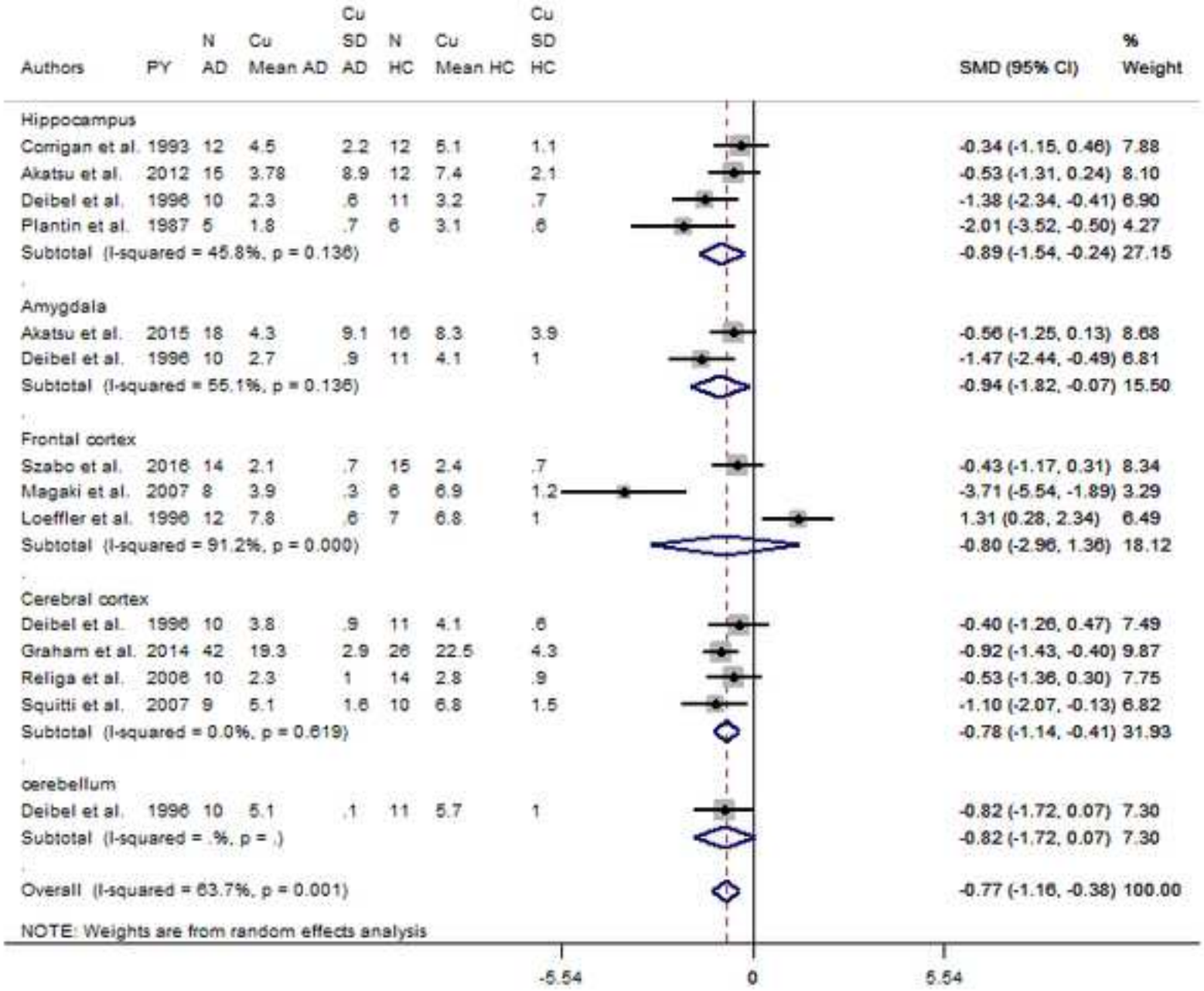

Figure 2

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Authors & PY	N AD	Cu Mean AD	Cu SD AD	N HC	Cu Mean HC	Cu SD HC	SMD (95% CI)	% Weight
Shore et al. 1984	10	18.3	4.2	10	18.6	1.6	-0.09 (-0.97, 0.78)	2.18
Kapaki et al. 1989	5	14.2	3.8	28	16.2	2.2	-0.81 (-1.78, 0.16)	2.10
Jeandel et al. 1989	55	22	6.1	24	21.2	4.1	0.14 (-0.34, 0.62)	2.46
Basun et al. 1991	24	17	3.9	28	16.7	3	0.09 (-0.46, 0.63)	2.42
Mattiello et al. 1993	21	18.8	.2	10	18.8	.2	0.00 (-0.75, 0.75)	2.28
Molaschi et al. 1996	31	18.9	3.4	421	19.3	3.8	-0.11 (-0.47, 0.26)	2.52
Molina et al. 1998	26	15.1	3.5	28	14.5	4.1	0.16 (-0.38, 0.69)	2.43
Snaedel et al. 1998	44	19.1	3.9	44	19.4	2.2	-0.09 (-0.51, 0.32)	2.50
Gonzalez et al. 1999	51	16.6	.5	40	15.4	.4	2.62 (2.05, 3.18)	2.41
Squitti et al. 2002	79	18.3	5.7	76	13.7	2.6	1.03 (0.70, 1.37)	2.54
Ozcakaya and Delibas 2002	27	12	.2	25	12.1	.2	-0.50 (-1.05, 0.05)	2.42
Smorgon et al. 2004	8	22.9	3.9	11	16.7	1.3	2.30 (1.11, 3.50)	1.91
Bocca et al. 2005	60	15.2	3.8	44	14.3	3.1	0.26 (-0.14, 0.65)	2.51
Sedighi et al. 2006	50	21.7	3.1	50	20.8	2.5	0.32 (-0.07, 0.71)	2.51
Sevym et al. 2007	98	16.7	2.9	76	15.4	2.1	0.50 (0.20, 0.81)	2.55
Zappasodi et al. 2008	54	15.1	3.4	20	12.9	3	0.67 (0.14, 1.19)	2.44
Agarwal et al. 2008	50	24.6	4.8	50	21.2	5	0.69 (0.29, 1.10)	2.50
Baum et al. 2010	44	16.2	3.5	41	15.3	2.7	0.29 (-0.14, 0.71)	2.49
Brewer et al. 2010	28	17	2.4	29	18.4	3.1	-0.50 (-1.03, 0.02)	2.43
Vural et al. 2010	50	20.7	2.9	50	22.5	2.9	-0.62 (-1.02, -0.22)	2.51
Arnal et al. 2010	110	15.7	.8	79	12.7	.9	3.56 (3.10, 4.02)	2.47
Mueller et al. 2012	14	19.1	1	19	16.9	.6	2.78 (1.80, 3.75)	2.09
Huang et al. 2013	28	15.9	3.5	19	16.4	3	-0.15 (-0.73, 0.43)	2.40
Lopez et al. 2013	36	15.8	2.9	33	13.8	3.7	0.60 (0.12, 1.09)	2.46
Azhdarzadeh et al. 2013	30	17	3.1	20	15.6	2.2	0.50 (-0.07, 1.08)	2.40
Azhdarzadeh et al. 2013	50	15.8	1.5	50	15.1	1.9	0.41 (0.01, 0.81)	2.51
Rembach et al. 2013	152	13.9	2.7	716	14.6	2.9	-0.24 (-0.42, -0.07)	2.59
Gonzalez-Dominguez et al. 2014	30	17.5	4.2	30	16.5	3.2	0.27 (-0.24, 0.78)	2.45
Singh et al. 2014	100	18.3	.5	100	14.9	.3	8.25 (7.39, 9.10)	2.19
Park et al. 2014	89	18	3	118	17	3.2	0.32 (0.04, 0.60)	2.56
Wang et al. 2015	83	19	3.6	83	16.1	2.8	0.90 (0.58, 1.22)	2.54
Koc et al. 2015	45	14.2	10.5	33	15.9	11.7	-0.15 (-0.60, 0.30)	2.48
Siotto et al. 2016	84	15.5	3.3	58	14	2.3	0.51 (0.17, 0.85)	2.53
Paglia et al. 2016	34	12.8	3.2	40	11.1	3.8	0.48 (0.02, 0.94)	2.47
Pu et al. 2017	125	19.3	11.5	40	16.3	6.5	0.29 (-0.07, 0.64)	2.53
Squitti et al. 2017	89	15.9	3.59	147	16.04	6.39	-0.03 (-0.29, 0.24)	2.56
Talwar et al. 2017	108	22.1	9.6	159	18.7	7	0.42 (0.17, 0.66)	2.57
Squitti et al. 2018	385	15.2	1	336	13.2	.925	2.07 (1.89, 2.25)	2.59
Shere et al. 2018	44	16.1	3.71	52	18.2	4.4	-0.51 (-0.92, -0.10)	2.50
Rozzini et al. 2018	44	18.3	3.5	28	16	3.3	0.67 (0.18, 1.16)	2.46
Present study	97	15.8	3.76	70	14.6	2.95	0.35 (0.04, 0.66)	2.55
Overall (I-squared = 96.3%, p = 0.000)							0.64 (0.32, 0.96)	100.00

NOTE: Weights are from random effects analysis

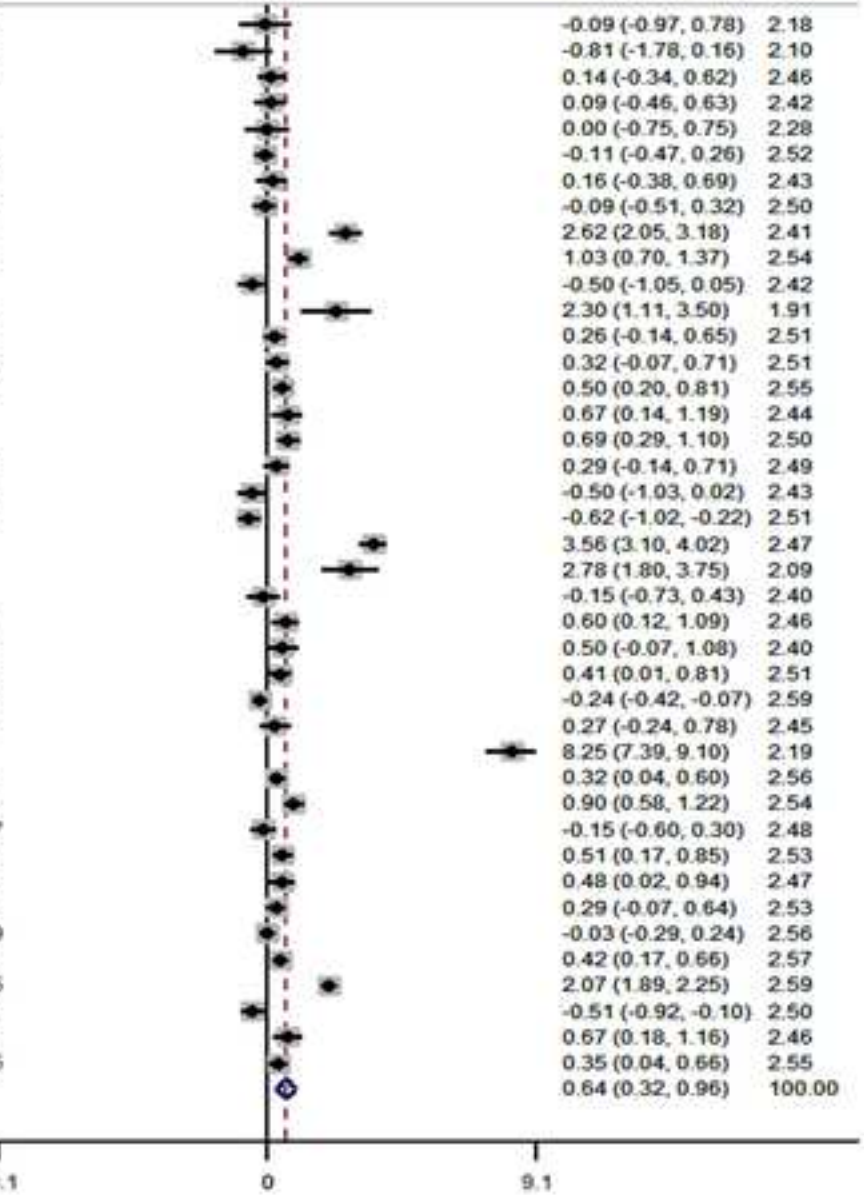

Figure 3

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Authors & PY	N AD	Non-Cp Cu Mean AD	Non-Cp Cu SD AD	N HC	Non-Cp Cu Mean HC	Non-Cp Cu SD HC	SMD (95% CI)	% Weight
Molaschi et al. 1996	31	.5	2.7	421	.1	3.6	0.11 (-0.25, 0.48)	5.59
Snaedel et al. 1998	44	1.1	2.9	44	1.3	3	-0.07 (-0.49, 0.35)	5.39
Sedighi et al. 2006	50	8.6	3.3	50	6.2	2.2	0.86 (0.45, 1.27)	5.42
Zappasodi et al. 2008	54	2.1	2.5	20	.2	2.5	0.76 (0.23, 1.29)	4.97
Agarwal et al. 2008	50	4.9	3.7	50	7.5	4.1	-0.67 (-1.07, -0.26)	5.45
Brewer et al. 2010	28	5.5	1.8	29	6.3	2.5	-0.37 (-0.89, 0.16)	4.98
Arnal et al. 2010	110	3.8	4.5	79	.2	2.8	0.93 (0.62, 1.23)	5.79
Lopez et al. 2013	36	3.6	2.1	33	1.6	3	0.78 (0.29, 1.27)	5.12
Rembach et al. 2013	152	-1	2.7	716	-.6	3.1	-0.13 (-0.31, 0.04)	6.13
Park et al. 2014	64	7.04	1.9	67	6.73	2.15	0.15 (-0.19, 0.50)	5.66
Siotto et al. 2016	84	2.1	1.8	58	1.5	1.5	0.36 (0.02, 0.69)	5.68
Squitti et al. 2017	89	2.31	1.64	147	1.68	2.46	0.29 (0.02, 0.55)	5.91
Talwar et al. 2017	108	5	5.2	159	1.4	1.91	0.99 (0.74, 1.25)	5.92
Pu et al. 2017	125	10.7	34.37	40	4.972	21.4	0.18 (-0.18, 0.54)	5.62
Rozzini et al. 2018	44	.57	.34	28	.3	.11	0.98 (0.48, 1.48)	5.07
Shere et al. 2018	44	5.37	1.8	52	7.25	2.3	-0.90 (-1.32, -0.48)	5.38
Squitti et al. 2018	385	2.2	2.12	336	.4	2.08	0.86 (0.70, 1.01)	6.17
Present study	97	1.98	2.29	70	.87	1.59	0.55 (0.23, 0.86)	5.76
Overall (I-squared = 91.5%, p = 0.000)							0.32 (0.06, 0.57)	100.00

NOTE: Weights are from random effects analysis

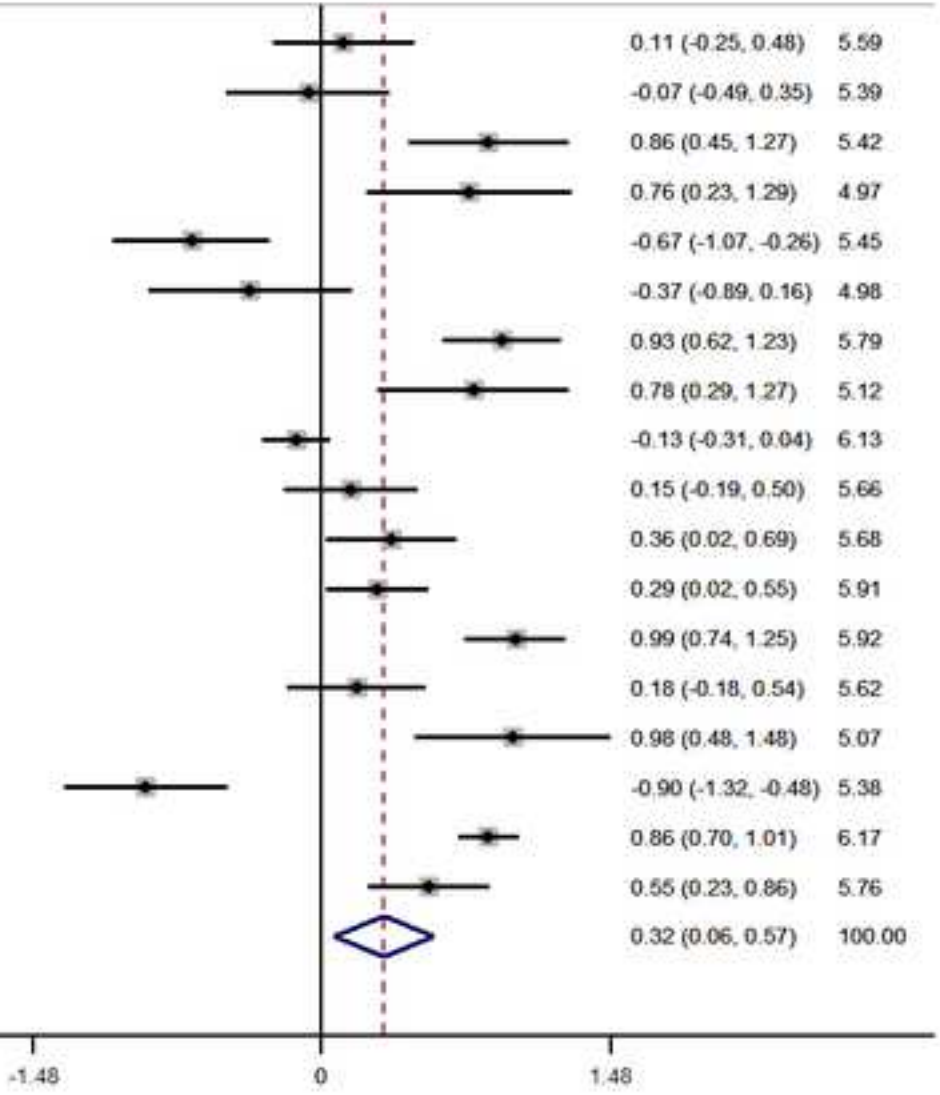

Figure 4

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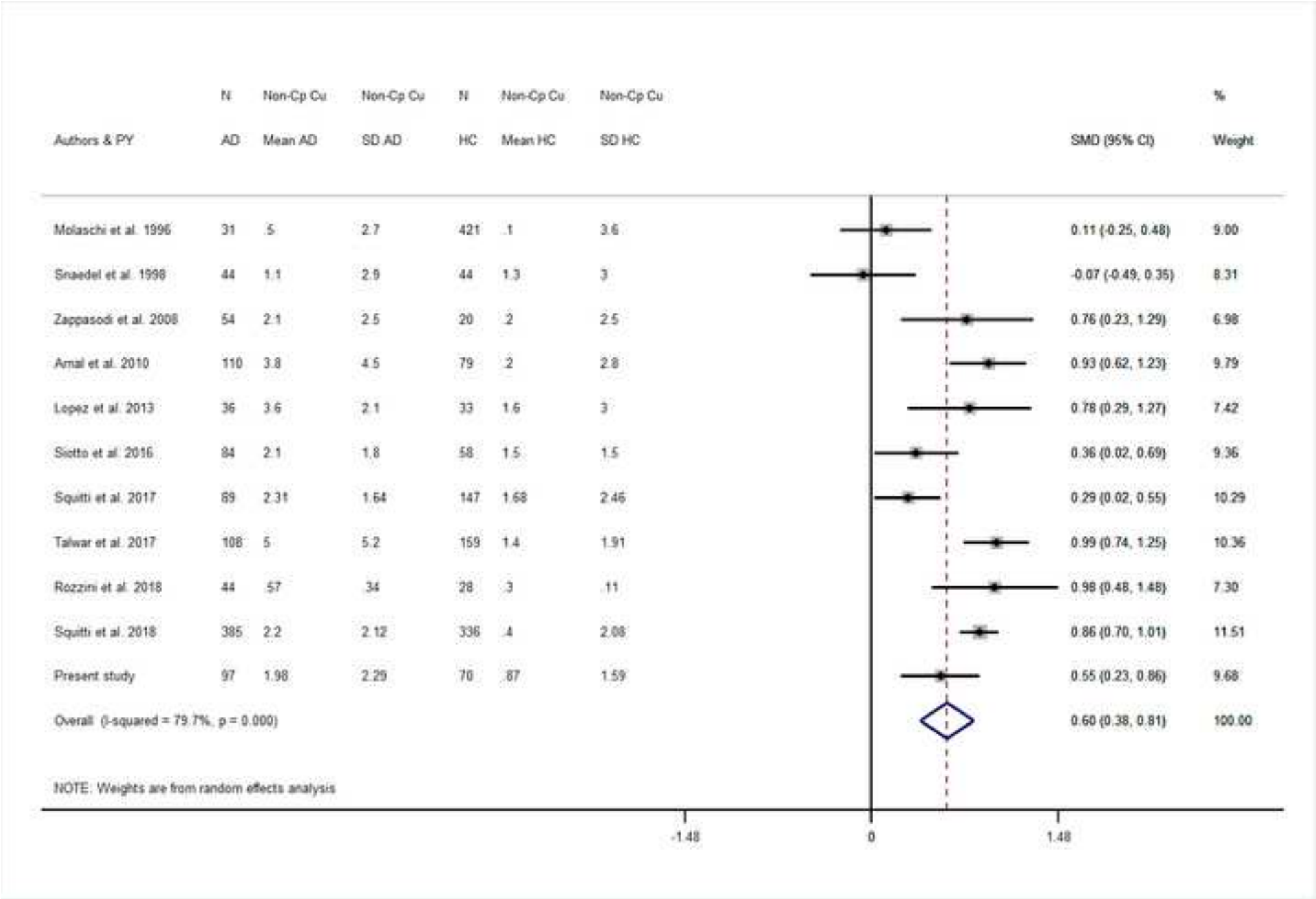

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METHODS AND MATERIALS

Meta-analyses

Search strategy

To identify appropriate studies for meta-analysis, we followed the procedural steps indicated by Cochrane (http://www.cochrane-handbook.org). We entered the keywords "Alzheimer's disease", "copper", "serum", "plasma", "brain", "metals" and their combinations in PubMed and selected studies from 1984 to 2019. We also identified other studies by using "Scopus" and "ISI Web of Knowledge" databases. Besides, reviewing the contents of this first selection, we also evaluated their reference lists to search for additional studies via Google Scholar. We considered only papers showing comparative analyses of AD and healthy populations, presenting original results and published in peer-reviewed journals. In most studies, the severity of the AD patients' cognitive decline had been assessed by a Mini-Mental State Examination (MMSE, (1)). The target of our meta-analysis was the comparison, between AD patients and controls, of the Cu weighted means reported in the selected papers.

Our first selection consisted of 54 studies. At this point, we further required that manuscripts explicitly present Cu mean and standard deviation values for both patient and control groups. From the first selection, one study that did not report standard deviation values (2) has been excluded and one that did not report the exact value of Cu levels (3) was dropped. We excluded the study by Alimonti et al. (4) because it was conducted partially on the same sample as the study by Bocca and colleagues (5). We also included data from 721 subjects investigated in our previous studies (6) excluding the studies from our laboratory in which the patient samples partially were overlapped. At this point, our selection had been reduced to 51 studies: 10 on Cu in brain (**Table 1**), 37 on Cu in serum and 4 in plasma (**Table 1 and Table 2 Supplementary material**); 18 studies of the latter section reported data on non-ceruloplasmin Cu in serum and on ceruloplasmin (**Table 2)**]. We

performed separate meta-analyses of: Cu in brain, Cu in serum and plasma pooled together, non-ceruloplasmin Cu and ceruloplasmin in serum plasma pooled together.

Of these 10 studies in brain, we also performed a meta-analysis stratified on the basis of the functions of different areas of brain: hippocampus, amygdala, frontal cortex, cerebral cortex, cerebellum.

Data extraction for Meta-analysis

To performed the meta-analyses, from each selected study mean and standard deviation (SD) of the parameters of interest in AD and control groups were extracted. Data manipulation was necessary when median and IQR or Min Max were extracted. From these values, the mean and standard deviation applying Hozo et al.'s methods (7) or Wan et al.' method (8) were calculated. The standard deviations were also calculated following the indication of the Cochrane Handbook (9).

Statistical procedures applied to run Meta-analyses

Five meta-analysis were performed considering: Cu in brain, Cu, non-ceruloplasmin Cu and ceruloplasmin in serum/plasma.

Meta-analysis was performed applying a random effects model to obtain a pooled estimate of Standardized Mean Difference (SMD). The heterogeneity was evaluated via the Cochran's Q test and quantified through the I^2. The I^2 describes the rate of variation across studies due to heterogeneity rather than chance, ranging from 0 (no heterogeneity) to 100 (maximal heterogeneity).

Funnel plot was used to investigate publication bias. The Egger's test for funnel plot asymmetry was used only when there were at least 10 studies included in the meta-analysis (9).

In meta-analysis of Cu in brain, subgroup analysis was performed stratified for hippocampus, amygdala, a not well defined 'cerebral cortex' area, and frontal cortex.

In meta-analysis of non-ceruloplasmin Cu, a sensitivity analysis was performed excluding studies that have a Cu:Cp ratio in healthy controls lower that 6 and higher than 8.

In meta-analysis of Cu and non-ceruloplasmin Cu and ceruloplasmin in serum/plasma, meta regression analysis was performed to evaluate the effect on the pooled SMD of the difference in mean age between healthy controls and AD patients.

A p value<0.05 was considered statistically significant. All statistical analyses were performed with STATA v10.

Replication study

Subjects analyzed in the Replication study

The study was approved by the Local Ethics Committee (approval number 53/2016). Subjects signed informed consent (approval number 2/1992; 26/2014). Nighty-seven AD patients and 70 cognitively normal individuals were included in a replication study (**Table 3**). Alzheimer's disease patients [(10, 11) and Mini-Mental State Examination (MMSE) score of 25 or less (1)], were enrolled by two specialized Neurological and Dementia care Centers in Italy: the Memory Clinic of the IRCCS Istituto Centro San Giovanni di Dio, Fatebenefratelli, Brescia, Italy, and Dipartimento di Scienze del Sistema Nervoso e del Comportamento, Università di Pavia, Istituto Neurologico Nazionale IRCCS C. Mondino, using common standardized clinical protocols and guidelines. Participants to the Replication study underwent to blood sampling at fast, analyses of an extensive panel of Cu metabolism markers composed by serum Cu, non-ceruloplasmin Cu, ceruloplasmin concentration, ceruloplasmin activity, ceruloplasmin specific activity (iCp/eCp), ceruloplasmin to Cu ratio (Cu:Cp) ratio, DNA extraction and genotyping of *ATP7B* rs1061472 and rs732774.

Sample collections

Fasting blood samples were collected in the morning and sera samples were separated by centrifugation (3000 rpm, 10 min, and 4 °C). They were then divided into 0.5 mL aliquots and rapidly stored at -80°C. Biological samples, isolated according to standard procedures, were stored at Fatebenefratelli Biobank (IRCCS Centro San Giovanni di Dio Brescia, Italy) and IRCCS Mondino. The subjects' samples were shipped to Ospedale Fatebenefratelli 'San Giovanni Calibita', Isola Tiberina, Rome, Italy for blinded biochemical analyses.

Biochemical investigation applied for the Replication study

The aliquots were thawed just before the assay. Concentration of Cp was measured with immunoturbidimetric assay (Futura System SRL, Rome, Italy) and was calibrated against the international reference preparation (ERM 470) (12), performed in duplicate on the multiple biochemical analyzer Horiba Pentra 400 (ABX Diagnostic, Montpellier, France).

The activity eCp was measured following an automation of the Schosinsky o-dianisidine eCp assay (13) adapted from our laboratory for multiple biochemical analyzers Horiba Pentra 400 (ABX Diagnostic, Montpellier, France) and described in detail elsewhere (14). Serum Cu concentration was estimated with the colorimetric

assay of Abe et al. (Randox Laboratories, Crumlin, UK) (15) and confirmed in 30% of samples by atomic absorption spectrophotometry measurements using an AAnalyst 600, Perkin-Elmer, Norwalk, CT, USA, equipped with graphite furnace, as described in details elsewhere (6).

Non-ceruloplasmin Cu was calculated from the equation provided by Walshe (appendix of (16)), based on the measures of concentration of total Cu and Cp in serum. Equivalent data can be obtained calculating non-ceruloplasmin Cu from mg/L of ceruloplasmin and considering the conversion of 3.15 µg/Cu for mg of ceruloplasmin (16).

Cu:Ceruloplasmin ratio (Cu:Cp) (17) was calculated as reported in Twomey et al. (18). These authors provided the equation as follows:

[Cu µmol/L]* [132000 (g/mol)] / [Cp (mg/dL)*10].

As we previously discussed in details (17), the plausible theoretical values of Cu:Cp, which can be effectively measured in specimens of healthy controls, should range between 6–8, even though this ratio can yield diverse values. A 6-8 range obtained in healthy controls for Cu:Cp assures to obtain a more reliable non-ceruloplasmin Cu value when applying the Walshe's formula (16, 17), since Cu and ceruloplasmin are in the in the correct stoichiometry for Cu and ceruloplasmin, based on the evidence that each ceruloplasmin molecule has 6 to 8 sites for Cu atom binding.

Statistical analyses applied to the Replication study

Continuous data were presented in terms of mean (Standard deviation, SD) or, if they were not normally distributed, of median (IQR: Q1-Q3). Logarithmic transformation of non-ceruloplasmin Cu values were applied, because of the presence of negative values.

Categorical data were presented as frequencies (percentage, %). Differences between healthy controls and AD patients in Sex or Haplotype AG were evaluated by Chi-square test. Difference in age between the two diagnosis groups was tested by T-test; differences in biochemical variables were evaluated by ANCOVA model adjusting for sex and age. Pearson's correlation coefficient was calculated to quantify relation between age and biochemical variables.

Procedures applied to run the Genetic studies

Genomic DNA was purified from peripheral blood using the conventional method for DNA isolation (QLAamp DNA Blood Midi kit). Genotyping of rs1061472 and rs732774 was performed by the TaqMan allelic discrimination assay as described in (19). The predesigned SNP genotyping assay IDs are ID_C_1919004_30 (rs1061472) and ID_C_938208_30 (rs732774) (Applied Biosystems, Inc). DNA extraction from 4 AD patients and genotyping of additional 2 AD patients and 1 control failed.

Quanto program showed that the study had an 85% power to detect an effect of OR ≥ 2.0 for a MAF ranging between 0.440 and 0.46 considering a general (co-dominant) model, based on an alpha of 0.05 and assuming AD prevalence 5.0% in the general population.

Statistical Analyses applied for the Genetic studies

Demographic and clinical characteristics in our patient sample were described either in terms of mean± SD, if quantitative, or in terms of proportions. The student's t test and the chi-square (χ^2) test were used to compare the characteristics of patient and control groups using the package SPSS 21.0. To calculate the power of our sample, we used the Quanto 1_2_4 program (Gauderman WJ, Morrison JM 2006); http://hydra.usc.edu/gxe)

Allele, genotype and haplotype frequencies along with the Hardy-Weinberg equilibrium were evaluated using Plink (Plink v1.07; http:// pngu.mgh.harvard.edu/~purcell/plink/) and SPSS 21.0. Permutation tests were used to correct multiple testing errors with 1,000 simulations. Haplotypes with frequencies greater than 5 % were considered in the analyses.

Table 1 Supplementary material. Demographic data of the eligible studies for meta-analysis of copper markers in serum/plasma

	Alzheimer's disease				Healthy controls			
References (Authors, year)	**N°**	**Sex (%F)**	**Mean age (SD)**	**MMSE Mean (SD)/ Median (25-75th percentile)**	**N°**	**Sex (%F)**	**Mean age (SD)**	**MMSE Mean (SD)/ Median (25-75th percentile)**
Rozzini et al. 2018 (20)	44	61%	77.1 (7.6)	18.1 (5.6)	28	43%	76.1 (8.4)	29.3 (1.0)
Squitti et al. 2018 (6)	385	73%	76 (2.5)	≤25	336	60%	65 (3.5)	
Squitti et al. 2017 (21)	89	67.5%	73 (8.5)	19 (4.7)	147	52%	49 (12.7)	29 (1)
Shere et al. 2018 (22)	44	54%	68.3 (9.7)	13.7 (6.6)	52	48%	65.7 (7.6)	29.5 (1.1)
Talwar et al. 2017 (23)	108	39%	68.8 (9.2)	13.5 (5.7)	159	48%	63.2 (7.5)	28.2 (1.5)
Pu et al. 2017 (24)	28	46%	73.4 (6.0)	19.8 (3.3)	40	47%	74.2 (6.5)	24.2 (3.3)
Pu et al. 2017 (24)	42	45%	75.8 (6.7)	13.5 (3)				
Pu et al. 2017 (24)	55	45%	77.8 (6.5)	5.6 (1.1)				
Siotto et al. 2016 (25)	84	69%	77 (8.7)	<25	58	47%	64.5 (18)	<25
Paglia et al. 2016 (26)	34	74%	72.4 (7.5)	12.3 (8.2)	40	63%	65.5 (6.4)	29.6 (0.7)
Koc et al. 2015 (27)	45	49%	77.7 (9.3)		33	51%	73.2 (10.6)	
Wang et al. 2015 (28)	83	64%	74 (7.1)		83	63%	72.2 (7.5)	
Gonzalez-Dominguez et al. 2014 (29)	30	60%	80.9 (4.5)		30	57%	74 (5.7)	
Singh et al. 2014 (30)	100	39%	62.7 (7.2)		100	39%	59.7 (8.1)	
Park et al. 2014 (31)	89	54%	77.8 (6.6)		118	58%	69.9 (5.9)	
Azhdarzadeh et al. 2013 (32)	30				20			
Azhdarzadeh et al. 2013 (32)	50				50			
Lopez et al. 2013 (33)	36	55%	77.7 (5.3)	20.7 (4.4)	33	64%	74 (5.0)	28.9 (1.3)
Rembach et al. 2013 (34)	152	59%	77 (7.9)	18.9 (5.3)	716	58%	69 (6.8)	28.9 (1.2)
Huang et al. 2013 (35)	28	86%	83 (6.8)	15.6 (4.2)	19	74%	79.9 (7.0)	23.1 (5.5)
Mueller et al. 2012 (36)	14	64%	80.6 (2.7)		19	47%	75.6 (4.0)	
Brewer et al. 2010 (37)	28	46%	76.2	24 (3.9)	29	69%	68.6	29.8 (0.7)
Baum et al. 2010 (38)	44	66%	74.3 (8.7)	<30	41	49%	79.1 (6.0)	<30
Arnal et al. 2010 (39)	110	56%	70 (5.6)	22.2 (3.1)	79	48%	77.8 (3.7)	27.8 (1.1)
Vural et al. 2010 (40)	50	54%	72 (6.8)		50	52%	65.1 (7.1)	

Agarwal et al. 2008 (41)	50	38%	59.9 (11.6)	14.1 (7.6)	50	34%	55.3 (10.9)	
Zappasodi et al. 2008 (42)	54	81%	73.7 (8.7)	19.5 (3.8)	20	65%	71.5 (9.2)	28 (1.7)
Sevym et al. 2007 (43)	98	66%	72.1 (6.7)		76	59%	70.3 (5.7)	
Sedighi et al. 2006 (44)	50	48%	76.4	14.3 (4.6)	50	50%	67.8	25.8 (1.5)
Bocca et al. 2005 (5)	60	67%	74.6 (6.4)	2-28	44	75%	>45	
Smorgon et al. 2004 (45)	8		79 (5)		11		78 (9.0)	
Ozcakaya and Delibas 2002 (46)	27	29%	72.3 (6.5)	16.8 (1.3)	25	36%	64.4 (7.2)	28.2 (2.4)
Squitti et al. 2002 (47)	79	68%	74.5 (7.4)	17.3 (4.9)	76	57%	70.1 (10.8)	27.7 (2.2)
Gonzalez et al. 1999 (48)	51	71%	74.5 (2.3)		40	45%	70.3 (4.0)	
Molina et al. 1998 (49)	26	46%	73.1 (8.2)	13.2 (5.7)	28	43%	70.8 (7.3)	
Snaedel et al. 1998 (50)	44	73%	74.3 (53-89)	15.9 (6-28)	44	matched	matched	
Molaschi et al. 1996 (51)	31	100%	77.2 (2.4)		421	100%	77.6 (2.3)	
Mattiello et al. 1993 (52)	21	71%	81 (6)		10	50%	82 (2.0)	
Basun et al. 1991 (53)	24	71%	75 (8)		28	57%	78 (3.0)	
Jeandel et al. 1989 (54)	55	73%	81.7 (5.7)	<25	24	40%		
Kapaki et al. 1989 (55)	5	20%	54		28	36%	46	
Shore et al. 1984 (56)	10	30%	63.7 (8.4)		10	70%	61.9 (8.0)	
Present study	97	64%	70.4 (7.0)	17.9 (5.8)	73	0.77	67.1 (9.2)	28.3 (1.9)

Table 2 Supplementary material. Serum or plasma Cu levels in Alzheimer's disease patients and in healthy controls

		Alzhimer's disease		Healthy controls	
Study	**Sample**	**N°**	**Cu, µmol/L (SD)**	**N°**	**Cu, µmol/L (SD)**
Rozzini et al. 2018 (20)	serum	44	18.3 (3.5)	28	16.0 (3.3)
Squitti et al. 2018 (6)	serum	385	15.2 (13-17)*	336	13.2 (11.3-15)*
Squitti et al 2017 (21)	serum	89	15.9 (3.59)	147	16.04 (6.39)
Shere et al. 2018 (22)	serum	44	16.1 (3.7)	52	18.2 (4.4)
Talwar et al. 2017 (23)	serum	108	22.1 (9.6)	159	18.7 (7.0)
		28	16.3 (6.4)		
Pu et al. 2017 (24) **	serum	42	20.3 (6.7)	40	16.3 (6.5)
		55	21.3 (6.9)		
Siotto et al. 2016 (25)	serum	84	15.5 (3.3)	58	14.0 (2.3)
Paglia et al. 2016 (26)	serum	34	12.8 (3.2)	40	11.1 (3.8)
Koc et al. 2015 (27)	serum	45	14.2 (10.5)	33	15.9 (11.7)
Wang et al. 2015 (28)	serum	83	19.0 (3.6)	83	16.1 (2.8)
Gonzalez-Dominguez et al. 2014 (29)	serum	30	17.5 (4.2)	30	16.5 (3.2)
Singh et al. 2014 (30)	serum	100	18.3 (0.5)	100	14.9 (0.3)
Park et al. 2014 (31)	serum	89	18.0 (3.0)	118	17.0 (3.2)
Azhdarzadeh et al. 2013 (32)	serum	30	17.0 (3.1)	20	15.6 (2.2)
Azhdarzadeh et al. 2013 (32)	serum	50	15.8 (1.5)	50	15.1 (1.9)
Lopez et al. 2013 (33)	serum	36	15.8 (2.9)	33	13.8 (3.7)
Rembach et al. 2013 (34)	serum	152	13.9 (2.7)	716	14.6 (2.9)
Huang et al. 2013 (35)	serum	28	15.9 (3.5)	19	16.4 (3.0)
Mueller et al. 2012 (36)	serum	14	19.1 (1.0)	19	16.9 (0.6)
Brewer et al. 2010 (37)	serum	28	17.0 (2.4)	29	18.4 (3.1)
Baum et al. 2010 (38)	serum	44	16.2 (3.5)	41	15.3 (2.7)
Arnal et al. 2010 (39)	plasma	110	15.7 (0.8)	79	12.7 (0.9)
Vural et al. 2010 (40)	plasma	50	20.7 (2.9)	50	22.5 (2.9)
Agarwal et al. 2008 (41)	serum	50	24.6 (4.8)	50	21.2 (5.0)
Zappasodi et al. 2008 (42)	serum	54	15.1 (3.4)	20	12.9 (3.0)
Sevym et al. 2007 (43)	serum	98	16.7 (2.9)	76	15.4 (2.1)
Sedighi et al. 2006 (44)	serum	50	21.7 (3.1)	50	20.8 (2.5)
Bocca et al. 2005 (5)	serum	60	15.2 (3.8)	44	14.3 (3.1)
Smorgon et al. 2004 (45)	serum	8	22.9 (3.9)	11	16.7 (1.3)
Ozcakaya and Delibas 2002 (46)	serum	27	12.0 (0.2)	25	12.1 (0.2)
Squitti et al. 2002 (47)	serum	79	18.3 (5.7)	76	13.7 (2.6)
Gonzalez et al. 1999 (48)	serum	51	16.6 (0.5)	40	15.4 (0.4)
Molina et al. 1998 (49)	serum	26	15.1 (3.5)	28	14.5 (4.1)
Snaedel et al. 1998 (50)	serum	44	19.1 (3.9)	44	19.4 (2.2)
Molaschi et al. 1996 (51)	serum	31	18.9 (3.4)	421	19.3 (3.8)
Mattiello et al. 1993 (52)	plasma	21	18.8 (0.2)	10	18.8 (0.2)
Basun et al. 1991 (53)	plasma	24	17.0 (3.9)	28	16.7 (3.0)
Jeandel et al. 1989 (54)	serum	55	22.0 (6.1)	24	21.2 (4.1)
Kapaki et al. 1989 (55)	serum	5	14.2 (3.8)	28	16.2 (2.2)

Shore et al. 1984 (56)	serum	10	18.3 (4.2)	10	18.6 (1.6)
Present study	serum	97	16.3 (4.6)	73	14.8 (3.2)

*25-75th percentiles. **Data were pooled for the analyses. SD: standard deviation

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Figure 1 Supplementary material. Funnel plot suggested no presence of publication bias in the studies of Cu in serum/plasma evaluated in Alzheimer's disease and healthy controls.

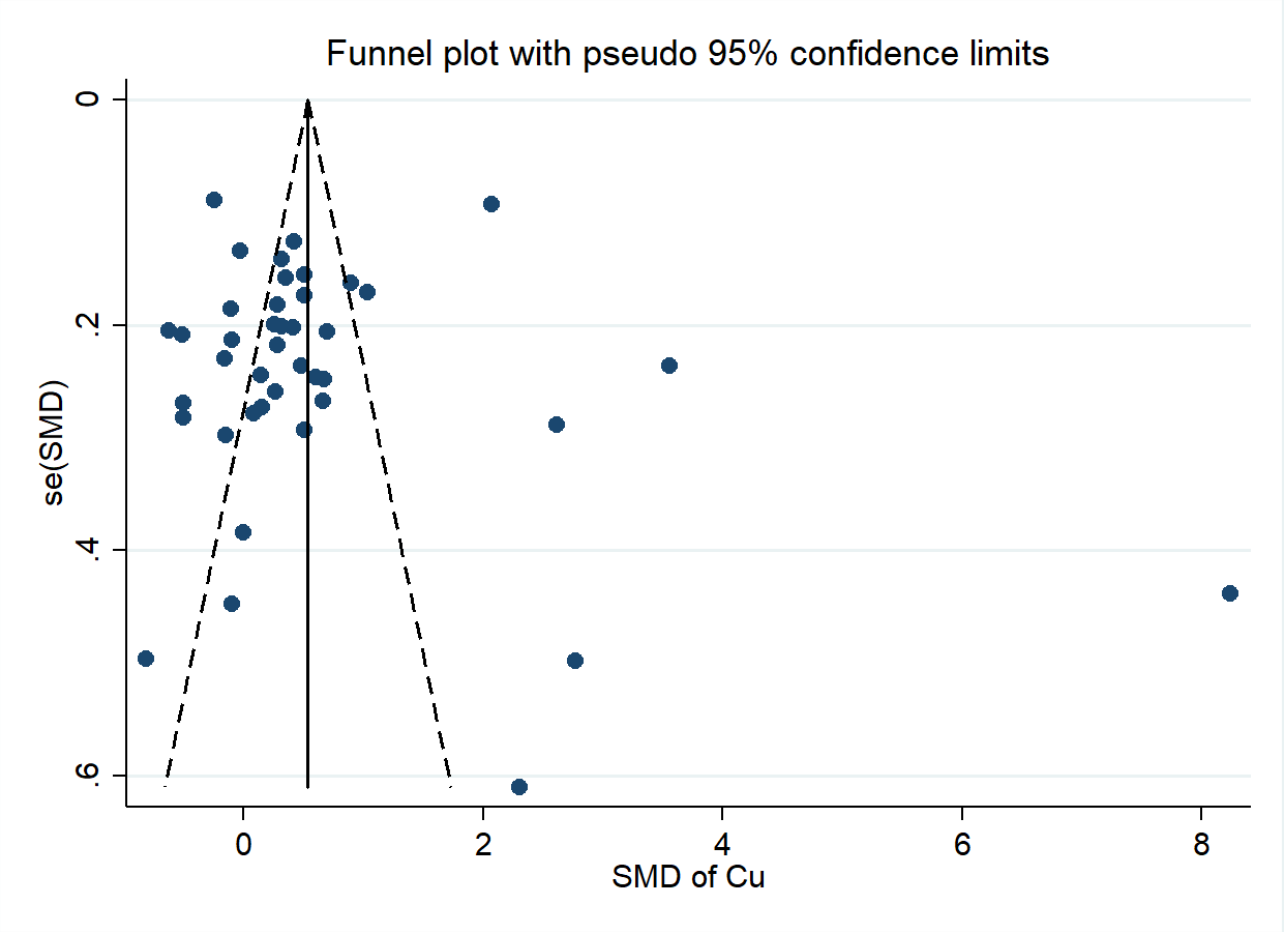


Figure 2 Supplementary material: Funnel plot suggested no presence of publication bias in the studies of non-ceruloplasmin Cu in serum/plasma evaluated in Alzheimer's disease and healthy controls.

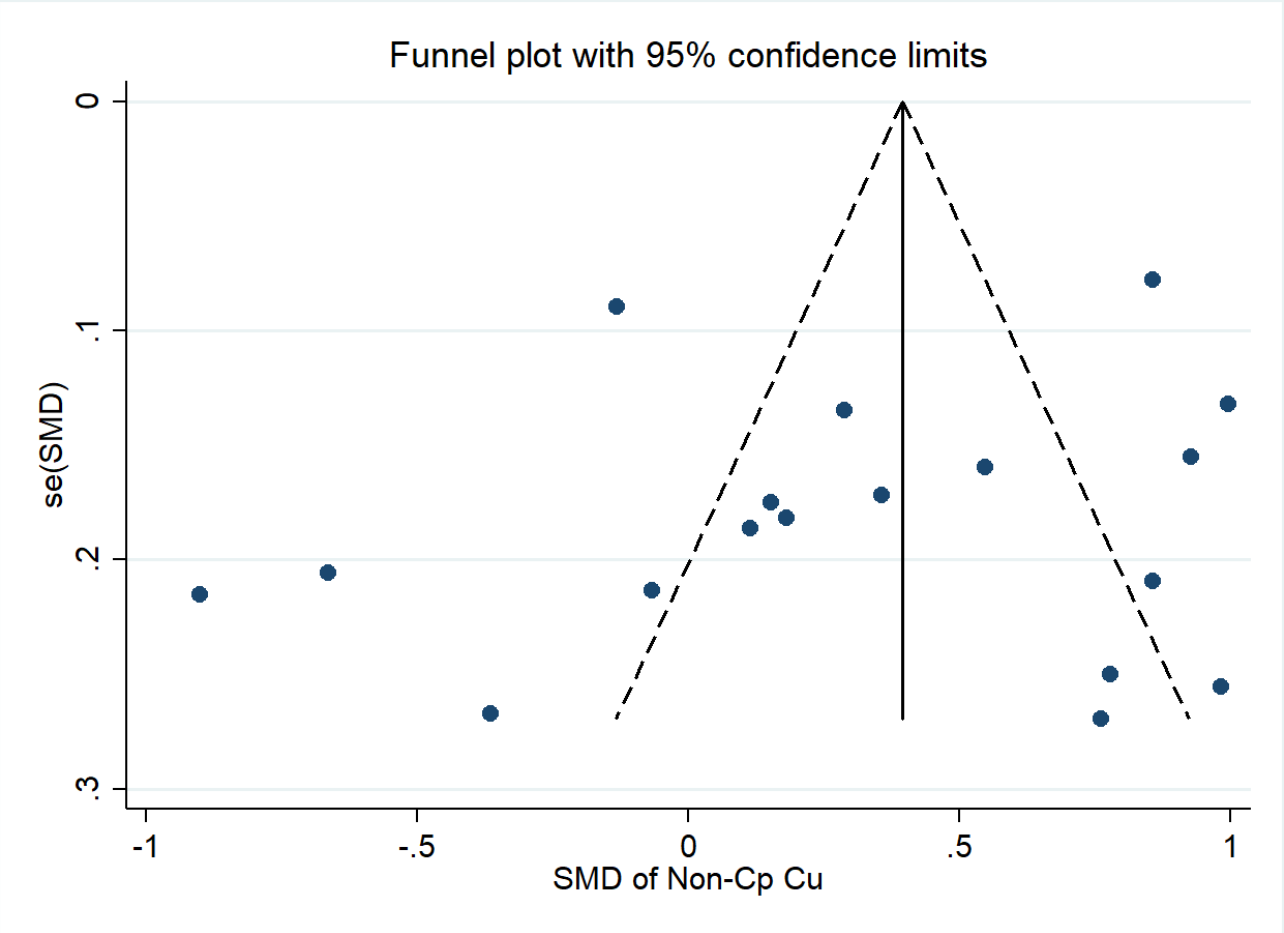
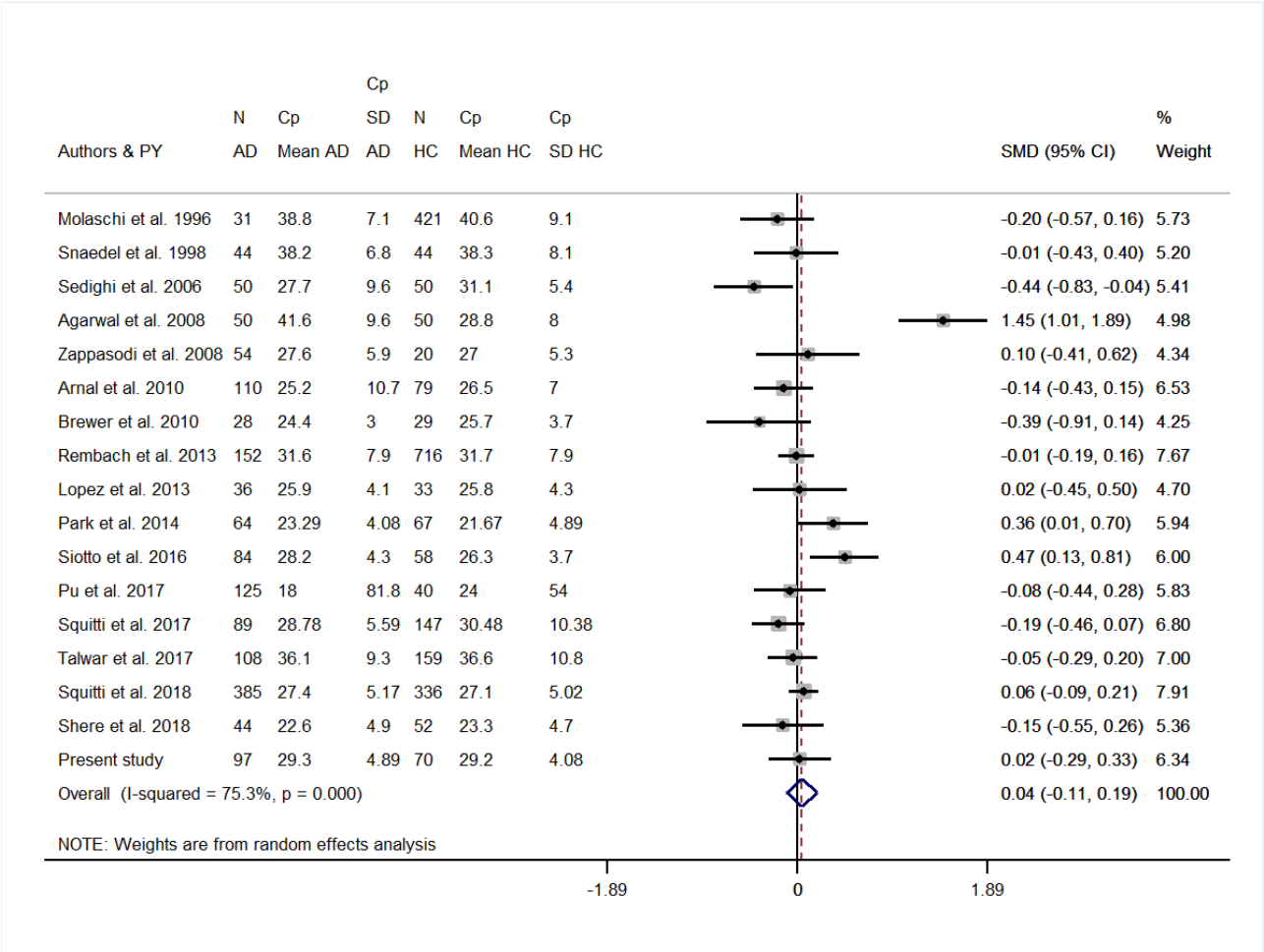


Figure 3 Supplementary material. Standardized mean difference (SMD) computed from the studies on ceruloplasmin (mg/dL) in Alzheimer's disease patients and healthy controls.



SMDs between AD subjects and controls are represented by squares, whose sizes are proportional to the sample size of the relative study. The whiskers represent the 95% confidence interval (CI). The diamond represents the pooled estimate based on the random effects model, with the centre representing the point estimate and the width the associated 95% CI.

Most of the ceruloplasmin studies carried out in serum/plasma demonstrated no variation (15 studies), while 2 studies reported increases and a study decreased values of ceruloplasmin in AD patients in comparison with healthy controls. As a whole, the forest plot of the meta-analysis reveals no variation of ceruloplasmin in serum/plasma of AD patients in comparison with healthy controls as indicated by the diamond that touch the zero line. This result further confirm that the increase in serum/plasma Cu can be explained by the increased levels of non-ceruloplasmin Cu fraction, as depicted in previous studies and in the current dedicated meta-analyses.

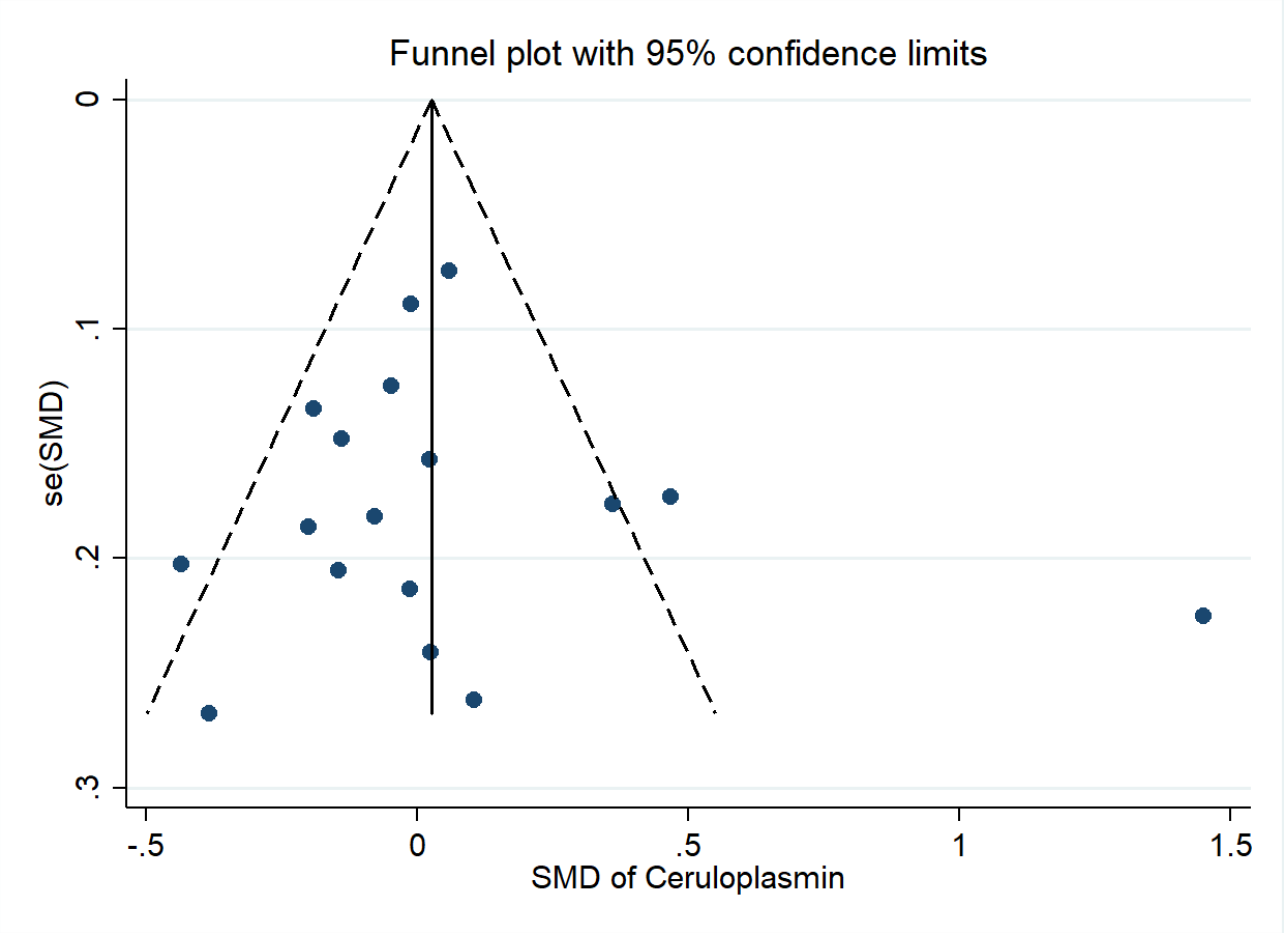



Figure 4 Supplementary material. Funnel plot suggested no presence of publication bias in the studies of ceruloplasmin in serum/plasma evaluated in Alzheimer's disease and healthy controls.