Biomarker Qualification Program: Free copper in serum assessed by inductively coupled plasma mass spectrometry (ICPMS)

Letter of Intent

***Free Copper (Free Cu)* or *Copper not bound to ceruloplasmin in serum* or *non-ceruloplasmin Copper*.**

Version 2.0 dated June 11, 2020

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SECTION 1 ADMINISTRATIVE INFORMATION

1. SUBMISSION TITLE:

Free copper in serum assessed by inductively coupled plasma mass spectrometry (ICPMS)

2. REQUESTING ORGANIZATION:

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3. SUBMISSION DATE:

- LOI submission date: April 9, 2020(v1); June 11, 2020 (v2)

SECTION 2 DRUG DEVELOPMENT NEED STATEMENT

Describe the drug development need that the biomarker is intended to address, including (if applicable) the proposed benefit over currently used biomarkers for similar context of uses (COUs).

Alzheimer's Disease (AD) is the most common cause of dementia. The disease is typified by misfolding of specific proteins that are associated with extracellular deposits of the beta-amyloid protein (Aβ) [1, 2] and aberrant aggregation of pathological forms of the intraneuronal protein Tau [3]. Despite a wealth of knowledge on the Aβ cascade mechanisms involved in AD, current treatments fail to show undoubtedly full efficacy. Reconsideration of the multifactorial nature of the disease is needed to develop effective prevention and drug strategies.

A recent study by Boyle and colleagues [4] shows the relative contributions of specific neuropathologies to cognitive loss at the individual level, indicating that many neurodegenerative processes synergistically take place in the brain of individuals affected by dementia, and suggests the urgent need for precision medicine approaches that embrace the heterogeneity of disease to target specific biological subsets rather than broad clinical entities.

In their analysis of primary prevention of AD, Norton et al. excluded from their estimation of a population-attributable risk based on relative risks from existing meta-analyses [5] some potentially modifiable risk factors. Among these excluded risks were dietary factors and metal imbalances, whose data about prevalence were missing in literature. However, at least seven meta-analyses (*Table 1*) demonstrated that

AD patients fail to maintain a physiological balance of copper (also reported as Cu through the text), showing excess copper and Free Copper in the serum [6-11] and copper depletion in the brain [8].

TABLE 1 MOST UPDATED META-ANALYSES OF COPPER IN ALZHEIMER'S DISEASE

Reference (Author, Journal, year)	**Copper Biomarker**	**Sample Sources**	**Studies Analyzed**	**Number of AD Cases**	**Number of Controls**	**Trend**	**p Value**
Li et al., Front Aging Neurosci 2017 [6]	Copper	serum	35	2128	2889	increase	<0.0001
Squitti et al., J Alzheimer's Dis 2014 [9]	Free Copper	serum/plasma	10	599	867	increase	<0.001
Bucossi et al., J Alzheimer's Dis 2011 [7]	Copper	serum/CSF*	5	116	129	no diff in CSF, increase in serum	
Schrag et al., Prog Neurobiol 2011 [12]	Copper	brain	7	115	123	decrease	=0.0003

*CSF: cerebrospinal fluid

These studies revealed a composite pattern of copper imbalance with plasmatic increases of Free Copper higher than normal values. As it is becoming increasingly appreciated, many diseases have subtypes with markedly different prognoses or responses to a specific treatment. The critical determination of whether a patient has a particular medical condition for which treatment may be indicated or whether an individual should be enrolled in a clinical trial studying a particular disease, is among the scopes of biomarkers directed to detect subtypes of diseases. New therapeutic strategies with the highest potential to generate advances in clinical practice for multifactorial chronic diseases, including multi- or comorbidity, including AD, ready for further development are those based on precision medicine, with treatments, being tailored to the individual patient. The summary report of the "Stratification biomarkers in personalized medicine" workshop, Brussels 10-12 June 2010 states that: '*…Personalized medicine may be defined as "a medical model using molecular profiling technologies for tailoring the right therapeutic strategy for the right person at the right time, and determine the predisposition to disease at the population level and to deliver timely and stratified prevention". In this context, the use of biomarkers can help identifying patients that are more likely to respond favorably to a given therapy. Until now, biomarkers have been used in clinical practice to describe both normal and pathological conditions. Increasingly, they find application in order to stratify different patient groups in terms of clinical response, to develop personalized, preventive or therapeutic strategies…*'. In this regard, data from our group supports the idea that Free Copper can be employed, on the basis of a specific cut-off, as biomarker instrumental for the stratification of AD patients in two subsets of AD individuals (*Table 2*) [9, 13-17].

TABLE 2 CLINICAL AND GENETIC EVIDENCE AT THE BASIS OF FREE COPPER AS A STRATIFICATION MARKER THAT IDENTIFY A COPPER AD (CUAD) SUBTYPE

Study Design and Reference	AD Patients	High Cu	Normal Cu AD	results
AD patients were stratified based on a Free Copper cut off (1.6 µmol/L) and compared for resting EEG-derived eLORETA cortical rhythms. (Tecchio et al., Curr. Alzheimer Res. 2016) [17]	67	31	36	The High Free Copper group had a lower power in all bands (0.2-48 Hz) in the parietal cortices (p=0.019) and a more limited alpha band (8-13 Hz) power in the sensory lobes (temporal, occipital, and parietal p>0.05 consistently) than the Normal noncerulopplasmin-Cu AD group. When corrected for MMSE, the Free Copper levels correlated with a reduction of high-frequency brain activity (from high alpha to gamma, 10.5-48 Hz).
AD patients were stratified based on a Free Copper cut off (1.6 µmol/L) and compared for medial temporal lobe atrophy, global atrophy periventricular hyperintensities, deeper hyperintensities, and infratentorial hyperintensities indices. (Squitti et al., Curr. Alzheimer Res. 2017) [15]	66	39	27	The two groups differed for global atrophy. More specifically, AD patients within the high Free Copper group had a less severe burden of global atrophy.
AD patients were stratified based on a Free Copper cut off (1.9 µmol/L) and compared for single-locus and haplotype of rs1801243, rs1061472, and rs732774 ATP7B genetic variants and the APOE4 genotype (Squitti et al., Mol. Neurobiol. 2017) [16]	287	176	111	The two AD subgroups did not differ regarding APOE4 frequency allele, while they did differ for allele, genotype, and haplotype frequencies of rs1061472 A > G and rs732774 C > T. The haplotype containing the AC alleles appeared more frequently in AD patients with normal Free Copper (43 vs. 33%), while the haplotype containing the GT risk alleles appeared more frequently in the higher Cu AD subtype (66 vs. 55%).
147 healthy subjects, 36 stable MCI, and 89 AD patients were analyzed for Cu abnormalities and sensitivity and specificity of Free Copper was evaluated through ROC* curves. (Squitti et al., Clin. Chem. Lab. Med. 2017) [18]	89	51	38	Free Copper was 1.5 times higher in AD patients (regarding the upper value of the reference interval) than in healthy controls. Healthy, MCI, and AD subjects were differentiated through a direct Free Copper method [area under the curve (AUC)=0.755]. Considering a 95% specificity and a 1.91 µmol/L cut-off, the sensitivity of noncerulopplasmin-Cu for distinguishing AD patients from healthy controls was 48.3% (confidence interval 95%: 38%-58%).

*ROC, receiver operating characteristic curves depict the likelihood of a given test to be excellent, good, or worthless. The accuracy of the test depends, in this case, on the probability that a subject randomly selected from AD group has a nonceruloplasmin-Cu value higher than that of a subject randomly selected from the healthy control group. The accuracy is measured by the AUC of the ROC curve. An AUC=1 represents a perfect test, a value of 0.5 represents a worthless test, and values in the 0.7–0.8 range considered fair.

These AD sub-sets differ in terms of:

i) frequencies of the functional single nucleotide polymorphisms (SNPs) [19] rs1061472 A > G and rs 732774 G > A, as well as frequency of derived GA risk haplotype [16];
ii) electroencephalographic (EEG)-derived brain cortical rhythms, with the High Free Copper group showing a lower power in all bands (0.2-48 Hz), in the parietal cortices ($p=0.019$) and a reduced alpha band (8-13 Hz) power in the sensory lobes (temporal, occipital, and parietal; $p<0.05$) when compared to the normal Free Copper AD group [17];
iii) neuroimaging indices of cerebral atrophy and neurovascular burden, having AD patients within the High Free Copper group a less severe burden of global atrophy ($p=0.042$) [15].
iv) Additionally, other authors showed a peculiar case-report of an AD patient with 11C-labeled Pittsburgh Compound-B PET positive and a [18F] fluorodeoxyglucose PET altered with a typical AD topography and carrying Kayser-Fleischer rings - pathognomonic of Wilson Disease – that strongly supports the existence of a Copper metabolic subtype of AD [20] (herein indicated as Cu-AD subtype).

In close analogy, we propose the same rationale for the stratification of patients with mild cognitive impairment due to AD (MCI), a prodromal stage of AD, with the aim at predicting the conversion to AD [21]. The stratification of MCI individuals in two groups on the basis of a cut-off revealed that subjects with values higher than a designed Free Copper cut-off had a hazard conversion rate (50% of conversion in 4 years) that was ~3x higher than those with values lower than the cut-off (<20% in 4 years).
On this basis, Free Copper can be employed, in association with the standard criteria, as a minimally invasive and cost-effective prognostic biomarker for AD, meeting all the requirements of the “precision medicine” manifesto. It can serve as a prognostic biomarker [21-24] aimed at identifying MCI individuals who are more likely to develop symptomatic AD [7, 21, 22, 25, 26] and can be used as an inclusion criterion for eligibility assessment in clinical trials testing anti-copper based therapy to delay or prevent AD [27].
As it will be discussed later, the proposed prognostic biomarker has demonstrated a fair sensitivity in detecting which individuals with MCI will convert to Cu-AD [21] and high specificity (95%) in detecting patients affected by Cu-AD due to copper exposure [18]. Qualifying Free Copper as a prognostic biomarker for AD will support future drug development programs for prevention of the Cu-AD subtype [15-17].
Free Copper test as an inclusion criterion for phase II clinical trial employing anti-copper based drugs is intended to set the innovation in drug development program based on biomarker science applied to Cu-AD subtype. On this side, therapeutic interventions, as for example anti-copper based therapy already in the market for Wilson disease, the paradigmatic disease of Free Copper accumulation and intoxication, can be re-purposed, for potential beneficial effects on MCI people exhibiting higher than normal values of Free Copper.
Free Copper, as inclusion criterion for patient selection in early clinical trial, will pave the way for new anti-copper-based drug developmental programs, exclusively dedicated to individuals with copper homeostasis

dysfunction who will likely benefit of regaining copper balance in their body. Furthermore, people at high risk of dementia with a normal value of Free Copper can be excluded early from such therapeutic or prevention interventions, avoiding undesired side effects, and waste of precious time with a treatment unlikely to succeed. Innovation will be the precise definition of target population for the treatment with anti-copper based therapies.

The likelihood of success of this drug development program is sustained by evidence-based medicine, which supports the use of knowledge acquired by clinical investigations (*Table 3*), large populations studies, (please refer *Table 4*) and meta-analyses (please refer to *Table 1)*

TABLE 3. CLINICAL STUDIES ANALYZING CU AND ATP7B GENE VARIANT LINK TO ALZHEIMER'S RISK AND THE ASSOCIATION WITH THE SUBJECTS' CLINICAL STATUS

Serum Copper and Risk for Alzheimer's Disease	**Subjects**	**Risk (OR, RR, HR)**	**CI 95 %**	**p Value**
Copper level was higher in subjects with AD than in control subjects and correlated with poor neuropsychological performance and medial temporal lobe atrophy. (Squitti et al., Neurol. 2002) [28]	76 AD vs 79 healthy subjects	**1.8**	1.36 - 2.43	p<0.05
Copper level was higher in subjects with AD than in patients with vascular dementia (VaD) (Squitti et al., Neurol. 2003) [29]	48 AD vs 20 VaD subjects	**2.06**	1.28 - 3.31	p<0.003
Free Copper was higher in AD than in healthy controls and VaD and correlated with poor neuropsychological performance. (Squitti et al., Neurol. 2005) [30]	47 AD vs 44 healthy subjects and 24 VaD subjects			P<0.001
Free Copper was higher in AD than in healthy controls; cerebrospinal (CSF) β-amyloid and h-Tau correlated with serum Free Copper; copper in the CSF was partially dependent on the serum Free Copper (t = 2.2, p = 0.04). Mini-Mental State Examination (MMSE) and verbal memory scores correlated positively with β-amyloid (r = 0.46, p = 0.002) and inversely with nonceruloplasmin-Cu (=0.45, p = 0.003). (Squitti et al., Neurol. 2006) [25]	28 AD vs 25 healthy subjects			P<0.001
Free Copper predicted the annual change in MMSE; when the annual change in MMSE was divided into <3 or ≥3 points, Free Copper was the only predictor of a more severe decline. (Squitti et al., Neurol. 2009) [22]	81 AD subjects, 1-year longitudinal study	**1.23**	1.03-1.47	p<0.022
Significant inverse correlation of the serum levels of Free Copper with both MMSE and attention-related neuropsychological tests scores. (Salustri et al., Clin Neurophysiol. 2010) [31]	64 healthy subjects			p<0.05
Free Copper was higher in MCI than in healthy subjects. (Squitti et al., J. Alzheimer Dis. 2011) [23]	83 MCI subjects, 100 healthy subjects	**1.22**	1.05-1.41	p<0.01

Copper level showed a significant increase in the serum of AD and MCI compared to control (p=0.038). (Lopez et al., J. Alzheimer's Dis. 2013) [32]	36 AD, 18 MCI vs 25 healthy subjects			p<0.05
Free Copper increased the risk of having AD; when combined in an algorithm with sex, APOE, Cp/Tf, TAS, the ability to discriminate AD patients vs. controls was high (ROC*, AUC=0.9). (Squitti et al., Curr. Alzh. Res. 2013) [33]	93 AD, 45 VaD, 48 healthy subjects	**3.21**	1.53-6.71	p<0.002
Free Copper was a predictor of conversion to AD: MCI subjects with nonceruloplasmin-Cu levels >1.6 µmol/L had a hazard conversion rate (50% conversion in 4 years) that was ~3 higher than those with values ≤1.6µmol/l (<20% in 4 years). (Squitti et al., Ann. Neurol. 2014) [21]	131 MCI subjects, 6 years longitudinal study	**3.3**	1.21-9.24	p=0.02
Free Copper levels higher in MCI and AD with respect to control (p<0.0001). (Rozzini et al., J. Alzheimer's Dis. 2018) [24]	44 AD and 36 MCI vs 28 healthy subjects			p<0.001
Free Copper and Cu:Cp resulted higher in AD and in Wilson disease than in healthy controls; while nCp-Cu was similar between AD and Wilson disease, Cu:Cp was higher in Wilson disease. 24 h urinary copper excretion in AD patients (12.05 µg/day) was higher than in healthy controls (4.82 µg/day); 77.8% of the AD patients under D-penicillamine treatment had a 24 h urinary excretion higher than 200 µg/day, suggestive of a failure of copper control. (Squitti et al., J. Trace Elem. Med. Biol. 2018) [26]	385 AD, 9 Wilson disease, 336 healthy subjects			p<0.0001
Free Copper does not change in frontotemporal lobar degeneration (FTLD) (Squitti et al., J. Alzheimer's Dis. 2018) [34]	85 FTLD, 55 healthy subjects			p < 0.001
ATP7B gene variants and risk for Alzheimer's disease				
Specific genetic variants in the ATP7B gene, namely rs1801243 (OR = 1.52, 95% CI = 1.10–2.09), rs2147363 (OR= 1.58, 95% CI = 1.11–2.25), rs1061472 (OR= 1.73, 95% CI = 1.23–2.43), and rs732774 (OR= 2.31, 95% CI = 1.41–3.77) increased the risk of having AD. (Squitti et al., Rejuvenation Res. 2013) [35]	285 AD vs 230 healthy subjects	**2.3**	1.41-3,77	p<0.001
Wilson disease-causing variant rs7334118 in linkage disequilibrium with the intronic rs2147363 (associated with AD risk) was detected in two AD patients but in no healthy individuals. However, this Wilson disease mutation did not explain the observed genetic association of rs2147363. Conversely, *in silico* analyses of rs2147363 functionality highlighted that this variant is located in a binding site of a	286 AD vs 283 healthy subjects	**1.3**	1.06-1.69	p=0.015

transcription factor and is associated with regulatory functions. (Bucossi et al., J. Alzheimer's Dis. 2013) [36]				
Haplotype TGC in specific genetic variants in the ATP7B gene, namely rs1801243, rs1801249, rs1801244, and rs1801243 was associated with an increased risk of having AD. (Liu et al., J. Biol. Regul. Homeost. Agents 2013) [37]	120 AD vs 111 healthy subjects	**5.16**	2.54-10.5	p<0.001

*ROC, receiver operating characteristic curves depict the likelihood of a given test to be excellent, good, or worthless. The accuracy of the test depends, in this case, on the probability that a subject randomly selected from AD group has a nonceruloplasmin-Cu value higher than that of a subject randomly selected from the healthy control group. The accuracy is measured by the AUC of the ROC curve. An AUC=1 represents a perfect test, a value of 0.5 represents a worthless test, and values in the 0.7–0.8 range are fair.

TABLE 4. EPIDEMIOLOGICAL STUDIES OF COPPER AND COGNITION

Reference (Author, year)	**Type of analysis**	**Number of Cases analyzed**	**Results**	**p value**
Morris et al. 2006 [38]	Longitudinal, 9 years follow-up	3718 (CHAP study)	Among people whose diets were high in saturated and trans fats, higher copper intake was associated with a faster rate of cognitive decline (those Participants in the highest fifth of copper intake combined with a diet high in saturated fats, lost cognition at a rate of 19 years in a period of 6 years, thus the cognitive decline was three times higher than expected)	<0.01
Lam et al. 2008 [39]	Cross-sectional, and cores on 11 of 12 cognitive function tests Plasma analysis	1551 (Rancho Bernardo Study)	In women, copper concentrations had inverse linear associations with Buschke total, long and short-term recall and Blessed scores (cognitive test).	<0.001
Shen et al. 2014 [40]	Copper concentration in soil in relation to annual mortality of AD concentration	Chinese general population, from 1991-2000, in 26 provinces, 3 districts	Relative risk of mortality in the regions with the highest copper concentrations (60-80 mg/kg) was high [OR: 2.63 (95% CI: 2.626-2.642)]	<0.001

Zhou et al. 2016 [41]		826 children	Significant association between high copper levels and poorer working memory in boys (unstandardized regression coefficient (b) = -0.099, robust standard error (s.e.) = 0.009, t = -10.99, p = 0.008, 95% CI (-0.138, -0.060).	
Kicinski et al. 2015 [42]			A two-fold increase in blood Cu was associated with a 0.37 standard deviation decrease in sustained attention (95% CI: -0.67 to -0.07, p=0.02) and 0.39 standard deviation decrease in short-term memory (95% CI: -0.70 to -0.07, p=0.02).	
Pujol et al. 2016 [43]		2836 children aged from 8 to 12 years.	The results establish an association between environmental copper exposure in children and alterations of basal ganglia structure and function.	
Takeuchi et al. 2019 [44]	gray matter volume (rGMV), mean diffusivity (MD), and cognitive performance	924 healthy young adults	Cu in hair associates with aberrant cognitive functions, greater rGMV in extensive areas, greater MD (which are indicative of low density in brain tissues) in subcortical structures in healthy young adults	
Alemany et al. 2017 [45]		1,645 scholar children from the BREATHE project	*ATP7B* rs1061472 (P for interaction 0.016) and *ATP7B*-rs1801243 (P for interaction 0.003) polymorphisms modified the association between indoor copper exposure and inattentiveness.	

Furthermore, results from studies using experimental models (*Table 5*), and pilot clinical trials (*Table 6*) challenging anti-copper-based intervention point out to the biological plausibility of a copper imbalance linked to cognitive decline.

TABLE 5 ANIMAL MODELS OF COPPER NEUROTOXICITY INDUCED BY ALTERED DIET

Authors, year	**Animal model**	**Dose and route**	**Duration**	**Effects**

Sparks and Schreurs 2003 [46]	New Zealand white rabbits	12 mg/L Cu in DW + 2% cholesterol - oral	10 weeks	Accumulation of Aβ in brain; deficit in complex memory acquisition
Sparks et al. 2006 [47]	Beagle dogs	200 mg/L $CuSO_4$ in DW + high fat diet - oral	4 months	Extracellular Aβ deposits
Lu et al. 2006 [48]	Kumming strain mice	0.21 mg/L Cu in DW + 2% cholesterol – oral	8 weeks	Cognitive deficits; neuronal apoptosis
Arnal et al. 2013 Arnal, 2013 #1024}	Wistar rat	3 mg/L Cu in tap water + 2% cholesterol – oral	2 months	Increased oxidative stress in brain; increased Free copper in hippocampus; increased Aβ (1-42)/ Aβ (1-40) in cortex and hippocampus
Arnal et al. 2013 [49]	Wistar rat	3 mg/L Cu in tap water + 2% cholesterol – oral	8 weeks	Slight nut noticeable change in visuo-spatial memory
Yao et al. 2018 [50]	Tg2567 mouse	0.1 mg/L copper in drinking water and 2% cholesterol in the food	3 months	Significant deposit of Aβ and senile-plaque formation in hippocampus and temporal cortex regions
Abolaji et al. 2020 [51]	D. melanogaster flies	Cu2+ (1 mM)	7 days	reduced survival
Lamtai et al. 2020 [52]	Rat	$CuCl_2$ (0.25 mg/kg, 0.5 mg/kg and 1 mg/kg) injected intraperitoneally	8 weeks	Working memory, spatial learning and memory were significantly impaired in rats treated with Cu at dose of 1 mg/kg

Models of copper neurotoxicity in genetically compromised animals				
Authors, year	**Animal model**	**Dose and route**	**Duration**	**Effects**
Sparks et al. 2006 [47]	Watanable rabbits	0.13 mg/L Cu in DW - oral	10 weeks	Accumulation of Aβ in superior temporal cortex and hippocampus
Sparks et al. 2006 [47]	PS1/APP transgenic mice	0.12 mg/L Cu in DW- oral	6 weeks	Deposition of Aβ
Singh et al. 2013 [53]	APP sw/0 mice	0.13 mg/L Cu in DDW- oral	90 days	Increase brain Aβ production; increased neuroinflammation; memory impairment; increased Cu levels in brain capillaries and parenchyma
Yu et al. 2014 [54]	3xTg-AD	250 mg/L $CuSO_4$ in drinking water	6 months	Memory impairment

TABLE 6. CLINICAL TRIAL TESTING ANTI-COPPER-BASED THERAPIES ON COGNITION AND ALZHEIMER'S DISEASE

Authors, year	**Treatment**	**Sample size**	**Design**	**Clinical outcomes**
Van Rhijn et al. 1990 [55]	Zinc-sulphate and sodium selenite	A total of 15 AD	Dietary supplementation study	Improved performance on the anomalous sentences repetition test, colored progressive matrices, graded naming test and digit copying test
Crapper McLachlan et al. 1991 [56]	Desferrioxamine mesylate (metal chelator)	A total of 48 AD	Single-blind trial	Slowing of clinical deterioration, increased activities of daily living in the treatment group
Constantinidis 1992 [57]	Zinc-hydrogenaspartate	5 presenile AD 5 senile AD	Follow-on study	Improved memory, understanding, communication, and social interaction in 8 patients
Potocnik et al. 1997 [58]	Zinc-methionine	4 AD	Open-labelled pilot study (12 months)	Improved performance on the cognitive tests
Squitti et al. 2002 [59]	D-penicillamine (Copper chelator)	A total of 18 AD (treated: placebo 1:1)	Double-blind, placebo-controlled 6 months trial	No widespread benefit on cognitive outcomes; placebo patients did not worsen in the 24 weeks follow-up; peroxides in serum from patients taking D-penicillamine decreased by

				29% with respect to their t0 evaluation found (F1,16 = 4·52; P = 0·049).
Ritchie et al. 2003 [60]	Clioquinol	A total of 36 probable AD (treated: placebo 1:1)	Double-blind, placebo-controlled, parallel group randomized 36 weeks study	No effect on cognitive outcomes at any of the time points assessed. Significant improvements in MMSE when the groups were stratified by their level of impairment at baseline; plasma Aβ declined in the treated group and increased in the placebo group
Maylor et al. 2006 [61]	Zinc-gluconate (Zenith)	387 healthy older adults	Randomized double-blind placebo-controlled 6 months study	Few significant benefits in visual memory, working memory, attention and reaction time were obtained using the Cambridge Automated Neuropsychological
Lannfelt et al. 2008 [62]	PBT2 (copper/zinc ionophore)	A total of 78 Early AD (treated: placebo 1:1	Phase II, double-blind, randomized, placebo-controlled 12 months trial	No significant difference in the Neuropsychiatric Test Battery (NTB); dose-dependent reduction of Aβ concentrations in the CSF and positively impacted on two executive function component tests showing significant improvement over placebo in the PBT2 250 mg group: category fluency test (2.8 words, 0.1 to 5.4; p=0.041) and trail making part B (–48.0 s, –83.0 to –13.0; p=0.009).
Faux et al. 2010 [63]	PBT2 (copper/zinc ionophore)	40 AD	Phase II double-blind, randomized, placebo-controlled 12 weeks trial	Improvement on NTB Composite or Executive Factor z-scores

Brewer 2012 [64]	Zinc (Adeona) 150 mg/day,	A total of 42 AD mild-moderate AD patients (treated: placebo 1:1)	Phase II double-blind, randomized, placebo-controlled 12 weeks trial. Primary outcome: ADAS-Cog	No significant effect on primary clinical outcome; post hoc analyses limiting the analysis to those patients aged 70 years and older (14 zinc treated patients vs. 15 placebo patients) revealed statistically significant better cognition scores in the zinc-treated patients vs controls in ADAS-Cog (p= 0.037) and CDR SOB (P ¼ 0.032), with near significant results in MMSE (p= 0.07)
Villemagne et al 2017 [65]	PBT2 (copper/zinc ionophore)	40 AD (12-month double-blind phase) (placebo = 15, PBT2 = 25), and 27 subjects 12-month (placebo = 11, PBT2 = 16)	A randomized, exploratory molecular imaging study targeting amyloid beta with PBT2 in AD, 12-month phase in a double-blind and a 12-month open label extension phase trial design.	There was no significant difference between PBT2 and controls at 12 months, likely due to the large individual variances over a relatively small number of subjects

Qualifying Free Copper as a prognostic biomarker will support future drug development for prevention and treatment of the Cu-AD subtype [15-17].

SECTION 3 BIOMARKER INFORMATION AND INTERPRETATION

Alzheimer's disease is one of the most common forms of dementia in the elderly. A bulk of evidence connects copper abnormalities to AD pathogenesis. A subset of patients affected by AD, or its prodromal form known as MCI, fails to maintain a physiological balance in the metabolisms and distribution of copper, thereby generating Free Copper increases in serum. The evaluation of Free Copper in serum is employed as a biomarker for Wilson disease, a paradigmatic condition of Cu-driven toxicosis. In close analogy with Wilson disease, assessment of Free Copper can be exploited as a cost-effective prognostic biomarker useful for the identification of individuals at-risk of Cu-related abnormalities that can facilitate AD pathogenesis. This approach can be employed as an eligibility criterion for clinical trials aimed at testing Cu-related interventions against AD.

1. BIOMARKER NAME:

- **Biomarker name:** Free Copper
- **Abbreviated short name for biomarker (or names if multiple):** Free Copper (Free Cu) or Copper not bound to ceruloplasmin in serum or non-ceruloplasmin Copper.
- **Identify biomarker type:** Molecular
- **For molecular biomarkers, please provide a unique molecular ID:** Free Copper

2. ANALYTICAL METHODS:

Name and briefly describe analytical methods used in raw measurement(s) of the biomarker(s).

The IGEA Free Copper test is a method for direct measurement of Free Copper in serum ultrafiltrate by inductively coupled mass spectrometry (ICPMS) as described in [66]. A two-step approach is employed to assess Free Copper: the first step separates the copper bound to ceruloplasmin and other cuproenzymes from the pool of loosely bound species of copper (procedure depicted in SOP in Attachment B). The second involves the measurement of copper levels in the filtrate by ICPMS, obtaining ICPMS reports that are used without any transformation.

3. MEASUREMENT UNITS AND LIMIT(S) OF DETECTION:

Free Copper measurements units are µmol/L. Also, µg/dL are reported (being µmol/L= 0.1574 µg/dL). Free Copper IGEA is accurate (95% recoveries), linear (nine concentration levels and 2 replicates at each level was tested 1.1–20 µg/dL [1.8-3.2 µM µmol/L]), and precise (coefficient of variation, <6.8%); the limit of black (LOB) estimated is 0.0002 µmol/L and the limit of detection (LOD) is 0.0397 µmol/L. Details about LOB and LOD performances are depicted in *Section 11*.

4. BIOMARKER INTERPRETATION AND UTILITY

Describe the application/conversion of the raw biomarker measurement in order for the biomarker outcomes to be used for the COU and provide the description and derivation of clinical interpretative criteria used to include:

a. Post-analytical application/conversion of biomarker raw measure to the applied measure: briefly describe how the raw biomarker measurement is used/applied. Describe if the raw measure is used directly or if there is further processing of the raw measurement into a multi-component panel, a scoring system, or alternatively; further manipulation or transformation of the raw biomarker measurement using modeling, simulation, application of formula(e), other algorithms, or combination with other clinical information. Describe how the process is designed, including software. List the elements, inputs and output(s) of the conversion, including a description of units, if applicable.

   Biomarker raw values are used directly, without any transformation. Raw measurements indicate quantitative level expressed in µmol/L of Free Copper in serum.

b. Describe rationale for post-analytical elements used as inputs in application or conversion of the raw biomarker measurement.

   Not applicable

c. Clinical Interpretive Criteria: describe the cut-off values, cut points/thresholds, boundaries/limits or other comparators used in the interpretation of the biomarker measurement or its applied/converted form to draw an actionable conclusion based on the biomarker result.

Since the early 90s, the clinical implications of different Free Copper circulating in serum have been evaluated for their implications in health and disease [67]. The main clinical application

has been in Wilson disease, the paradigmatic disease of Free copper toxicosis or accumulation and cut-offs have been identified [18, 68-70] (*Table 7*).

TABLE 7 INTERPRETATION OF FREE COPPER IN SERUM AND CLINICAL IMPLICATIONS IN HEALTH AND DISEASE

Cut-off	Range	Study population	Interpretation	Article citation	Similar range citations
	0.1-1.6 µM	Healthy controls	Normal reference intervals	[18, 68-70]	[23, 33, 68, 71, 72]
1.6 µM	1.61- 2.4 µM	Copper subtype of Alzheimer	MCI and Alzheimer's disease patients with copper subtype	[15-17, 21, 26]	[16, 18]
2.31 µM	2.31-3.9 µM	n.a	Probable Wilson disease;	[68]	[73]
3.9 µM	>3.9 µM	n.a.	Wilson disease	[68]	[73]

Free copper cut-off values for discriminant analyses of Alzheimer's disease versus healthy controls, of Wilson disease patients versus healthy controls, and of stable mild cognitive impairment (MCI) versus MCI converter.

Reference intervals have been established in healthy controls. For example, a method for the direct measurement of Free Copper in serum ultrafiltrate by ICPMS, developed and validated for commercial use for Wilson disease in 2009 [69], identified a reference interval of 0.1–1.6 µmol/L. This reference interval was determined parametrically with 137 healthy adult (20–59 years) blood donors. Moreover, the method was accurate (94%–109% recoveries), linear (0.08-126 µmol/L]), and precise (coefficient of variation, <15% over the analytic measurement range). Another fluorescent direct method (IGEA Pharma NV patent) was developed in 2017 for AD [18]. It was linear (0.9-5.9 µM), precise (CV of 9.7% for low and 7.1 % for high measurements) and had a good recovery. A reference interval of 0-1.9 µM was determined in 147 healthy adult blood donors. The Intraclass correlation with a gold standard method for Free Copper, namely Free copper calculated Walshe's index was 0.75 (CI 95% 0.69-0.80). Free Copper was 1.5 times higher in AD patients than in healthy controls. Healthy, MCI and AD subjects were differentiated through this Free Copper

method [AUC=0.755; cut-off 1.91 µmol/L, 95% specificity, sensitivity 48.3% (CI 95%: 38%-58%)]. The likelihood ratio was 9.94 for positive test results (LR+) and 0.54 for negative test result (LR-) [18]. The low sensitivity of Free Copper may limit its utility in drug development. However, a drug development program based on the presence of Free Copper above a designated cut off will have a low chance of including patients for whom that strategy would be inappropriate. Even if the low sensitivity might mean that not every patient for whom that strategy would be beneficial would be captured, it would prevent the exposure to treatment risks (especially in a clinical trial setting) to patients who might otherwise not expect the same benefits based on the Free Copper stratification.

As summarized in *Table 7*, in healthy and MCI patients, Free Copper concentrations of 1.6 µmol/L through 2.4 µmol/L indicate a risk for development of AD [21]. In contrast, values below this range do not associate with increased AD risk in these populations, and values above it are associated with Wilson Disease [26, 68, 73].

SECTION 4 CONTEXT OF USE STATEMENT (500 CHARACTERS)

Serum Free Copper is intended to serve as a prognostic biomarker for conversion from mild cognitive impairment (MCI) [21-24] to symptomatic Alzheimer's disease (AD) typified by copper imbalance [7, 21, 22, 25, 26]. In association with the standard amnestic MCI criteria, it can serve as a minimally invasive and cost-effective prognostic biomarker for MCI that may be used as an inclusion criterion for eligibility assessment in clinical trials testing anti-copper based therapy to delay or prevent AD [27].

SECTION 5 ANALYTICAL CONSIDERATIONS

General description of what aspect of the biomarker is being measured and by what method (e.g., lesion number or specific measure of organ size by imaging, serum level of an analyte, change in the biomarker level relative to a reference such as baseline).

Free Copper concentration in serum, measured *in vitro*, is the proposed basis for assessing increased risk for AD and specifically for as predictive of the conversion from MCI to full AD. In serum, most of the copper is structurally bound to ceruloplasmin, the main body copper protein, which is generally thought to account for 85-95% of human serum copper and other proteins that as a whole constitute a pool of firmly-bound species of copper [74]. The remaining is historically defined as 'Free' Copper and is a pool of loosely bound species of copper in serum composed of different compounds (*Table 8*).

TABLE 8 COPPER DISTRIBUTION IN HUMAN SERUM

Pool of copper in serum		**Serum proteins or compounds**	**Notes**
		Ceruloplasmin	
		Extracellular Superoxide Dismutase (SOD3)	
Pool of firmly bound species of copper in serum	Cuproenzymes: proteins that bind copper with high affinity in high-coordinate binding site	Ferroxidase II	
		Factor V and VIII (blood coagulation protein)	
		Amine oxidases	
		Albumin	Albumin has a high affinity copper binding sites (Kd 10^{-12}- 10^{-17}), but it can easily exchange copper with other protein or chelator
Pool of loosely bound species of copper in serum	Proteins and compounds that bind copper with low affinity or low-coordinate flexible geometries that enable transfer	Alpha-2-macroglobulin	Alpha-2-macroglobulin has an affinity higher than albumin
		Small Copper Carriers	
		Cu-Metallothionein	Not always present (Kd 10^{-17} – 10^{-19})

Genetic, physiological, and pathological conditions, as well as drug therapy, can alter the concentration of the pool of loosely bound species. Accordingly, an accurate measurement of Free Copper could represent a significant diagnostic tool for those conditions in which dysregulation of the cation is critical for disease progression. As a matter of fact, novel quantitative approaches should aim at avoiding misleading conclusions based on 'total', bulk copper measurements that disregard abnormalities in the concentrations of the carrier proteins. In agreement Free Copper is a key biomarker for Wilson disease but has recently acquired additional clinical applications. Direct method developed for the experimental determination of Free Copper is generally performed in two steps: the first consists of the separation of the copper bound to ceruloplasmin and the other cuproenzymes of the pool of firmly-bound species of copper from the pool of loosely-bound species of copper (solid phase extraction or ultrafiltration). The second involves the measurement of copper levels in the filtrate. Further methodological differences are based on the copper measurement technique employed *(Table 9*) and on the procedures set to isolate the pool of loosely bound species. *Table 10* provides details on the different methods employed so far.

TABLE 9 ANALYTICAL METHODS FOR COPPER MEASUREMENTS

Analytical methods for copper measurements	**Advantages**	**Disadvantages**
Atomic absorption spectrometry (AAS) [75] AAS [75]relies on Beer-Lambert law: the electrons of the atoms in the atomizer can be promoted to higher orbitals for a short amount of time by absorbing a set quantity of energy provided by the light at a given wavelength. This wavelength is specific to a specific electron transition for a particular element; consequently, each wavelength corresponds to only one element. This gives the technique its elemental selectivity. From the differences between the quantity of energy (the power) applied to the flame (the atomizer), and the remaining quantity transmitted to the detector, it is possible to calculate how many of these transitions took place, and thus get a signal that is proportional to the concentration of the element analyzed.	Low interference, easy to use, low cost analysis (apart from instrument cost)	High precision, one metal at a time detection, not useful for isotope analysis, sample is totally disrupted
Inductively coupled plasma-mass-spectrometry (ICP-MS) [76] ICP-MS is a highly sensitive mass spectrometry technique that can measure a range of metals and several non-metals at concentrations below one part in 10^{12}. An inductively coupled argon plasma is employed to atomize and ionize the sample. For coupling to mass spectrometry, the ions from the plasma are extracted through a series of cones into a mass spectrometer, usually a quadrupole. The ions are separated based on their mass-to-charge ratio and a detector receives an ion signal proportional to the concentration. ICP-MS is also capable of monitoring isotopic speciation for the ions of choice. The concentration of a sample can be determined through calibration with certified reference material such as single or multi-element reference standard. ICP-MS is used in the medical and forensic field, specifically toxicology.	High power detection (ppb), multiple metals detection, useful for isotope analysis, metal image option	High skill technical level required, sample is totally disrupted, high cost analysis
Colorimetric method (Abe) [77] Automated colorimetric methods use the chemical reaction of serum copper with organic reagents. The Abe method [77]is an automated colorimetric method based on the use of the water-soluble chromogenic color reagent, 4-(3,5-dibromo-2-pyridylazo)-*N*-ethyl-*N*-sulfopropylaniline (DiBr-PAESA). This compound forms a chelate with copper (II) in Sodium Dodecyl Sulfate	Applied on commonly used automatic analyzers, easy to use, low cost	not for isotope analysis, sample is totally disrupted

solution, that is measured by a spectrophotometer at 580 nm. The method is linear (at least 5 mg/L), the lower limit of detection is 0.1mg/L; within-run coefficient of variation CVs are 1.6% and 3.3% for copper concentrations of 1.03 and 0.72 mg/L, respectively (n = 10 each); between-run CV is 2.8% at 1.22 mg/L (n = 14). Results of Abe method (y) correlated well with those determined by standard AAS techniques (x): y = 0.99x - 0.02 mg/L; S_{yx} = 0.08; r = 0.977; n = 56. Squitti *et al.* [28] reported good correlation of copper concentrations between Abe and AAS methods (Pearson's correlation, r= 0.89, p<0.001). Iron, zinc, cadmium, cobalt, and lead do not interfere		

TABLE 10 COMPARISON OF CURRENTLY EXISTING METHOD FOR FREE COPPER MEASUREMENTS

Authors (year)	*Method*	*Easiness*[1]	*Reliability*	*Affordability*[2]	*Disease Application*	*Results*	*Limitation*	*Related articles*
Noubah et al 1990 [78]	Serum, ultrafiltration (10 KDa) + ETAAS[3]	Moderate difficult	Good Precision, CV < 11.5%	n.a.	Pregnancy (Normal, pregnant and cord blood)	Free copper was not different	Obsolete method	
Kulipa-Rantala et al 1996 [79]	Plasma, EDTA incubation, ultrafiltration (10 KDa) +PIXE[4]	Moderate difficult	Not reported	n.a.	Breast cancer	Authors report that the method is sensitive and precise	Time consuming, academic setting	
Favier et al 1998 [80]	Serum, ultrafiltration (25KDa, EDTA[5]) + AAS[6]	Moderate difficult	Not reported	n.a.	Healthy controls	Reference values	Obsolete method	
Bohrer et al 2004 [81]	Serum, solid phase exctraction+AAS (compared with ultrafiltration 20 kDa +AAS	Difficult	Not reported	Moderate-Utility Moderate-Expense	Wilson disease	The solid phase extraction method performed better, and two methods have a good agreement	Time consuming, academic setting	
Inagaki et al 2000[82]	Serum pre-treatment with Chelex-100[7] +ICP-MS[8]	Easy		n.a.	Healthy controls	Author report the method is simply and reliable	Not direct method[7]; time consuming, academic setting	[83]

Squitti et al 2006 [25]	Indirect method, known as Walshe's index [70] based on the subtraction of the quantity of the copper bound to ceruloplasmin from the total concentration of copper in the blood serum	Easy	n.a. reliability relate to the Ceruloplasmin and Copper measurement methods	Higher-Utility Moderate-Expense	Alzheimer's disease. Mild cognitive impairment. Wilson disease Frontotemporal Lobar Degeneration. Healthy controls	Free copper is higher in Alzheimer's disease, Wilson disease and Mild Cognitive Impairment individuals than in healthy controls and frontotemporal Lobar degeneration individuals	NDML[9]	[18, 23, 26, 31, 84, 85] [9, 15, 21, 33, 86-88] [89-94]
Buckley et al 2008 [95]	Plasma, ultrafiltration (10KDa) + ICP-MS with isotope composition analysis	Difficult	Repeatable and stable	Higher-Utility Higher-Expense	6 lactating cows, 2 humans	The method yields reliable, physiologically meaningful results	Time consuming, academic setting	[95]
McMillin et al 2009 [69]	plasma/serum, ultrafiltration (30KDa) + ICP-MS	Moderate difficult	Good precision CV <15%	Higher-Utility Moderate-Expense; Clinical pathology Lab setting	Wilson disease	Free copper increases in Wilson disease patients	Not more in the market	
El Balkhi et al 2011[71]	Plasma heparin, EDTA incubation, ultrafiltration (30 KDa) + ETAAS	Moderate difficult	Repeatable and stable	Higher-Utility Moderate-Expense	Wilson disease	Free copper increases in Wilson disease patients	ETAAS is time consuming	[96]

Lai et a 2016 [97]	Plasma, Ultrasonic Oscillation Dialysis membrame (25 KDa) + AAS	Moderate difficult	Not reported	Moderate-Utility Moderate-Expense	Cerebral ischemic stroke patients	Free copper increases in ischemic stroke patients	Dialysis membrane was made in house	[98]
Squitti et al 2017 [99]	Direct method: size exclusion solid phase extraction + coumarin fluorescent probe specific to Cu (II)	Moderate difficult	Good, Precision, Repeatability level high CV =5.7%	Higher-Utility Moderate-Expense. Clinical pathology Lab setting	Alzheimer's disease; stratification Alzheimer's disease; Type II diabetes	Free Copper in increased in Alzheimer's disease patients and in patients affected by Copper AD that is characterized by higher frequency of rs1061472 and rs732774 of the ATP7B gene [35, 98, 100, 101]	Fluorescent probability	[16, 99]
Rozzini et al 2018 [24]	Serum, ultrafiltration (30KDa) + AAS	Moderate difficult	Good Precision, CV < 3%	Moderate-Utility Moderate-Expense	Alzheimer's disease, Mild cognitive Impairment	Free copper increases in Alzheimer's disease and Mild Cognitive impairment individuals	AAS imply time consuming	[102]
Lauwens et al 2018 [103]	Copper Speciation in Serum and EDTA incubation ultrafiltration (30KDa) +ICP-MS with isotopic composition analysis	Challenging	Not reported	Higher-Utility Higher-Expense	Alcoholic Cirrhosis	Free copper increases in Alcoholic Cirrhosis	Time consuming, academic setting	[104]

Guillaud et al 2018 [105]	Plasma EDTA, EDTA incubation ultrafiltration (30KDa) + ETAAS	Moderate difficult	Good, Precision Repeatability level high CV =5.1%	Moderate-Utility Moderate-Expense	Wilson disease	Relative exchangeable Copper higher in Wilson disease	Time consuming, academic setting	
Squitti et al 2019 [66]	Serum, EDTA incubation, ultrafiltration (100KDa) + ICP-MS	Moderate difficult	Good Precision, CV < 5%	Higher-Utility Moderate-Expense; Clinical pathology Lab setting	Diabetes Type I	Free copper is increased even though not reaching the statistical threshold (p<0.003)	Favorable cost-effective	[106]
Ajsuvakova et al 2019 [107]	Copper speciation serum using HPLC-ICP-MS[10]	Challenging	Not reported	Higher-Utility Higher-Expense	Parkinson disease	0.18-0.3 KDa of Free copper increases in Parkinson's disease	Time consuming, academic setting	n.a.
Solovyev et al 2020 [108]	Copper speciation serum and ultrafiltration EDTA 30KDa + SAX-ICP-MS-MS[11]	Challenging	Not reported	Higher-Utility Higher-Expense	Wilson disease	Free copper increases in Wilson disease	Time consuming, academic setting	n.a.

Notes of the Table:

[1] Easiness scale: challenging, difficult, moderate difficult, easy.

[2] Affordability scale: Lower-Utility Lower-Expense; Moderate-Utility Moderate-Expense; Higher-Utility Moderate- Expense; Higher-Utility Higher-Expense

[3] ETAAS: Electrothermal Atomic Absorption Spectroscopy

[4] EDTA: ethylenediaminetetraacetic acid. The incubation of the serum with EDTA for 1 hour followed by ultrafiltration of the diluted serum includes both small amino acid complexes fraction copper and the labile fraction of copper complexed to albumin and transcuprein. This copper has been firstly reported by El Balky and colleagues as exchangeable copper (CuEXC) and corresponds to non-ceruloplasmin copper. EDTA effectively chelates copper, including that loosely bound to ceruloplasmin, albumin, transcuprein and amino acids and form copper chelates that are small enough to be easily transferred to ultrafiltrate, while the rest of macromolecules are excluded. Moreover, the addition of EDTA prevents the adsorption of Cu

on the ultrafiltration membranes since in the EDTA-Cu chelate the copper ion is located in the center of the complex. The amount of EDTA used does not disturb the structural copper in ceruloplasmin.

[5] PIXE: proton-induced X-ray emission

The method used for measuring Free Copper concentration in serum, briefly reported in [66] and depicted in the details in the standard operating procedure (*section 11*), consists in filtering serum samples through an Amicon Ultra centrifugal filter unit after incubation for 1 hour with Ethylenediaminetetraacetic acid (EDTA)-disodium salt dehydrate (Sigma, cat. Number E5134-50G), according to previous published methods [69, 96].
In previous studies of ours we have confirmed data provided by literature concerning the change in the biomarker levels relative to a reference such as baseline [18, 21, 33]. More specifically, we gained that in AD and MCI patients, Free Copper concentrations of 1.6 µM through 2.4 µM indicate a risk for development of AD [14] (*Table 7*).

If this biomarker involves an index/scoring system, please provide information about the elements and weighting of the elements. Include a rational for how the index/scoring system was developed.

Not applicable

Brief description of sample source, matrix (base material and any additives), stability and composition of biomarker.

We used commercial serum samples, a Pooled Human AB Serum Off the clot, catalog number 20636, from Innovative Research, Inc. USA and internal healthy human standards purchased from SIGMA-ALDRICH (Catalog number H4522). Laboratory serum samples were collected from donors into BD Vacutainers Rapid Serum tubes with thrombin and in BDMicrotainer collection tubes with Gold closure (with clot activator/SST gel). The usual volume of finger-dropped blood was around 500 µL. Tubes were immediately centrifuged for 15 minutes at 2500 rpm (1321 g) at 4°C. Serum (200-250 µL) was collected and stored refrigerated for same or next day processing.
Stability experiments were carried out for 3 different Free Copper concentrations after storing them at room temperature, refrigerated temperature, -80°C and at 40°C are detailed in *Table 11*.

TABLE 11 IGEA FREE COPPER TEST STABILITY

Stability at room temperature									
Room Temperature	**Sample**	**Mean**	**% Recovery**	**Sample**	**Mean**	**% Recovery**	**Sample**	**Mean**	**% Recovery**
	Low conc			**Medium conc**			**high conc**		
Neat	**0.62**	**0.63**		**1.46**	**1.45**		**1.83**	**1.85**	

	0.63			1.43			1.87		
4 hours	0.59	0.59	94	1.49	1.48	102	1.86	1.85	100
	0.59			1.47			1.84		
24 hours	0.57	0.58	92	1.46	1.47	101	1.88	1.88	102
	0.59			1.47			1.87		
48 hours	0.59	0.59	94	1.63	1.6	110	1.95	1.97	106
	0.59			1.56			1.98		
96 hours	0.7	0.71	112	1.76	1.77	122	2.24	2.29	123
	0.71			1.77			2.34		
1 week	0.61	0.62	98	1.45	1.47	101	1.89	1.9	102
	0.63			1.48			1.91		

Stability in the refrigerator (2-7°C)

Refrigerator	Sample Low conc	Mean	% Recovery	Sample Medium conc	Mean	% Recovery	Sample High conc	Mean	% Recovery
Neat	0.6	0.61		1.46	1.45		1.83	1.85	
	0.62			1.43			1.87		
4 hours	0.6	0.6	98	1.49	1.48	102	1.86	1.85	100
	0.6			1.47			1.84		
24 hours	0.57	0.58	95	1.45	1.45	100	1.85	1.84	99
	0.58			1.44			1.82		
48 hours	0.59	0.59	97	1.49	1.51	104	1.93	1.93	104
	0.59			1.53			1.93		
96 hours	0.62	0.62	102	1.61	1.62	111	2.03	2.02	109
	0.61			1.62			2.01		
8 days	0.56	0.57	93	1.57	1.58	109	2.02	2.03	110
	0.58			1.58			2.03		

14 days	**0.73** **0.72**	**0.73**	**119**	**1.8** **1.87**	**1.84**	**127**	**2.31** **2.39**	**2.35**	**127**
21 days	**0.88** **0.91**	**0.9**	**147**	**2.03** **2.08**	**2.06**	**142**	**2.6** **2.66**	**2.63**	**142**
28 days	**1.14** **1.11**	**1.13**	**185**	**2.34** **2.27**	**2.31**	**159**	**2.84** **2.96**	**2.9**	**157**

Stability at -80°C (freezer; start 10/8/2019)

-80°C freezer	**Sample** **Low conc**	**Mean**	**% Recovery**	**Sample** **Medium conc**	**Mean**	**% Recovery**	**Sample** **High conc**	**Mean**	**% Recovery**
baseline	**0.57** **0.57**	**0.57**		**1.43** **1.43**	**1.43**		**1.79** **1.81**	**1.8**	
1 week	**0.61** **0.63**	**0.62**	**109**	**1.45** **1.48**	**1.47**	**103**	**1.89** **1.91**	**1.9**	**105**
2 weeks	**0.7** **0.71**	**0.71**	**125**	**1.42** **1.47**	**1.45**	**101**	**1.9** **1.91**	**1.91**	**106**
1 month	**0.68** **0.63**	**0.66**	**116**	**1.57** **1.46**	**1.52**	**106**	**1.73** **1.82**	**1.78**	**99**
3 months	**0.72** **0.71**	**0.72**	**126**	**1.51** **1.36**	**1.44**	**101**	**1.7** **1.78**	**1.74**	**97**
6 months	**Value rep 1** **Value rep 2**			**Value rep 1** **Value rep 2**			**Value rep 1** **Value rep 2**		
12 months	**Value rep 1** **Value rep 2**			**Value rep 1** **Value rep 2**			**Value rep 1** **Value rep 2**		

18 months	**Value rep 1**			**Value rep 1**			**Value rep 1**		
	Value rep 2			**Value rep 2**			**Value rep 2**		
Stability at 40°C									
40°C	**Sample L**	**Mean**	**% Recovery**	**Sample M**	**Mean**	**% Recovery**	**Sample H**	**Mean**	**% Recovery**
Neat	**0.62**	**0.63**		**1.46**	**1.45**		**1.83**	**1.85**	
	0.63			**1.43**			**1.87**		
4 hours	**0.6**	**0.6**	**95.23**	**1.51**	**1.5**	**103**	**1.93**	**1.92**	**103**
	0.6			**1.49**			**1.91**		
24 hours	**0.64**	**0.66**	**105**	**1.88**	**1.89**	**130**	**2.3**	**2.28**	**123**
	0.68			**1.9**			**2.26**		
48 hours	**0.76**	**0.78**	**123**	**2.12**	**2.08**	**143**	**2.57**	**2.6**	**140**
	0.79			**2.04**			**2.62**		
96 hours	**1.06**	**1.09**	**173**	**2.79**	**2.81**	**193**	**3.57**	**3.54**	**191**
	1.12			**2.82**			**3.51**		

Analytical validation plan: description of measurement tool and device calibrations, validation study design with statistical analysis plan (SAP) or performance data (e.g. sensitivity, specificity, accuracy, and/or precision of the assay or method).

Procedures from Clinical and Laboratory Standards Institute (CLSI) were employed for the validation of the IGEA Free Copper test. In particular, the EP05-A3 (Evaluation of Precision of Quantitative Measurements Procedures; Approved Guideline – Third Edition), the EP06-A method evaluation (Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline), EP17-A (Protocols for determinations of Limit of Detection and Limit of quantitation; approved guidelines) and Recovery were applied. StatisPro Methods evaluation software (from CLSI version 3.02.2) was used for these analyses. Results of these testing procedures are reported in detail in the Attachment B together with the SOP of the method reporting details about test procedures and reference materials for calibrations.

Once the SOP and analytical validation plan is finalized, describe how you will use this process to validate the final version of the measurement tool.

We are planning to complete CLSI validation of the Free Copper test for verification of performance for precision and trueness, applying the testing procedures, as follow:

EP09c (Measurement of Procedure Comparison and Bias Estimation Using Patient Samples), EP24-A2 (Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves; Approved –Second edition) and EP28-A3c (Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline –Third Edition)

More specifically our validation plan includes:

1. Comparison of the Free Copper Test with the standard/reference method (EP9A3).

2. Definition of the confidence intervals (28A3C).

3. Discrimination test to distinguish between diseased (AD patients) and non-diseased individuals to identify separation cut offs. To identify the discriminating value we need to test non-diseased subjects and subjects with AD (EP24-A2). Our plan is to enroll at least the same number of non-diseased subjects and subjects with AD. As indicated in article [109], the sample size for each group of diseased and non-diseased in estimating the accuracy (area under the curve, AUC) changes in dependence of the configurations of AUC and marginal errors with 95% confidence level. Hypothesizing an AUC=0.85 with errors d=0.07 or d = 0.10, 77 or 38 AD patients would be needed, respectively. However, a more plausible scenario based on our previous results could be an AUC of 0.65 with an error d = 0.10: in this case, 59 patients with AD and 59 non-diseased individuals would be needed. This scenario would be in agreement with previous reports of ours showing Free Copper as a stratification biomarker [18, 23, 33].

SECTION 6 CLINICAL CONSIDERATIONS

Describe how the biomarker measurement is used to inform drug development. Please provide a decision tree to guide how the biomarker information would be used in drug development or a clinical trial.

Free Copper is intended to serve as a serum prognostic biomarker that indicates the potential for individuals to develop symptomatic AD typified by copper imbalance [7, 9, 15-18, 21, 22, 25, 26] and for the eligibility assessment for clinical trials testing anti-copper based interventions to delay or prevent AD [27]. Free Copper can be considered an efficacy biomarker in early clinical trials employing anti-copper-based therapies, as for example Zinc therapy. During Zinc therapy, measurement of serum Free Copper, which should approach normal range (lower than 1.6 µmol/L) will ensure the biochemical efficacy of the treatment. To monitor Zinc overtreatment, ceruloplasmin (Cp in *Figure 1*) level, that is considered a good surrogate measure of body copper status, and blood cell counts can be employed as markers of treatment tolerability, since the initial indication of Zinc overtreatment is a mild anemia and/or leukopenia. *Figure 1* depicts how Free Copper can be employed either as a biomarker for eligibility assessment or as an efficacy biomarker in decision tree to guide information during drug development in clinical trials testing anti-copper based interventions to delay or prevent AD. As inclusion criterion, Free copper will set the stage for precision medicine drug development program (*Figure 1*).

FIGURE 1 DECISION TREE WITH FREE COPPER AS TARGET OF EFFICACY AND CP AS TOLERABILITY

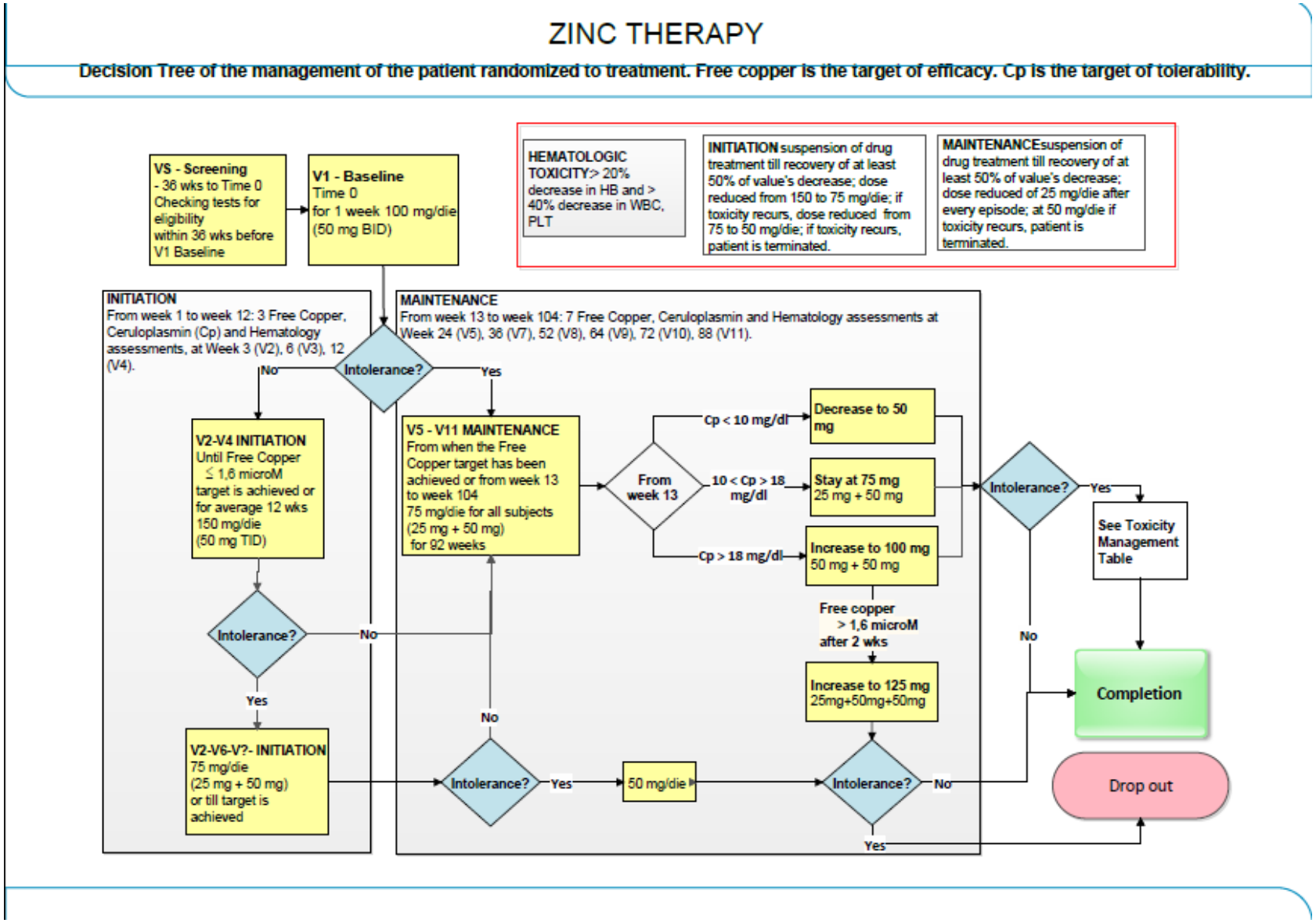


Figure 1: The diagram above depicts decision tree. Free Copper is intended as a serum prognostic biomarker for eligibility assessment for clinical trials testing interventions in MCI individuals to delay or prevent AD: 1.6 µmol/L cut-off is an inclusion criterion for eligibility. The decision tree show how Free Copper can be employed as an efficacy biomarker in early clinical trial in Zinc therapy. During Zinc therapy, the measurement of serum Free Copper, which should approach normal range (lower than 1.6 µmol/L) will ensure the biochemical efficacy of the treatment. To monitor Zinc overtreatment, ceruloplasmin (Cp in *Figure 1*) level, that is considered a good surrogate measure of body copper status, can provide information about a possible copper deficiency occurring in the subject. A value lower than 10 mg/dL will not be allowed. Ceruloplasmin will be considered a tolerability marker to monitor copper deficiency during Zinc therapy: low levels of ceruloplasmin precede anemia (normal values of ceruloplasmin: 20-50 mg/dL). A third type of measurement informative about the safety of Zinc therapy is blood cell counts. The initial indication of Zinc overtreatment is a mild anemia and/or leukopenia, that is easily correctable by lowering the dose, or if more severe, temporarily stopping drug administration. This is rarely seen at ceruloplasmin levels over 10 mg/dL but is more frequently seen between 5 and 10 mg/dL, and is much more common at ceruloplasmin levels below 5 mg/dL. Thus, during Zinc therapy, ceruloplasmin levels lower than 10 mg/dL should not be allowed.

Describe patient population or drug development setting in which the biomarker will be used.

The Free Copper biomarker can be used as inclusion criterion in phase II clinical trial aimed at demonstrating that anti-copper therapy can delay or arrest cognitive decline progression in MCI individuals exhibiting values of Free copper higher than 1.6 µmol/L [7, 9, 15-18, 21, 22, 25, 26]. To this regard we are setting a phase II clinical trial funded by the Alzheimer's Association (number PTC-19-602325; EudraCT No.: 2019-000604-15) employing a zinc-based therapy.

The patient population description:

i) **Individuals with MCI:** Eligible individuals for phase II clinical trial testing anti-copper-based treatments are MCI subjects according to current diagnostic criteria that are summarized herein. The inclusion and exclusion criteria for amnesic MCI are based on previous studies [110-117] and are as follows:

a) Inclusion criteria for MCI:

1. Objective memory impairment on neuropsychological evaluation, as identified by the partner, with or without complaints by the participant.
2. Memory impairment as indicated by scores below the cut-off level on The Free and Cued Selective Reminding Test (FCSRT). FCRST is a memory test that allows to evaluate "amnestic syndrome of the hippocampal type" and successfully differentiate patients with AD from any other memory disorders that are seen in depression, frontal lobe dysfunction, subcortical dementias, or vascular dementias [118].
3. Normal activities of daily living as documented by the history and evidence of independent living.

4. A Clinical Dementia Rating Score of 0.5 [119];
5. MMSE scores between 24 and 30 [120].

b) Exclusion criteria for MCI:

1. Mild AD.
2. Evidence of concomitant dementia, such as frontotemporal form, vascular dementia, reversible dementias (including pseudo-depressive dementia), fluctuations in cognitive performance, and/or features of mixed dementias.
3. Evidence of concomitant extra-pyramidal symptoms.
4. Clinical and indirect evidence of depression, as revealed by Geriatric Depression Scale scores higher than 13.
5. Other psychiatric diseases, epilepsy, drug addiction, alcohol dependence, and use of psychoactive drugs.
6. Current or previous uncontrolled or complicated systemic diseases (including diabetes mellitus), tumors or traumatic brain injuries

In addition to MCI criteria reported above, eligible subjects have to comply with **inclusion criteria** of 'amyloid positivity' (positive to Aβ PET [18F] florbetapir, or [18F] florbetaben or [18F] flutemetamol) or positive to Aβ 1-42, holo-Tau (h-Tau) and phosphorylated-Tau (p–Tau) cerebrospinal (CSF) markers] and 'Free copper positivity' (Free Copper >1.6 µmol/L) and others as depicted below:

6. 50-80 years old. If Women, in menopause for at least 2 years.
7. Free Copper serum concentration > 1.6 µmol/L ;
8. Stable presence of the informant family member.
9. Release of written informed consent prior to participation in the study.
10. Capacity of full compliance with the protocol requirements (i.e.: assumption of the medicine per os, etc.).
11. Brain MRI performed within 12 months preceding or at the Screening Visit.
12. Evidence of cerebral amyloidosis by scanning with Florbetapir (18F)-PET within 12 months or positive for CSF biomarkers of AD.

Clinical validation: provide information to support biological and clinical relevance of the biomarker as applied in the COU:

Free Copper is already recognized as a key diagnostic marker for Wilson disease [26, 68, 73] and is also associated with a worse cognitive performance in normalcy [31] and in MCI [18, 21, 23, 24, 32, 121].

In two different follow-up studies, we reported that subjects with values of Free Copper higher than 1.6 µmol/L had a higher risk for a more severe cognitive decline and a faster rate of deterioration with respect to subjects with normal Free Copper [122, 123], demonstrating Free Copper utility to individuate people with the potential to develop symptomatic AD and a worse prognosis. The proposed prognostic biomarker has demonstrated a fair sensitivity in detecting which individuals with MCI will convert to Cu-AD [21] (*Table 12*)

TABLE 12 SENSITIVITY AND SPECIFICITY OF FREE COPPER FOR THE CONVERSION FROM MCI TO AD

Cut off	SE	SP	corrected classified	AUC	CI95%
Total sample				0.68	0.56-0.79
>1.6	72.70%	55.90%	62.00%		
≥2.0	63.60%	64.40%	64.10%		
≥2.4	51.50%	72.90%	65.20%		
APOE E4 neg				**0.71**	0.56-0.86
≥1.6	77.80%	54.80%	61.70%		
≥2.0	66.67%	64.30%	65.00%		
≥2.4	61.10%	73.80%	70.00%		
APOE E4 pos				0.65	0.45-0.85
≥1.6	66.67%	58.80%	62.50%		
≥2.0	60.00%	64.7%	62.50%		
≥2.4	40.00%	70.60%	56.30%		

Based on data published on [21]; SE: sensitivity; SP: specificity; AUC: area under the curve of of the ROC curve. An AUC=1 represents a perfect test, a value of 0.5 represents a worthless test, and values in the 0.7–0.8 range considered fair

Existing meta-analysis supported that 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 imbalance can be considered as an AD risk factor, has never been performed.
To fill this gap we are in the process of analyzing through meta-analyses multiple AD studies performed on copper in serum/plasma and in brain specimens from 1984 [124]. To identify appropriate studies for our meta-analysis, we followed the procedural steps indicated by Cochrane (http://www.cochrane-handbook.org). *Figure 2* shows that in AD patients brain copper deficiency (about 14% less than normal, *Figure 2A*) coexists with copper excess in the bloodstream (about 10% than normal, *Figure 2B*) that is explained by increased levels of Free copper (*Figure 2C*). These observational data show an overall copper imbalance in AD that support metal changes as one of

the drivers of AD pathology. On the basis of the standardized mean difference (SMD), gained from the meta-analysis, applying the

$$SMD = \sqrt{lnOR}$$

formula, serum/plasma Free copper generates 3-fold higher odds in AD patients as compared to healthy controls [124].

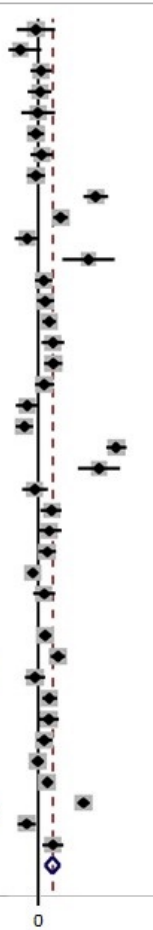
FIGURE 2. OVERALL META-ANALYSES OF COPPER IN AD.

A

Study (Authors, year)	Brain region	Alzhimer's disease N°	Alzhimer's disease Cu µg/g (SD)	Healthy controls N°	Healthy controls Cu µg/g (SD)	SMD	(95% CI)	Weight
Corrigan et al 1993	Hippocampus	12	4.5 (2.2)	12	5.1 (1.1)	-0.34	(-1.15, 0.46)	7.88
Akatsu et al 2012	Hippocampus	15	3.78 (8.9)	12	7.4 (2.1)	-0.53	(-1.31, 0.24)	8.10
Akatsu et al 2012	Amygdala	18	4.3 (9.1)	16	8.3 (3.9)	-0.56	(-1.25, 0.13)	8.68
Szabo et al 2016	Frontal cortex	14	2.1 (0.7)	15	2.4 (0.7)	-0.43	(-2.44, -0.49)	8.34
Deibel et al. 1996	Temporal cortex	10	3.2 (0.6)	11	4.0 (0.6)	-1.38	(-2.34, -0.41)	6.81
Deibel et al. 1996	Inferior parietal	10	3.8 (0.9)	11	4.1 (0.6)	-0.40	(-1.26, 0.47)	7.49
Deibel et al. 1996	Cerebellum	10	5.1 (0.1)	11	5.7 (1.0)	-0.82	(-1.72, 0.07)	7.30
Graham et al 2014	Brodman area 7	42	19.3 (2.9)	26	22.5 (4.3)	-0.92	(-1.43, -0.40)	9.87
Religa et al 2006	Neocortical	10	2.3 (1.0)	14	2.8 (0.9)	-0.53	(-0.36, 0.30)	7.75
Magaki et al 2007	Frontal cortex	8	3.9 (0.3)	6	6.9 (1.2)	-3.71	(-5.54, -1.89)	3.29
Plantin et 1987	Temporal lobe	5	1.8 (0.7)	6	3.1 (0.6)	-2.01	(-3.52, -0.50)	4.27
Squitti et al 2007	Cortical tissue	9	5.1 (1.6)	10	6.8 (1.5)	-1.10	(-2.07, -013)	6.82
Loeffler et al. 1996	Frontal cortex	12	7.8 (0.6)	7	6.8 (1.0)	1.13	(0.28, 2.34)	6.49
Overall (I-squared=63.7%, p=0.0001)						**-0.77**	**(-1.16, -0.38)**	**100**

B

Study (Authors, year)	Alzhimer's disease N°	Alzhimer's disease Cu µmol/L (SD)	Healthy controls N°	Healthy controls Cu µmol/L (SD)	SMD	(95%CI)	Weight
Shore et al. 1984	10	18.3 (4.2)	10	18.6 (1.6)	-0.09	(-0.97, 0.78)	2.24
Kapaki et al. 1989	5	14.2 (3.8)	28	16.2 (2.2)	-8.81	(-1.78, 0.16)	2.16
Jeandel et al. 1989	55	22.0 (6.1)	24	21.2 (4.1)	0.14	(-0.34, 0.62)	2.53
Basun et al. 1991	24	17.0 (3.9)	28	16.7 (3.0)	0.09	(-0.46, 0.63)	2.49
Mattiello et al. 1993	21	18.8 (0.2)	10	18.8 (0.2)	0.00	(-0.75, 0.75)	2.34
Molaschi et al. 1996	31	18.9 (3.4)	421	19.3 (3.8)	-0.11	(-0.47,0.26)	2.59
Snaedel et al. 1998	44	19.1 (3.9)	44	19.4 (2.2)	0.16	(-0.38, 0.69)	2.49
Molina et al. 1998	26	15.1 (3.5)	28	14.5 (4.1)	-0.09	(-0.51, 0.32)	2.56
Gonzalez et al. 1999	51	16.6 (0.5)	40	15.4 (0.4)	2.62	(2.05, 3.18)	2.48
Squitti et al. 2002	79	18.3 (5.7)	76	13.7 (2.6)	1.03	(0.70, 1.37)	2.60
Ozcakaya et al. 2002	27	12.0 (0.2)	25	12.1 (0.2)	-0.50	(-1.05, 0.05)	2.48
Smorgon et al. 2004	8	22.9 (3.9)	11	16.7 (1.3)	2.30	(1.11, 3.5)	1.97
Bocca et al. 2005	60	15.2 (3.8)	44	14.3 (3.1)	0.26	(-0.14, 0.65)	2.57
Sedighi et al. 2006	50	21.7 (3.1)	50	20.8 (2.5)	0.32	(-0.07, 0.71)	2.57
Sevym et al. 2007	98	16.7 (2.9)	76	15.4 (2.1)	0.50	(0.20, 0.81)	2.61
Zappasodi et al. 2008	54	15.1 (3.4)	20	12.9 (3.0)	0.67	(0.14,1.19)	2.50
Agarwal et al. 2008	50	24.6 (4.8)	50	21.2 (5.0)	0.69	(0.29, 1.10)	2.57
Vural et al. 2010	50	20.7 (2.9)	50	22.5 (2.9)	0.29	(-0.14, 0.71)	2.55
Arnal et al. 2010	110	15.7 (0.8)	79	12.7 (0.9)	-0.50	(-1.03,0.02)	2.50
Baum et al. 2010	44	16.2 (3.5)	41	15.3 (2.7)	-0.62	(-1.02, -0.22)	2.57
Brewer et al. 2010	28	17.0 (2.4)	29	18.4 (3.1)	3.56	(3.10, 4.02)	2.54
Mueller et al. 2012	14	19.1 (1.0)	19	16.9 (0.6)	2.78	(1.80, 3.75)	2.16
Huang et al. 2013	28	15.9 (3.5)	19	16.4 (3.0)	-0.15	(-0.73, 0.43)	2.46
Rembach et al. 2013	152	13.9 (2.7)	716	14.6 (2.9)	0.60	(0.12, 1.09)	2.52
Lopez et al. 201315	36	15.8 (2.9)	33	13.8 (3.7)	0.50	(-0.07, 1.08)	2.47
Azhdarzadeh et al. 2013	50	15.8 (1.5)	50	15.1 (1.9)	0.41	(0.01, 0.81)	2.57
Azhdarzadeh et al. 2013	30	17.0 (3.1)	20	15.6 (2.2)	-0.24	(-0.42, -0.07)	2.65
Park et al. 2014	89	18.0 (3.0)	118	17.0 (3.2)	0.27	(-0.24, -0.07)	2.51
Singh et al. 2014	100	18.3 (0.5)	100	14.9 (0.3)	8.25	(7.39, 9.10)	2.26
Gonzalez et al. 2014	30	17.5 (4.2)	30	16.5 (3.2)	0.32	(0.04, 0.60)	2.62
Wang et al. 2015	83	19.0 (3.6)	83	16.1 (2.8)	0.90	(0.58, 1.22)	2.61
Koc et al. 2015	45	14.2 (10.5)	33	15.9 (11.7)	-0.15	(-0.60, 0.30)	2.54
Paglia et al. 2016	34	12.8 (3.2)	40	11.1 (3.8)	0.51	(0.17,0.85)	2.60
Siotto et al. 2016	84	15.5 (3.3)	58	14.0 (2.3)	0.48	(0.02, 0.94)	2.54
Pu et al. 20176	125	19.3 (11.6)	40	16.3 (6.5)	0.29	(-0.07, 0.64)	2.59
Talwar et al. 2017	108	22.1 (9.6)	159	18.7 (7.0)	-0.03	(-0.29, 0.24)	2.63
Shere et al. 2018	44	16.1 (3.7)	52	18.2 (4.4)	0.42	(0.17, 0.66)	2.63
Squitti et al 2017	89	15.9 (3.59)	147	16.04 (6.39)	2.07	(1.89, 2.25)	2.65
Squitti et al. 2018	385	5.2 (13-17)	336	13.2 (11.3-15)	-0.51	(-0.92, -0.10)	2.56
Rozzini et al. 2018	44	18.3 (3.5)	28	16.0 (3.3)	0.67	(0.18, 1.16)	2.52
Overall (I-squared=96.4%, p=0.0001)					**0.65**	**(0.32, 0.98)**	**100**



C

Study (Authors, year)	Alzhimer's disease N°	Alzhimer's disease Non-Cp Cu µmo/L (SD)	Healthy controls N°	Healthy controls Non-Cp Cu µmo/L (SD)	SMD	(95% CI)	Weight
Molaschi et al. 1996	31	0.5 (2.7)	421	0.1 (3.6)	0.11	(-0.25, 0.48)	9.99
Snaedel et al. 1998	44	1.1 (2.9)	44	1.3 (3.0)	-0.07	(-0.49, 0.35)	9.29
Zappasodi et al. 2008	54	2.1 (2.5)	20	0.2 (2.5)	0.76	(0.23, 1,29)	7.90
Arnal et al. 2010	110	3.8 (4.5)	79	0.2 (2.8)	0.93	(0.62, 1.23)	10.78
Lopez et al. 2013	36	3.6 (2.1)	33	1.6 (3.0)	0.78	(0.29, 1.27)	8.36
Siotto et al. 2016	84	2.1 (1.8)	58	1.5 (1.5)	0.36	(80.02, 0.69)	10.35
Squitti et al 2017	89	2.31 (1.64)	147	1.68 (2.46)	0.29	(0.02, 0.55)	11.26
Talwar et al. 2017	108	5.0 (5.2)	159	1.4 (1.9)	0.99	(0.74, 1.25)	11.35
Rozzini et al. 2018	44	0.57 (0.34)	28	0.3 (0.11)	0.98	(0.48, 1.48)	8.23
Squitti et al. 2018	385	2.2 (2.1)	336	0.4 (2.1)	0.86	(0.70, 1.01)	12.47
Overall (I-squared=81.5%, p=0.0001)					**0.60**	**(0.37, 0.83)**	**100**

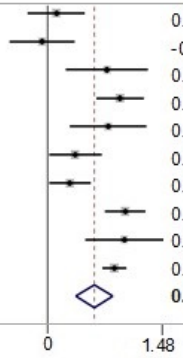


FIGURE 2. OVERALL META-ANALYSES OF COPPER IN AD. Standardized mean difference (SMD) computed from the studies on copper brain levels (µg/g; panel A), on copper serum levels (µmol/L; panel B) and on serum Free copper (µmol/L; panel C) in Alzheimer's disease patients and healthy controls. SMDs between 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.
Panel A, copper in brain specimens: Results indicate that AD subjects had lower levels of copper in the brain than healthy controls [SMD=-0.77 (95% CI -1.16 to -0.38); p<0.001]; there was substantial heterogeneity among the included studies (I2=63.7%; p<0.001). Panel B, copper in serum/plasma: AD subjects had higher levels of copper in serum than healthy controls [SMD =0.65 (95% CI 0.32 to 0.98); p< 0.001]; there was considerable heterogeneity among the included studies (I2=96.4%; p<0.001). Panel C, Free copper in serum: Results indicate that AD subjects had higher levels of Free copper in serum/plasma than healthy controls (SMD =0.60 (95% CI 0.37 to 0.83; p<0.001); there was considerable heterogeneity among the included studies (I2=81.5%; p<0.001).

Copper changes in the brain may be associated with dysregulation of copper metabolism in blood. More specifically, 20% labile copper elevation in the brain [125] has been attributed to bloodstream excess Free copper [25, 26]. Free copper is redox active and if the pool is expanded in bloodstream because of a disorder in copper metabolism, it becomes toxic, since it crosses the BBB. In AD patients, it reaches values commonly found in Wilson disease [26], a rare inborn error of copper metabolism and a paradigm of Free copper toxicosis and accumulation as discussed above. Wilson disease is caused by loss of function mutations in both copies of the *ATP7B* gene encoding for the ATPase7B protein, a copper-transporting ATPase essential for delivering copper to copper enzymes in Golgi apparatus (e.g. holo-ceruloplasmin) (reviewed in [14]) that results in a plethora of biochemical and clinical traits in the affected individuals (hepatic, neurologic or psychiatric symptom presentation). Some of these traits are also expressed in the Cu-subset of AD patients. Cu-AD subtype share some features with Wilson disease that can be summarized as follows:

a) Free copper in serum higher than 1.6 µmol/L (higher than the upper value of normal reference range 0.1-1.6 µmol/L [68]) [26];
b) Increased frequencies of some functional SNPs (rs732774 and rs1061472) and other gene mutations (rs2147363 rs7334118) associated with regulatory function in *ATP7B* and with excess Free copper [16, 19, 101];
c) Decreased values of ceruloplasmin specific activity [72];
d) Increased values of the inactive and copper-free form of ceruloplasmin (apo-ceruloplasmin) in the CSF [126], brain and in serum [127];
e) Increased values of copper excretion in the urine, thought sensitively lower than Wilson disease [26];
f) Abnormalities of liver function (though sensitively lower than Wilson disease) associated with increased Free copper levels [88];
g) Kayser-Fleischer rings - pathognomonic of Wilson Disease – that have been described in a peculiar case-report of an AD patient exhibiting 11C-labeled Pittsburgh Compound-B PET positive and a [18F] fluorodeoxyglucose PET altered with a typical AD topography [20];
h) Decreased values of copper in the brain [127](*Table 2*; *Figure 2*), in line with the Long Evan Cinnamon (LEC) rat, a preclinical model of Wilson disease that show decreased values of

copper in the brain coexisting with increased values of Free copper excess in the bloodstream [128].

Our hypothesis is that genetic heterogeneity (e.g. presence of the mutated alleles in the functional SNPs *ATP7B* rs732774, rs1061472 (their haplotypes) [19] and additional *ATP7B* undefined multiple rare mutations) associated to copper exposure might contribute to the global susceptibility for AD. In a theoretical paper we presented the hypothesis positing *ATP7B* gene as potential harbor of multiple rare variants that can account for the missing hereditability of sporadic AD [129]. A genome wide association study (GWAS) identified loci affecting essential metal metabolism [130] in two regions on chromosome 1 as having significant association on copper concentrations in erythrocytes, even though genes in this region do not code for proteins associated with copper metabolism. This GWAS did not find significant association between the above reported SNPs in the chromosomal region where ATP7B is located and AD as we anticipated by our theoretical article [129]. In fact, the GWAS outcome can be explained by the paradigm "common disease – common gene variants", which is currently the basis for GWAS. This paradigm hardly detects rare variants, which are instead supposed to account for the missing hereditability of complex diseases, especially if referred to the ATP7B gene, which is a high polymorphic gene, as previously described [129]. Subsequent studies of ours in 2013 [35, 36] indicated that SNPs in transmembrane domains detected in *ATP7B* gene may have a stronger association with AD risk than variants in copper-binding domains. The analysis of *ATP7B* informative SNPs confirms our previous hypothesis about the absence of *ATP7B* in the significant loci of GWAS of AD [129] and the genetic association study suggests that transmembrane and adenosine triphosphate (ATP) domains in the *ATP7B* gene may harbor variants/haplotypes associated with AD risk.

ATP7B mutations have a complex interaction with copper take-in from the diet (foods, supplements and drinking water) as documented by the study of Siaj and colleagues [131]. The study demonstrated that the preclinical model of Wilson disease LEC rat has an early and rapid onset of fulminant hepatitis on a high-copper dietary regimen, but it survives long term on a low-copper dietary regimen. This means that life style habits have an effect on *ATP7B* mutations penetrance. Furthermore, modifiers genes as *ATOX 1* and *COMMD-1* are known as influencer of copper status. Moreover, additional factors other than genetics have effects on the level of Free copper as it happens other clinical conditions (neurological conditions [97], but also breast cancer [79] and alcoholic cirrhosis [103], and particularly in diabetes [66, 99].
In diabetes, as well as in the AD brain, advanced glycation end-product (AGES) formation is facilitated by copper and iron: non-enzymatic protein glycation caused by glucose in Maillard reactions, when rearranged, generate Amadori compounds that facilitate oxidation, cyclization, and condensation reactions that generate the AGEs [132]. Copper accelerate AGEs formation that also promote protein glycoxidation [133]. AGEs damage arterial wall in diabetes and facilitate a progressive Cu-trapping potential [134] in a vicious cycle of oxidative stress contributing to the pathogenesis of diabetic complications. As discussed in a recent review [106], in AD and atherosclerosis, a large amount of Aβ in the plaques is found in the form of AGEs [135], and it has been demonstrated that Aβ, sugars, and Cu take part in oxidative stress mechanisms of AD: Aβ glycation by sugars generates the free radical superoxide anion ($O^{-\cdot 2}$), that reacting with Cu^{2+}, is converted to a hydroxyl radical (HO•), which is the source of oxidative stress in AD [136].
As a whole this evidence suggest that Free copper is a superior prognostic marker with respect to genetic analysis of the *ATP7B* gene at this stage of the research: detection of Free copper levels higher than the designed cut-off has an immediate clinical meaning (refer also to Wilson disease)

since it crosses the BBB and speeds some early events of AD changes that can be summarized as follows:

i) disturbance of the compartmentalization of metals in neurons and glia [137];
ii) accumulation of Aβ peptide and plaque formation, as well as copper can drive intracellular accumulation of h-Tau and p-Tau due to the presence of Cu-binding sites on these proteins [138];
iii) Metal-dependent protein aggregation that is one of the main pathways of abnormal protein handling [139]
iv) increased intra-synaptic copper, and lead to dys-regulation of the glutamatergic synapse due to binding of copper to the glutamate receptor: labile copper can disrupt amyloid precursor protein (APP) capacity to reduce Cu(II) to Cu(I) and enable Ctr1 to facilitate ATPase7B translocation of copper into synaptic vesicles, hampering copper released at the synaptic cleft as part of the glutamatergic transmission in the hippocampus at the pyramidal CA1 synapse (reviewed in [13]);
v) loss of functional copper in protein-bound pools that reduces energy production and oxidative stress control mainly into the brain (reviewed in [13]);
vi) oxidative stress, since copper and iron are the main elements associated with Haber-Weiss and Fenton chemistry, driving reactive oxygen species formation, including the highly reactive hydroxyl radical [140]; oxidative damage to lipids, proteins and other molecules is a major feature of the neurodegenerated brain [140];
vii) abnormal copper handling in mitochondria. Copper is critical to normal function (respiration) of mitochondria, essential for neuronal activity, and mis-appropriation of copper (either too little, or mis-localized) can lead to impaired electron transport chain function (cytochrome c) and diminished neuronal energy. Conversely, abnormal localization of copper can increase oxidative stress from mitochondria [141];
viii) Cu detrimental effects on neuro-immune function: copper is a key element for normal functional activation of microglia, the resident immune cell of the brain; recent studies have demonstrated an important role for abnormal microglia behavior in AD and other forms of neurodegeneration; excess copper can drive abnormal pro-inflammatory microglial activation; alternately, limiting copper can lead to impaired early (beneficial) responses of microglia; as described here, copper is central to many (most) of the key pathological changes that occur in the AD and abnormal copper metabolism likely has a major impact on these processes [142];
ix) AGES formation in the AD brain, as discussed above.

Describe how normal or other reference values are established, provide study design(s), analytical plan, etc.

Reference interval (0–1.6 µmol/L) was determined parametrically with 137 healthy adult (20–59 years) blood donors [69] and confirmed by another reference interval (0-1.9 µM) determined in 147 healthy adult blood donors [18]. Established literature reports normal values of Free Copper below 1.6 µmol/L (*Table 7*)

As depicted in Section 5 (analytical consideration) our analytical plan for Free Copper validation includes:

1. Comparison of the Free Copper Test with the standard/reference method (EP9A3).

2. Definition of the confidence intervals (28A3C).

3. Discrimination test to distinguish between diseased (AD patients) and nondiseased individuals to identify separation cut offs. To identify the discriminating value, we need to test nondiseased subjects and subjects with AD (EP24-A2).

Benefits and Risks of applying the biomarker in drug development or a clinical trial.

Patients with AD are heavy users of long-term care services, especially nursing homes, with estimated annual costs upward of tens of billions of dollars nationwide. Much of the fiscal burden is borne by state and federal governments — and thus taxpayers — through the Medicaid and Medicare programs. Disease prevention and treatment, even though targeting only a percentage of individuals, could reduce these costs and be beneficial in term of care for individuals and their families.

Free Copper is intended to be part of a drug development program tailored to individuals with the potential to develop AD on the basis of their susceptibility for copper imbalance: applying anti-copper based prevention programs or interventions could save millions or even billions of dollars while simultaneously improving care.

Among the anti-copper based treatment, Zinc therapy has replaced D-penicillamine as first-line therapy for Wilson disease, because of its safe profile. New guidelines reflect the paradigm shift in treatment that has occurred in recent years for Wilson disease [143], and has been recently foreseen for AD as well [144, 145].

The use of zinc in AD patients has been studied in several trials (*Table 6*) and has the ultimate goal of counteracting amyloid dysregulation by mobilizing copper from Aβ plaques, to promote the redistribution of metals from the brain to the bloodstream. Furthermore, zinc supplementation is a powerful driver for neurotrophic signaling and neuronal plasticity mediated by increased levels of the Brain-derived neurotrophic factor (BDNF) [146] and that AD patients suffer for a slight zinc deficiency, as per existing meta-analyses [11, 147].

Such a treatment will take advantage of the availability of zinc drugs in the market at low cost with beneficial effects in terms of timing of drug availability for the public and limited costs for the Health Care System.

The utility of our biomarker relays in the stratification of people with an increased susceptibility to the AD based on copper abnormalities who can benefit of specific preventive or treatment intervention directed to reduce the toxicity of copper in the body. In a drug developmental program focusing on the hypothesis that anti-copper based therapy might halt the development of dementia in early stage patients, even should such treatments become available, systematically screening the over-50 population for Free Copper would only become cost-effective.

Describe any current knowledge gaps, limitations and assumptions in applying the biomarker in drug development or a clinical trial.

Zinc therapy is based on the use of zinc compounds at a dosage of 100-150 mg/day that induces body copper balance to become negative, reverting copper compartmentalization and distribution from the blood to organs and tissues, including the brain [148, 149]. Zinc is an essential trace element that is tightly bound to proteins and peptides and acts as a crucial component for proper protein folding or as the catalytic/co-catalytic element required for the activity of many enzymes. Regardless of the ligand, zinc blocks the intestinal absorption of copper from the diet and reabsorption of endogenously secreted copper such as that from the saliva, gastric juice and bile, facilitating the production of metallothionein's in the enterocyte, a protein that binds copper. Zinc reduces copper absorption by potentiating 25-fold expression of metallothionein's, thereby preventing its serosal transfer into the blood, reducing Free Copper pool expansion in serum. Being a low molecular weight copper pool, excess Free Copper crosses the BBB, partaking into neurodegeneration processes. The metallothionein - bound copper in the enterocyte is then lost in the stool following desquamation of the intestinal cells. On this basis, Zinc (dosage 100-150 mg/day) has been used since 1958 in the treatment of Wilson disease, the paradigmatic disease of copper accumulation and intoxication, typified by increased levels of Free Copper.

Initial studies of Zinc supplementation in AD patients were originally undertaken by Constantinidis [57, 150, 151] and also later suggested by Burnet [152] (*Table 6*). A recent review [27] summarizes findings on the potential beneficial effect of Zinc therapy on cognitive decline, that derive essentially by studies with a small sample size. For example, a study evaluating 10 AD individuals treated in a period from three months to a year showed improvement in memory: for 8 patients, improvement of memory, understanding, communication, and social contact were evident [57, 150, 151]. Another study carried out on 4 individuals suggested modest improvements on MMSE after 3 months [58] and another on altered brain waves activities [153]. A subsequent phase II clinical trial carried out by George Brewer [64] on 42 AD patients revealed in a post-hoc subset analysis in the oldest three quartiles of the patient sample significant improvements in some cognitive domains [64]. Furthermore, the ZENITH study, a randomized double-blind placebo-controlled study carried out in 387 healthy older adults (aged 55–87 years) taking an oral dose of 0, 15, or 30 mg/day zinc, revealed an effect in both the treatment groups, consisting in a significant treatment vs. time interaction for spatial working memory errors (requiring frontal and temporal lobe activation), although long-term benefit was unclear [154, 155]. All the studies (depicted in *Table 6*) did not employed Free Copper as an inclusion criterion for eligibility to participate to the clinical trial, this representing a major limitation, and furthermore, they were hampered by having a small sample size and an advanced patient disease stage.
All these issues and shortcomings can be easily addressed and resolved by maximize the possibility of early intervention, investigating individuals diagnosed as MCI [110-117] and who have values of Free Copper higher than 1.6 µmol/L (normal reference range 0.1 - 1.6 µmol/L; [70]), and evaluating the power of the study and calculating the size of the sample on the basis of the primary clinical outcome.

As previously discussed, the low sensitivity of Free Copper may limit its utility in drug development. However, in a clinical trial setting, it would prevent the exposure to treatment risks to patients who

might otherwise not expect the same benefits based on treatments aimed at regaining copper balance in the body.

SECTION 7 SUPPORTING INFORMATION

Provide underlying biological process or supporting evidence of association of the biological process with the biomarker.

Copper is an essential trace metal controlling human physiology. Brain cells use copper during development, and it is indispensable in vital processes such as respiration, energy production, formation of myelin sheath around neurons, synthesis of neurotransmitters, immune system responses, collagen and pigment synthesis and wound healings.
The underlying biological processes of the association of Free Copper with AD becomes clearer when considering the biological processes underlying copper altered metabolism occurring in Wilson disease. Wilson disease is a paradigmatic disease of copper-related toxicosis. The *ATP7B* mutations result in the defective functioning of the ATPase7B copper pump affecting the loading of copper into the nascent hepatic ceruloplasmin [64, 148, 156, 157]. Wilson disease is also characterized by the increased extrusion of Free Copper from the liver. The increased levels of Free Copper promote metal accumulation in the liver, the cornea (where Cu produces the pathognomonic Kayser-Fleischer rings), the brain, and other organs of Wilson disease patients. The pool of Free Copper is exchanged with albumin, α2-macroglobulin, peptides, and low molecular weight amino acids (*Table 8*). Blood Free Copper levels above the physiological value of 1.6 µmol/L [68-70, 73] cross the BBB and accumulates in crucial brain areas such as the basal ganglia, thalamus, and upper brainstem [158]. The increased copper uptake in the brain is coupled with a decrease in cation extrusion by the endothelial cells of the BBB, thereby enhancing the copper dysregulation of the brain. The damaging effects of Free Copper occur primarily through the production of oxidative stress (reviewed in [14]) via Fenton-like and Haber-Weiss reactions [159, 160]. Copper is buffered by the astrocytes that belong to the neurovascular unit and are located in the proximity of the BBB (reviewed in [14]). Long-term exposure to high copper levels results in astrocytes impairment, which reverberates on nearby cells of the brain, including neurons and oligodendrocytes, and help in the production of neurodegenerative processes [158]. Similar mechanisms may occur in the subset of AD patients showing serum Free Copper excess, as detailed in *Section 6 Clinical considerations.*

A study, particularly, is of interest since it reported Wilson disease and AD share similarities achieved by a diresct comparison of serum copper, ceruloplasmin, Free Copper and Cu:Cp in 385 AD patients, 336 healthy controls and 9 newly diagnosed Wilson disease patients [26]. Moreover, data of 24h copper urinary excretion in 24 Wilson disease patients under D-penicillamine treatment and in 35 healthy controls were compared with results of AD patients participating to a D-penicillamine phase II clinical trial previously published [59]. After adjusting for sex and age, serum Free Copper and Cu:Cp resulted higher in AD and in Wilson disease patients than in healthy controls (both $p<0.001$). 24h urinary copper excretion in AD patients (12.05µg/day) was higher than in healthy controls (4.82µg/day; $p<0.001$). 77.8% of the AD patients under D-penicillamine treatment [59] had a 24h urinary excretion higher than 200 µg/day, suggestive of a failure of copper control [26].

Copper dyshomeostasis in AD is less severe compared to that in Wilson disease that shows increased levels of copper in the brain. However, this refers to the final stage of the disease when the patient has died, and post-mortem inspection is done. The number of studies investigating copper brain values in Wilson disease is scanty, mainly case reports, with only three bigger surveys [161-163]. Generally, it is reported that 40% of Wilson disease patients have levels of brain copper higher than normal [164, 165]. Long Evan Cinnamons rat and toxic milk mouse are two animal models of Wilson disease bearing natural mutations of the *ATP7B* gene [166]. They share many of the pathological and biological features of the human disease [166], expressively excess Free copper, even though they rarely show any apparent neurological abnormalities [164]. Never-the-less, they can be help to explain the dynamics and progression of copper imbalance and build-up in Wilson disease brain. In both LEC rat and toxic milk mouse brain copper is normal (toxic milk [166]) or significantly lower (LEC [128]) than in the wild-type animal up to the age of 20 (LEC) or 48 weeks (toxic milk), in line with decreased levels of brain copper in AD as shown in meta-analysis (*Table 1; Figure 2*). In animal models of Wilson disease, copper build up in the brain (about 6 times higher than normal) occurs in the final stages and corresponds to the most severe phase of the disease that precedes death, when huge quantities of copper are released from the damaged hepatocytes and then settles in the brain [166]. The picture gleaned from copper meta-analyses in AD (*Table 1; Figure 2*), consisting of Free copper excess in serum/plasma and decreased levels of copper in the brain finds biological plausibility in the models of Wilson disease depicted above that set copper imbalance in AD as a very early (mild severity) stage of the Wilson disease. This hypothesis is anecdotally substantiated by the case report of a patient diagnosed with probable AD and presenting copper build-up in Kayser-Fleischer rings [20].

Summary of existing preclinical or clinical data to support the biomarker in its COU (e.g., summaries of literature findings, previously conducted studies).

Searches performed on 'Scopus' research engine employing the terms «Copper» and «Alzheimer's disease» retrieved 3,009 documents (*Figure 3*); the stratification by 'subject area' reveals that 26% of the papers published come from the biochemistry area, 17 % from medicine, 16% from Neuroscience, 13% from chemistry and 9.2% from pharmacology. A brief summary of this literate is provided herein.

FIGURE 3: OVERVIEW OF THE LITERATURE ON THE TOPIC «COPPER» AND «ALZHEIMER'S DISEASE»

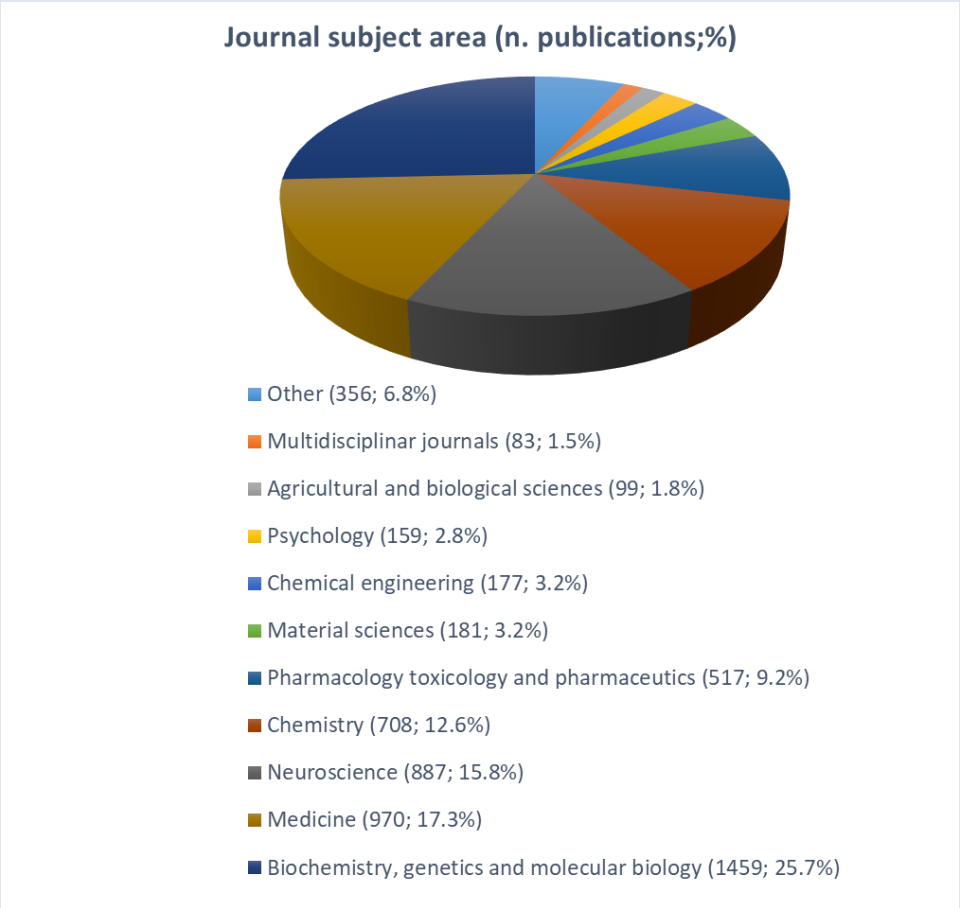


Figure 3: Pie chart illustrating the literature published on the topic «Copper» and «Alzheimer's disease». Search on the Scopus research engine retrieved 3,009 scientific articles; the stratification by 'subject area' reveals that 25.7% of the paper published comes from the Biochemistry area, 17.3 % from Medicine, 15.8 % from Neuroscience, 12.7% from Chemistry and 9.2% from Pharmacology.

The Alzheimer's disease and copper connection: review of the biochemistry literature

Biochemistry studies investigating copper imbalance in AD have been centered on inorganic and coordination chemistry and primarily focused on the interaction between copper and Aβ or the Aβ APP, a Cu-binding molecule [160]. The APP/Aβ system plays a critical role in the pathogenesis of AD. Recent evidence indicates that the proteins are also critically involved in the transport of neuronal copper at the synapse where the ion acts as modulator of learning and memory processes (reviewed in [13, 14, 167]). Loosely bound copper contributes to Aβ oligomer formation and its precipitation within amyloid plaques (reviewed in [13]). In parallel, the redox properties of the metal, are critically involved in the production of oxidative damage and lipid peroxidation [168-171]. Robust evidence indicates that: 1) APP is a copper protein that binds and reduces copper from Cu(II) to Cu(I) [160], thereby facilitating Cu-induced toxicity in cell cultures and oxidative stress through the production of H_2O_2 [172]; 2) Aβ plaques are highly enriched in metals like copper or zinc [160, 173, 174]; 3) in preclinical AD models, Aβ plaques can be dissolved by copper chelating agents [146, 173]. Of note, the interaction between copper and Aβ is also likely to occur strategically in the synaptic cleft where both are available at high concentrations (reviewed in [13]). Moreover, recent evidence implicates altered copper homeostasis in tau pathology, the other key hallmark of AD. *In*

vitro and *in vivo* findings suggest that copper promotes tau deposition by modulating kinases involved in peptide phosphorylation and by triggering self-aggregating conformational changes [175], thereby resulting in the formation of misfolded, high molecular weight tau adducts.

The Alzheimer's disease copper connection: a review of the medical literature

Most of the AD-Cu related studies published in the medical area are focused on the evaluation of copper levels in a variety of organs and biological matrices like serum, plasma, cerebrospinal fluid, brain, hair, or nails. Meta-analyses (*Table 1*) of these data have provided consistent results supporting the notion that AD patients fail to maintain a correct metabolic balance and distribution of copper in the body, mainly consisting in serum Free Copper excess [9], decreased levels of copper in the brain [12], along with copper increases in serum/plasma [6, 7, 9-11] (*Table 1*), despite the lack of concurring changes in ceruloplasmin levels [8]. The copper increase in serum can be explained by the occurrence of increased levels of Free Copper [26] (*Table 2*). These clinical findings led to the hypothesis that: '*…there are 2 neurodegenerative diseases with abnormalities in Cu metabolism: (a) the juvenile form with degeneration in the basal ganglia (Wilson disease) and (b) the age related form with cortical neurodegeneration (Alzheimer's disease)*…' [144].
In the AD brain, the progressive increase of labile (Free) copper pool is consistent with the parallel presence of an expanded pool of Free Copper in the blood [25, 125].

Copper disturbance in AD can be described by a loss of functional copper from protein-bound pools that reduces energy production and oxidative stress control, and a gain of redox-toxic function that is described by a bigger pool of copper loosely bound to proteins (reviewed in [13]).

As described before, in patients with AD, Free Copper correlates with the appearance of changes in brain activity [17, 85, 176], signs of brain atrophy [15, 30], pathological CSF levels of Aβ and tau protein [25], a worse prognosis [22] and it increases the susceptibility to AD approximately threefold [21, 33]. Increased levels of Free Copper have been also associated with worse cognitive performances in patients affected by MCI [21, 23] as well as deficits in cognition in healthy age-matched subjects [31]. Altered copper levels have been also linked to functional and structural changes like brain rhythms alterations assessed with EEG [21, 23, 24, 177] as well as atrophic [28] and cerebrovascular pathological burden [15]. Substantial evidence shows that changes in Free Copper levels in the serum, plasma, CSF, and brain tissue of AD patients are associated with the increased occurrence of cognitive deficits and faster transition from different stages of the AD continuum (reviewed in [14]).

Genetic evidence lends further support to the AD-Cu liaison. Free Copper is associated with higher frequency of specific variants of the ATP7B gene that increase the risk for AD [16, 19, 35-37, 98, 100, 129, 178-183] (*Table 2*, *Table 3*, *Table 4*). The gene variants rs1061472 and rs732774 of ATP7B are SNPs that modify expression and function of the ATPase7B protein inducing a decrease in the activity of ceruloplasmin [19, 72, 126, 180] and are associated with a higher risk of AD and a higher fraction of Free Copper in serum [19, 100, 180].

Alzheimer's disease copper connection: review of the neuroscience literature

A number of scientific articles evaluating measurements of electrical activities in cell cultures models have been included in this category (reviewed in [184] and in [167]). A bulk of evidence comes from studies *in experimental models* focused on the investigation of the causative correlation of copper and Free Copper in AD development and progression (reviewed in [185] and [186]; *Table 5*).

At least four lines of evidence converge towards a key role played by copper imbalance in shaping the course of the disease.

First, copper has been shown to be highly enriched in the core of senile plaques of AD transgenic models, a finding reminiscent of what observed in human brain samples [187].
In a seminal study published in 2003, Sparks and Schreurs demonstrated that, in a cholesterol fed rabbit model of AD, adding trace amounts of 0.12 ppm (0.12 mg/L) copper to distilled drinking water resulted in significantly enhanced cognitive waning. It also exacerbated amyloid plaque deposition to that of control animals [46]. This finding was somehow provocative, as it led to concerns about the content of copper in drinking water leached from copper pipelines. Other studies in the line, demonstrates that copper supplementation in drinking water worsen Aβ- and tau-pathology leading to deterioration of cognitive performances [188]. The study by Singh et al (2013) [53] provided specific emphasis on the causative role that Free Copper might play in AD onset and progression [53]. Singh et al. [53] studied normal mice (wild type) and a mouse model of AD (AβPP transgenic mice) exposed to 0.13 ppm of copper sulfate for 90 days levels of copper via drinking water, which doubled plasma concentrations of Free Copper. This caused either a reduction of CSF Aβ clearance across the BBB in wild-type mice, or an identical effect, along with an increase in Aβ production in the transgenic mice. This study searched for and provided evidence of an increase of Free Copper as a vehicle of this metal, able to pass through the BBB filter, reach the brain, and partake in the AD cascade, thus, proposing the concept that that Free Copper is a causative risk factor for AD. Mechanistically, increased Free Copper levels reduce Aβ clearance and trigger aberrant activation of CDK5, a kinase involved in tau hyperphosphorylation [46, 188].

Indirect evidence for an involvement of copper in AD pathogenesis comes from the use of anti-copper-based therapies as copper chelating agents and Zinc therapy (*Table 6*). *In vivo* administration of these compounds markedly and rapidly reduced Aβ accumulation and cognitive deficits in mouse models of AD [173, 174, 189]. Of note, a recent report showed that zinc administration, here employed as in Wilson disease to reduce systemic copper absorption, was effective in reducing tau phosphorylation in a transgenic mouse model of tau pathology [188]. The approach, although sub-optimal as does not account for the sole effect of zinc on brain functioning [146, 190], further support the idea that a drug development program focusing on anti-copper based treatments could be beneficial to counteract AD.

Finally, some reports, taking advantage of a PET-based copper tracer (^{64}Cu-GTSM), identified overt signs of altered copper metabolism in TASTPM and AβPP/PS1 transgenic mice, two widely employed models of AD-like amyloidosis [191]. The studies also provide the tools for testing the relevance of copper tracers in Neuroimaging settings.

Summary of any planned studies to support the biomarker and COU. How will these studies address any current knowledge gaps?

1) To expand on the findings above, we are conducting a meta-analysis of the available data and a replication study of Free Copper in AD accounting for ATP7B gene variants (Figure 2; [124]). The meta-analyses is based on published studies comparing Free Copper in AD patients to healthy controls in the following three sets: data on Free Copper in serum and plasma pooled together, Copper in brain tissue, and in serum alone. The replication study evaluates the Free Copper levels in AD patients and healthy controls in association with ATP7B rs1061472 and ATP7B rs732774 variant frequency, in order to evaluate the effect of Free Copper on the probability to have AD in carriers of the ATP7B risk haplotype. These studies will allow us to refine and reinforce our understanding of the connection between Free Copper, AD and the gene variants rs1061472 and rs732774 of ATP7B and will provide a basis for further prospective studies.

2) Outside her IGEA Research activity R. Squitti is engaged to an Italian research consortium to carry out a no-profit Phase II clinical trial proof-of-concept funded by the Alzheimer's Association (number PTC-19-602325; EudraCT No.: 2019-000604-15) to validate the hypothesis that regaining a normal level of Free copper (0.1-1.6 µmol/L) in MCI individuals exhibiting Free copper levels higher than 1.6 µmol/L will delay or stop the MCI progression to symptomatic AD. Results from this study will be hopefully available in 4 years and if successful immediately provide a cure for a Cu-MCI/AD subset of patients, since Zinc is already available in the market at a very low cost. Through a 24 months prospective, randomized, double blind, parallel, placebo controlled phase II clinical trial, with Zinc therapy in MCI, the proposal is aimed at:

Aim 1: demonstrating a reduction of the rate of conversion from MCI to full AD along with the clinical worsening in the Zinc treated subjects;

Aim 2: establishing Free copper as a biomarker of a specific Cu-MCI/AD subtype;

Aim 3: identifying genetic variants, which can predict response to Zinc treatment.

Inclusion criteria: MCI subjects who exhibit Free copper higher than 1.6 µmol/L and either positive AD-related biomarkers (Aβ peptides, h-Tau, p-Tau in CSF) or evidence of brain amyloidosis by Florbetapir (18F)-PET scanning.

Study design: Zinc dosage: 135 mg/day of elemental zinc for 11 weeks then 67.5 mg/day till the end of the trial. Sample size: 65 (placebo)+130 (Zinc treated) =195 MCI will allow to detect differences at two sided alpha level of 0.05 with a power of 0.80. 10% attrition so the sample size is 215 using Cognitive Composite 2 (CC2; [192]) as primary outcome at 0, 6, 12, 18 and 24 months. EFFICACY PRIMARY OUTCOME: CC2 [192]. Efficacy secondary outcomes: i) cognitive: a neuropsychological battery including revised ADAS-Cog, RBANS, MMSE, DAD, CDR-SoB e RUD), ii) biochemical (Free copper, zinc, iron, copper, transferrin, ceruloplasmin); iii) Rare variants as well as common polymorphisms in *ATP7B* and *MT* gene encoding for metallothioneins and *APOE4* will be related to the response to treatment.

Please describe alternative comparator, current standard(s), or approaches.

Several methods have been set up for the measurement of Free Copper in serum so far, based on different techniques (*Table 9*, *Table 10*). A first differentiation can be made based on not direct and direct methods. Not direct methods, as for example the widely used mathematical algorithm known as Walshe's index [70], are based on the subtraction of the calculated quantity of the copper bound to ceruloplasmin from the total concentration of copper in the blood serum; briefly: CB =

ceruloplasmin (mg/dL) * 10* n; n=0.0472 (µmol/mg); Free Copper = absolute serum copper – CB. This calculation expresses Free in µmol/L and is based on the fact that ceruloplasmin binds 6 atoms of structural copper, which is equivalent to ceruloplasmin containing 0.3% copper (weight percentage of copper in Cp): [6 (atoms of Cu) *63 mass atom U (Cu molecular weight)/132 KDa (Cp molecular weight)] * 100=0.3% [70]; this algorithm is applied in Wilson disease as per established guidelines [68].

Regarding the direct method, the experimental determination of Free Copper is generally performed in two steps: the first consists of the separation of the copper bound to ceruloplasmin and the other cuproenzymes of the pool of firmly-bound species of copper from the pool of loosely-bound species of copper [solid phase extraction or ultrafiltration). The second involves the measurement of copper levels in the filtrate.

Other differentiations can be made based on the copper measurement technique employed and, on the procedures, employed to isolate the pool of loosely bound species. *Table 9* provides details about the different methods employed so far. As reported in *Table 9*, the most used analytical methods for measuring total serum copper (bound and free) are: atomic absorption spectrometry (AAS), X-ray fluorescence spectrometry (XRF), ICP-MS, and colorimetric or fluorescent probe automated methods. Advantages and disadvantages of the metal assessment techniques are briefly discussed in *Table 9*. While AAS and ICPMS have high costs and need specialized technical personnel or staff and generally represent facilities in Department Academic infrastructures, the colorimetric methods have a higher affordability and easiness deriving from their application on automated biochemical analyzers used in a clinical setting, also in small size clinical pathology laboratories. On the other hand, AAS and ICMPS techniques provide very high sensibility and precision data, even further the clinical application needs.

The gap between the advantages and disadvantages has narrowed since the early 2000s, as much as ICP-MS equipment at relatively lower costs and with fewer needs for specialized personnel have been introduced on the market. Starting from the years 2005 several new methods have been developed for the measurement of Free Copper. Different groups faced this issue also focusing on different clinical applications (*Table 10*). Not only academic research but also the implementation of methods with potential clinical applications started to be pursed. The first device that has been proposed in the market has been the McMillin method in 2009 [69], focused on the clinical application of Wilson disease. If on the one hand, Free Copper as a biomarker of Wilson disease had had certainly a high impact on clinical management of a severe and insidious disease as Wilson disease that has a spread range of disease presentation (from hepatic to psychiatric and neurological symptoms), on the other hand, Wilson disease is a rare disease, so the method had low commercial revenues, and this impacted on resources and efforts spent on further commercial developments. Therefore, most of the interest in Free Copper implementation and applications came back to the academic setting with new developments starting from 2018, employing some highly sophisticated technique of copper speciation, able to be distinguished single copper components in the Free Copper pool. Most of these new techniques employing high-performance liquid chromatography (HPLC) - ICP-MS that are challenging in terms of amount of technical expertise needed to perform the method, and also in terms of high costs, even though the amount of data and information they

provide is very high level and exceeding the clinical application needs. In parallel to the technological evolution on the method for detection of Free Copper previously discussed, development in terms of clinical application has been implementing starting from 2005. A number of studies from different groups connected copper and Free Copper to the etiology of Alzheimer's disease and other neurological disorders [149]. In 2014, data about AD have been collected and evaluated through meta-analyses demonstrating an increase of Free Copper in the disease [9]. In most of these studies, the not direct methods, as for example the Walshe's index [70], has been employed, since they had a clinical setting basis, thus applied method inherited from Wilson disease clinical practice. These findings fueled interest in developing Free Copper methods with easiness and affordability advantages to allowing the clinical application, as for example a new fluorescent method [18] proposed in the market in Italy in 2015 (06 – *Dispositivo diagnostici in vitro 1102010600 -Copper Cu; CE certification*). The IGEA Free Copper tool has been implemented to overcome the fluorescent method drawback derived from shortage fluorescence decay and lability of the fluorescent probe. The IGEA Free Copper tool employs ICP-MS technology plus ultrafiltration and it has been adapted to assess Free copper in small serum volumes that can be easily collected and transported for testing in a centralized laboratory.

SECTION 8 PREVIOUS QUALIFICATION INTERACTIONS AND OTHER APPROVALS

We had applied previously in an obsolete format (#DDTBQP000098) that was subsequently withdrawn.

SECTION 9 MOST RELEVANT AND SELECTIVE PUBLICATIONS

Please provide a list of publications most relevant to this biomarker development proposal.

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SECTION 10 LONG TERM OBJECTIVES/GOALS

Please provide biomarker development effort as part of a longer-term goal, please summarize your long- term objectives.

We will spend efforts to set up a research network of centers among the best excellence in the field devoted to degenerative dementias. The network will be responsible for conducting future multicenter trials and represents an ideal setting to capture large volumes of invaluable biological and clinical, as well as neuroimaging data. Our perspective contrasts with the limited translational research use of biomarkers in most prior clinical trials, recognizing precision medicine as a unique opportunity to maximize data science and landmark investments with the long-term goal of tailoring preventive and treatment interventions on the individual needs of people at risk to develop AD (*Figure 3*).

PLANNED ACTIVITIES TOWARD LONG TERM OBJECTIVES

Our long-term objective include determination of a new therapeutic approach based on Zinc therapy to delay the insurgence of AD in MCI subjects susceptible to copper exposure.

Activity 1 (Obj 1.1), US based Phase II clinical trial with Zinc therapy: to demonstrate a reduction of the rate of conversion from MCI to full AD along with the a slowing of clinical worsening in MCI subjects treated with 100-150 mg/day Zinc, in a two years phase II, double blind, placebo controlled clinical trial of 215 MCI with Free Copper higher than 1.6 µMol; primary clinical outcome: Cognitive Composite 2 (CC2) [192]; inclusion criterion: CSF Aβ and Tau positive; Free Copper higher 1.6 µmol/L). Duration: 36 months divided in existing site activations (2 months); screening phase (10 months); Double blind phase 2 (24 months); statistical analyses (2 months).

Activity 2 (Obj 1.2): To confirm that Free copper as the elective biomarker of a specific MCI-AD subtype susceptible to copper exposure and responding to Zinc therapy (12 months).

Activity 3 (Obj 1.3): to identify genetic variants, which can predict response to the Zinc treatment, by employing a target sequencing approach; main candidates genes: ATP7B and MT genes encoding for proteins that control copper and zinc level/transport (copper chaperons/transporters, and metallothioneins) (12 months).

SCIENTIFIC SERVICE AND INFRASTRUCTURE DEVELOPMENT (SSID):

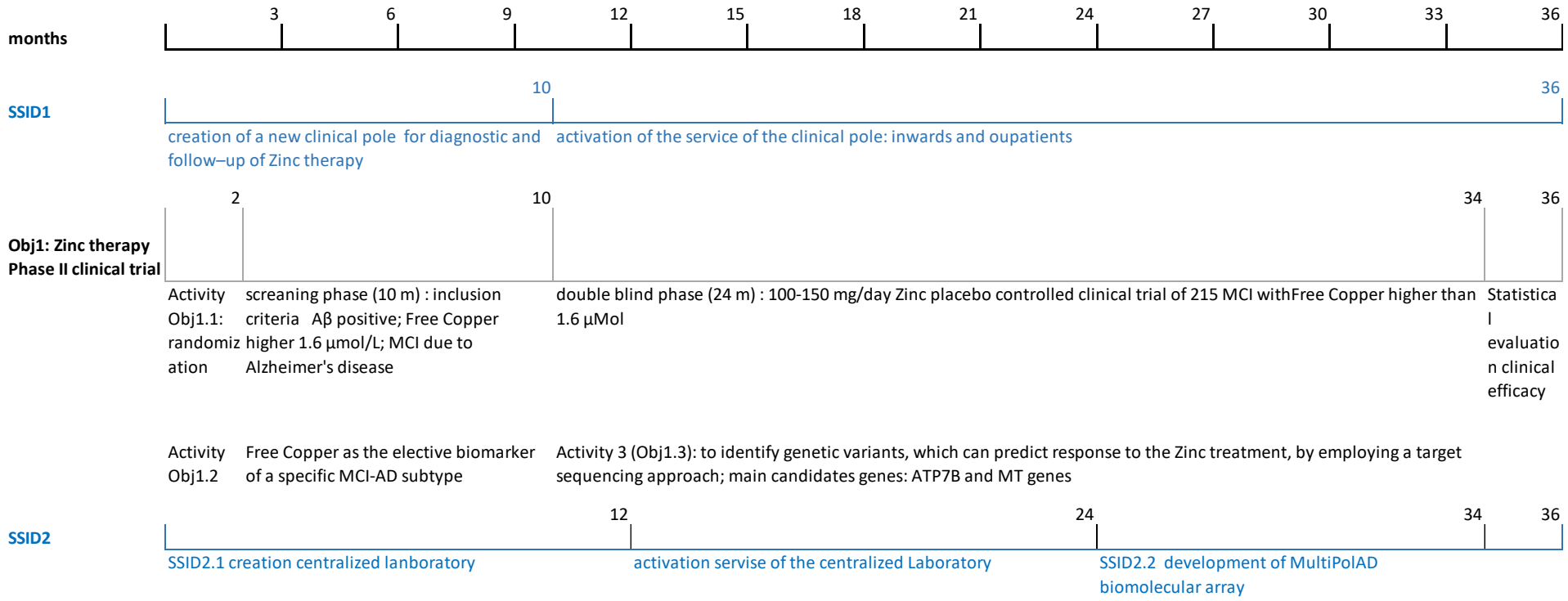
SSID1: creation and activation of a new clinical pole for performing Phase II clinical trial with Zinc therapy as anti-copper-based treatment in MCI subjects with Free copper >1.6 µmol/L

SSID2: creation and activation of a new Centralized Laboratory where to apply translational research applied to precision medicine

SSID2.1: creation and activation of a new Centralized Laboratory

SSID2.2: development of a new patentable product or array based on genetic and biochemical assessments to typify MCI-AD subjects affected by copper dyshomeostasis who can take clinical benefit from the administration of a Zinc therapy

FIGURE 3. LONG-TERM OBJECTIVE PLAN



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SECTION 11 ADDITIONAL INFORMATION

ATTACHMENT 1 - CLSI VALIDATION (FREE COPPER ICPMS IGEA METHOD)

ATTACHMENT 1

CLSI VALIDATION
Free Copper ICPMS IGEA method

Analysis was made by analyze-it as suggested by the Clinical and Laboratory Standards Institute (CLSI)

Direct Measurement of Free Copper in Serum Ultrafiltrate

IGEA Research Corporation
2600 SW 3rd Avenue 350,
Miami, FL 33129

Dr. Valentine Negrouk (Technical Expert)
Dr. Valentina Panetta (Statistical Expert)
Dr. Rosanna Squitti (Final Review and Approval Authority)

The CLSI validation section of free copper ICPMS IGEA method includes following sections as described below:

- Introduction
- Precision (EP05-A3)
- Linearity (EP06-A)
- Limit of detection (EP17-A)
- Recovery

INTRODUCTION

The IGEA Free Copper test is a method for direct measurement of Free Copper in serum ultrafiltrate by inductively coupled mass spectrometry (ICPMS). Procedures from Clinical and Laboratory Standards Institute (CLSI) were employed for validation of the Free Copper IGEA test. In particular, the EP05-A3 (Evaluation of Precision of Quantitative Measurements Procedures; Approved Guideline – Third Edition), the EP06-A method evaluation (Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline), EP17-A (Protocols for determinations of Limit of Detection and Limit of quantitation; approved guidelines) and Recovery were applied. StatisPro Methods evaluation software (from CLSI version 3.02.2) was used for these analyses.

PRECISION (EP05-A3)

A 20X2X2 design was used to evaluate precision, considering 20 non consecutive days, 2 runs per day and 2 replicates per runs.

Evaluation was made on

a) sample with “average” value with mean= 1.21 µmol/L
b) sample with low value with mean = 0.86 µmol/L
c) sample with high value with mean=2.08 µmol/L

A single reagent lot and a single calibrator lot are used, and all testing is performed with a single instrument at a single site

Two-way ANOVA show a good precision (CV<5%) in three groups with a higher precision in the group with higher values

Attachment 1 Table 1 Precision (A)

Precision

	Mean	Repeatability SD	Repeatability CV	Within Laboratory SD	Within Laboratory CV
	1.209	0.048	4.0%	0.061	5.0%

Component	% of Total	SD	Exact / MLS 95% CI	CV
Repeatability	63.8%	0.048	0.040 to 0.062	4.0%
Between Run	14.0%	0.023	0.000 to 0.049	1.9%
Within Day	77.8%	0.053	0.045 to 0.069	4.4%
Between Day	22.2%	0.029	0.000 to 0.052	2.4%
Within Laboratory	100.0%	0.061	0.053 to 0.077	5.0%

Attachment 1 Table 2 Precision (B)

Precision

	Mean	Repeatability SD	Repeatability CV	Within Laboratory SD	Within Laboratory CV
	0.862	0.035	4.1%	0.059	6.8%

Component	% of Total	SD	Exact / MLS 95% CI	CV
Repeatability	36.0%	0.035	0.029 to 0.045	4.1%
Between Run	18.3%	0.025	0.000 to 0.045	2.9%
Within Day	54.3%	0.043	0.036 to 0.057	5.0%
Between Day	45.7%	0.040	0.021 to 0.064	4.6%
Within Laboratory	100.0%	0.059	0.050 to 0.078	6.8%

Attachment 1 Table 3 Precision (C)

Precision

	Mean	Repeatability SD	Repeatability CV	Within Laboratory SD	Within Laboratory CV
	2.080	0.056	2.7%	0.092	4.4%

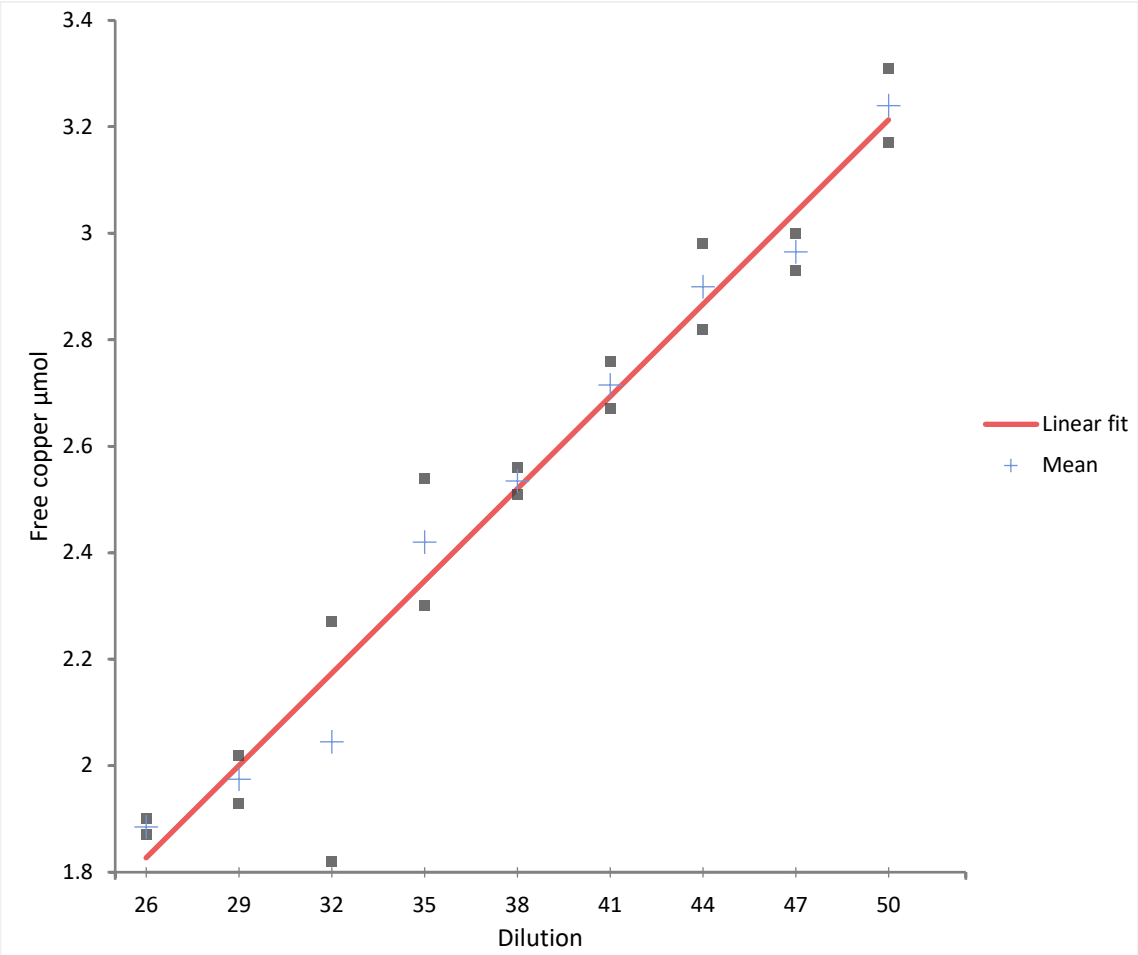
Component	% of Total	SD	Exact / MLS 95% CI	CV
Repeatability	37.8%	0.056	0.046 to 0.072	2.7%
Between Run	12.8%	0.033	0.000 to 0.063	1.6%

Within Day	50.6%	0.065	0.055 to 0.085	3.1%
Between Day	49.4%	0.064	0.038 to 0.102	3.1%
Within Laboratory	100.0%	0.092	0.077 to 0.122	4.4%

LINEARITY (EP06-A)

Nine concentration levels and 2 replicates at each level was tested

Attachment 1 Figure 1 and Attachment 1 table 4 show concentrations level used and data distribution. Regression models (table 5) show that factor of second or third order are not significant.



Attachment 1 Figure 1 Linearity

Attachment 1 Table 4 Concentration level and linearity

Dilution	Linear fit	Nonlinear fit	Nonlinearity	95% CI	Relative nonlinearity
26	1.827	-	-	- to -	-

29	2.000	-	-	- to -	-
32	2.174	-	-	- to -	-
35	2.347	-	-	- to -	-
38	2.520	-	-	- to -	-
41	2.693	-	-	- to -	-
44	2.867	-	-	- to -	-
47	3.040	-	-	- to -	-
50	3.213	-	-	- to -	-

No 2nd/3rd order polynomial fit is statistically better than a linear fit at the 5% significance level.

Attachment 1 Table 5 regression models

Fit Model

Linear fit

RMSE	0.121

Parameter	Estimate	SE	t	DF	p-value
Constant	0.3255	0.14239	2.29	16	0.0362
X	0.05775	3.6715E-03	-256.64	16	<0,0001

2nd order polynomial fit

RMSE	0.125

Parameter	Estimate	SE	t	DF	p-value
Constant	0.4129	0.78564	0.53	15	0.6069
X	0.05295	0.042550	-22.26	15	<0,0001
X^2	6.313E-05	5.5764E-04	0.11	15	0.9114

3rd order polynomial fit

RMSE	0.127

Parameter	Estimate	SE	t	DF	p-value
Constant	3.155	4.5581	0.69	14	0.5002
X	-0.1750	0.37550	-3.13	14	0.0074
X^2	0.006212	0.010078	0.62	14	0.5475
X^3	-5.393E-05	8.8262E-05	-0.61	14	0.5509

LIMIT OF DETECTION (EP17-A)

Sixty measurements (12 replications on 5 samples) on blank and 60 measurements (12 replications on 5 samples) on low-levels samples were carried out. Alpha and Beta error were fixed at 5%.

The LOB estimated is 0.0002 and the LOD is 0.0397

Attachment 1 Table 6 detection limits

Detection Decision

	N	Mean	SD	Alpha	Critical value (LoB)
Blank material	60	0.00010	0.00008	5%	0.0002

Detection Limit

	N	Pooled SD	Beta	Detection limit (LoD)
Non-blank material	60	0.02387	5%	0.0397

RECOVERY

Nine replications were used to evaluate recovery in samples added with 0.5 µmol/L, 1 µmol/L, 2 µmol/L, 3 µmol/L e 5 µmol/L, compared with the one added with only water. Recovery rate range between 95% and 96% with a median value of 95%

Attachment 1 Table 7 Recovery

	Recovery %
Standard Free Copper serum + 0.5 µmol/L	96
Standard Free Copper serum + 1 µmol/L	95
Standard Free Copper serum + 2 µmol/L	95
Standard Free Copper serum + 3 µmol/L	94.6
Standard Free Copper serum + 5 µmol/L	95
Mean Value	95

ATTACHMENT 2

Clinical Laboratory Improvement Amendments (CLIA)

IGEA Research Corporation
2600 SW 3rd Avenue 350,
Miami, FL 33129

By IGEA RESEARCH CORPORATION

CENTERS FOR MEDICARE & MEDICAID SERVICES

CLINICAL LABORATORY IMPROVEMENT AMENDMENTS

CERTIFICATE OF ACCREDITATION

LABORATORY NAME AND ADDRESS
IGEA RESEARCH CORPORATION
2600 SW 3RD AVE STE 350
MIAMI, FL 33129

CLIA ID NUMBER
10D2106042

EFFECTIVE DATE
03/22/2019

LABORATORY DIRECTOR
RAJA M GIDWANI M.D.

EXPIRATION DATE
03/21/2021

Pursuant to Section 353 of the Public Health Services Act (42 U.S.C. 263a) as revised by the Clinical Laboratory Improvement Amendments (CLIA), the above named laboratory located at the address shown hereon (and other approved locations) may accept human specimens for the purposes of performing laboratory examinations or procedures.

This certificate shall be valid until the expiration date above, but is subject to revocation, suspension, limitation, or other sanctions for violation of the Act or the regulations promulgated thereunder.

CMS
CENTERS FOR MEDICARE & MEDICAID SERVICES

Karen W. Dyer, Acting Director
Division of Laboratory Services
Survey and Certification Group
Center for Clinical Standards and Quality

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ATTACHMENT 3- STANDARD OPERATING PROCEDURE (SOP)

ATTACHMENT 3

Standard Operating Procedure (SOP)

Direct Measurement of Free Copper in Serum Ultrafiltrate

IGEA Research Corporation
2600 SW 3rd Avenue 350,
Miami, FL 33129

Dr. Valentine Negrouk (Technical Expert)
Dr. Valentina Panetta (Statistical Expert)
Dr. Rosanna Squitti (Final Review and Approval Authority)

The standard operating procedure for direct measurement of free copper in serum ultrafiltrate includes following sections as described below:

- Purpose
- Introduction
- Specific Procedure
    - Blood Samples and Serum
    - Electronic Registration
- Back End Specifications – Box Manager and Technician Workflow
    - Equipment and Materials
    - Reagents and Media
    - Preparation of Solutions
    - Procedure of Free Copper Measurement
- Protocol
    - Waste Disposal Procedures
    - Chemical Waste Consists of Two Types of Waste
    - Medical or Biohazardous Waste
    - Glass Recycling
- Emergency Procedures
    - Spills and Splashes
    - Biological Spill Kit
    - Sample Delivery Overload
    - Contingency Plan in the Case of Extraordinary Event
    - Earthquake, and Fire in the Immediate Vicinity
    - Health Emergencies (Ambulatory)
    - Health Emergencies (Unconscious)
    - Reminders
- References

Purpose

The presented SOP is required to establish a procedure of the direct measurement of Free Copper in patient's blood serum complying with The Good Clinical Practice (GCP) and other internal procedures and guidelines.

Introduction

Copper (also reported as Cu through the text) is an essential trace metal that is a required co-factor for the formation and function of many vital enzymes and pathways, such as for example

hemoglobin and carbohydrate metabolism. By redox cycling between Cu(II) and Cu(I), the metal facilitates numerous enzymes in their catalytic reactions [14]. Copper is crucial for central nervous system development and correct neurophysiology, taking part in brain homeostasis through pathways governed mainly by the two copper-transporting P-type ATPases (copper-ATPases), encoded by the genes *ATP7A* and *ATP7B.* ATPase7B is expressed in the endothelial cells of the blood cerebrospinal fluid (CSF) barrier where facilitates copper transport into the CSF, as well as copper sequestration into the choroid plexus [14].

Defects of *ATP7B* gene cause an inborn error of copper metabolism known as Wilson disease, a rare autosomal recessive disorder that leads to an altered distribution of copper in the body. Wilson disease is typified by a severe depletion of copper bound to proteins, the most relevant of which is ceruloplasmin, binding 70-95% of copper in the blood, and an increase in the fraction of copper not bound to proteins, referred as 'Free' Copper (also known as non-ceruloplasmin Cu). In Wilson disease, *ATP7B* mutations cause the failure of copper incorporation into ceruloplasmin and other copper proteins and enzymes [14], while the Free Copper in serum becomes expanded, bleeding from the liver, and accumulating in some brain areas, and in the cornea forming the Kayser-Fleischer rings in the eye. Free Copper a low molecular weight exchangeable copper pool (exchanged among albumin, α2 macroglobulin, peptides and amino acids) with a low molecular weight [193]. When it it exceeds the normal values of 1.6 μmol/L in the blood, it is toxic as it crosses the blood brain barrier (BBB) and causes oxidative stress and tissue damage (reviewed in [14]). Less outstanding Free Copper excess might also be associated with Alzheimer's disease (AD), cancer, preeclampsia, dilated cardiomyopathy, inflammation, and biliary obstruction, and total parenteral nutrition [14, 21, 69, 194-196]. A number of studies indicate that AD patients and mild cognitive impairment (MCI, a prodromal stage of AD) subjects suffer from a mild copper dysregulation, with some features shared with Wilson disease, even though the diseases have different aetiology [14]. In AD, neuronal damage is mainly associated with a peptide, named amyloid beta (Aβ) that forms plaques in specific areas of the brain, and with the presence of Tau proteins that form tangles of filaments within neurons preceding the Aβ extracellular plaque formation. Numerous *in vitro* and animal studies show that Free Copper facilitates the formation of Aβ plaques and oxidative stress in the brain associated with neurodegeneration (reviewed in [14]).

Furthermore, recent meta-analyses demonstrate that patients with AD fail to maintain a physiological balance and distribution of copper in the body and show increased copper levels in serum along with an overall copper depletion in the brain, associated to increased levels of Free Copper in serum (reviewed in [14]). In the AD brain, the progressive increase of the Free Copper pool is consistent with the parallel increase of the Free Copper pool in the blood, even though the total copper content, represented by copper bound to proteins, is decreased, especially in the cerebral cortex [14]. In patients with AD, Free Copper correlates with the appearance of changes in electroencephalography (EEG) brain activity, signs of brain atrophy, pathological CSF levels of Aβ and Tau protein, a worse prognosis and an increase in the susceptibility to AD of approximately threefold (reviewed in [14]).

Free Copper is also associated with higher frequency of specific variants of the *ATP7B* gene that increase the risk for AD [14]. The gene variants rs1061472 and rs732774 of *ATP7B* are functional single nucleotide polymorphisms (SNPs) that modify properties of ATPase7B protein and are

associated with a higher risk of having AD and a higher fraction of Free Copper in serum even in normalcy [197]. Increases in Free Copper pertains to a percentage of AD patients, a copper-related AD subtype called 'CuAD', characterized by high Free Copper levels, and showing peculiar clinical, genetic and neurophysiological characteristics [14].

Increased levels of Free Copper are also found in patients MCI [14]. During the very early stages of cognitive complaints, it accounts for an increased relative risk and for a faster rate of conversion to AD (in a 6-year survey period) [21]. Slightly elevation of Free Copper also in healthy controls associates with a worse cognitive performance [31].

The existence of CuAD is supported by EEG-derived brain cortical rhythms alterations, reduced in all bands in the parietal cortices, and in alpha band in sensory lobes in CuAD with respect to AD with non-ceruloplasmin copper within normal limits.

Direct measurement of the Free Copper concentrations in human blood serum may provide a useful tool for the screening, diagnosis, and monitoring of AD and the other pathologies associated with abnormal levels of Free Copper [69].

Specific Procedure

The presented SOP was based on the protocol established in the process of preliminary research and development conducted in IGEA Research Corporation. The whole protocol was developed according [69, 71, 79, 96] with some modifications.

BLOOD SAMPLES AND SERUM:

Blood samples should be delivered by FedEx mail in BDMicrotainer collection tubes with Gold closure (with clot activator/SST gel). The usual volume of finger-dropped blood used is around 500 µL. Tubes should be immediately registered and centrifuged for 15 minutes at 2500 rpm (1321 g) at 4ºC. Serum (200-250 µL) should be collected and stored at refrigerator for the same or next day processing). Preliminary research showed that serum stored in refrigerator up to 5 days gave us the same quantity of free cooper as samples processed at the day of delivery. As internal healthy human standard, we used serum purchased from SIGMA-ALDRICH (Catalog number H4522).

ELECTRONIC REGISTRATION:

The medical assistant (Box Manager) should be responsible for electronic and hard copy registration of the delivered blood samples. For the electronic registration, the following are required:

A. Date of a given sample.
B. Date of the day when a given sample was sent.
C. Check showing that this given sample was processed to serum the same day (show time of processing).

D. Check showing that this given sample was placed into the refrigerator in A rack which should be processed the same or next day (rack should be signed with an indication of the day when sample should be processed for ICP-MS.
E. Date of ICP-MS processing.
F. Result (concentration of free copper in patient's serum expressed in µg/dL).
G. Indication of sample serum leftover, volume, date of deposition and number of the box were sample was deposited.

Back End Specifications – Box Manager and Technician Workflow

a) Kit arrives at the laboratory.
b) Box Manager (BM) takes care of the kit management after the arrival at the lab and completes the kit registration process.
c) BM logs into his/her account with his/her username and password.
d) BM opens the envelop and checks the box integrity.
e) If the box is broken the BM selects the "Broken Kit Email" to be sent by the CRM system;
f) If the kit box is broken the Box goes into an "Invalid Kits Box".
g) If not "c", BM opens the kit. If the blood tube is missing or broken, BM selects the "Missing or Broken Blood Sample Email". The kit goes into the "Invalid Kits Box".
h) If not "e", BM checks consistency between the box and the tube's serial number.
i) If numbers are not consistent BM selects the "Inconsistent Serial Numbers email" to be sent to both serial number customers. The kit goes into the "Invalid Kits Box".
j) If not "g", and the serial numbers are consistent, BM controls that the tube contains at least 500 ml of blood (value to be verifies with biologist).
k) If the tube does not contain enough blood, the BM select the "Missing Sample Specification email". The kit goes into the "Invalid Kits Box".
l) If not "i", BM adds the kit into the system from his/her account by clicking on the "Add a Kit" button.
m) BM clicks on the "Add a Kit" button, inserts the kit's serial number, the receiving date and the customer's blood collection date. The system automatically inserts the "Expiring Date" as the Customer Collection Blood date + 72 hours. Box Manager clicks on "Register Kit" button.
n) If the kit has not been registered by the customer on Alz1.com/kit the BM will receive a "Warning Message" on the screen. The kit is be saved with status "Not Registered" and goes into a "Invalid Kits Box".
o) If the Blood Sample is expired the BM will receive a "Warning Message" on the screen. The Box Manager selects the "Blood Conditions Email" to be sent to the customer by the CRM system. kit is to be saved with status "Expired" and goes into a "Invalid Kits Box".
p) The Kit will be registered, and its status will be "To Be Centrifuged";
q) BM stores valid and registered kits into refrigerator (around $4^{o}C$) by the expiration date generated by the system.
r) After completing the registration process of all the "regular" kits the BM collects all the Invalid Kits for registration.

s) BM clicks on the "Add a Kit" button. BM inserts the kit serial number and the receiving date. He clicks on the button "Add". The system displays a pop up: "Do you want to register this kit as invalid". BM confirms and the kit enters into the system with the "Invalid" status.

All accounts of BMs have a username and password with secure logins. Accounts can be edited, blocked, deleted. Password can be retrieved. The system collects basic stats on the BM activities. Time of activity and number of kits processed, number of valid and invalid kits (with specific reasons.) Stats on total kits are available aggregated by date as well.

Invalid kits will be registered as valid kits, but will be saved as "Invalid"

EQUIPMENT AND MATERIALS:

A. EQUIPMENT:

1. Eppendorf Refrigerated Centrifuge 5810R
2. Eppendorf table-top centrifuge Mini-Spin Plus
3. Analytical Balance (Fisher Science Education)
4. Mini-Vortex (Fisher Scientific)
5. Micropipettes (1-10 µL, 10-100 µL, 100-1000 µL) (Eppendorf)
6. Millipore Water Purification System "Simplicity"
7. New Brunswick Premium -86°C Freezer
8. Refrigerator "Frigidaire"

B. SUPPLIES AND MATERIALS:

1. Storage bottles, 125 ml, 250 ml, 500 ml and 1000 ml (Fisher Scientific)
2. Parafilm
3. Eppendorf Tubes 1.5 ml
4. Eppendorf Tubes 2.2 ml
5. Eppendorf micropipettes tips (1-10 µL, 10-100 µL, 100-1000 µL)
6. Weigh Boats (Fisher Scientific)
7. 15 ml tubes for ICP-MS analysis (Thermo-Fisher, cat. number ASX-560)
8. Amicon Ultra 0.5 ml Ultracel 100k (Millipore Amicon, cat. Number UFC510096)

REAGENTS AND MEDIA:

1. Nitric Acid TraceMetal Grade (Fisher Scientific, cat. Number A509- P500)
2. EDTA-disodium salt dehydrate (Sigma, cat. Number E5134-50G
3. Copper standard 1000 µg/mL (SPEX CertiPrep, cat. Number CLCU2-2M)
4. Yttrium internal standard 10 µg/mL (SPEX CertiPrep, cat. Number CLY2-1AM)
5. Distilled Water, Type I (DW-1)

PREPARATION OF SOLUTIONS:

All solutions used for this procedure must be clearly labeled with name of the solution, the preparation and the expiration date.

1. Solution of EDTA – 3g/L or 8mM: Weighted 300 mg of EDTA-disodium salt and solved in 100 ml of DW-1.
2. Solution of 2% nitric acid: Added to 980 ml of DW-1 20 ml of Nitric Acid TraceMetal Grade. Mix well.
3. Solution of 2% nitric acid with Yttrium internal standard: Added 1 ml of Yttrium internal standard (10000 µg/L) to 99 ml of 2% nitric acid. Mix well.
4. 100 times concentrated standard copper solution 1 (10^4 µg/L): Mixed 10 µL of Copper standard (1000 µg/mL) with 990 µL of 2% nitric acid. Mix well.
5. 100 times concentrated standard copper solution 2 (10^3 µg/L): Mixed 100 µL of 100 times concentrated standard copper solution 1 with 900 µL of 2% nitric acid. Mixed well.
6. 100 times concentrated standard copper solution 3 (10^2 µg/L): Mixed 100 µL of 100 times concentrated standard copper solution 2 with 900 µL of 2% nitric acid. Mixed well.

PROCEDURE OF FREE COPPER MEASUREMENT:

The copper is determined using an iCAP Q ICP-MS manufactured by ThermoFisher, which is calibrated for copper measurements (copper 65) for the range from 0.1 to 10.0 µg/dL (0.0157-1.57 µmol/L). The technician should be dressed in a laboratory coat and required gloves.

Protocol:

1. Mix 50 µL of serum with 50 µL of EDTA solution (EDTA disodium salt dehydrate, conc. 3g/L) and incubated 1h at room temperature.
2. Add 400 µL of twice diluted EDTA solution, mix and load 500 µL of mixture into filtration insert of 100kD centrifugal filter unit Amicon Ultra-0.5 ml.
3. Centrifuge 30 min at 7000xg in Eppendorf Mini Spun plus at room temperature.
4. Volume of filtrate should be around 400 µL. Mix well.
5. 300 µL of filtrate mix with 900 µL of 2% nitric acid containing 100 µg/L of yttrium (as internal standard). Final dilution factor will be 40.
6. For background control sample instead of filtrate add 300 µL of water.
7. For standard curve take 3 background control samples and add 12 µL of concentrated solutions of copper in 2% nitric acid. For final concentrations:
   a) Standard 1 - 100 µg/L use 10000 µg/L.
   b) Standard 2 - 10 µg/L use 1000 µg/L.
   c) Standard 3 - 1 µg/L use 100 µg/L.
8. Every sample should be measured by ICP-MS 3 times. Final result used to be an average of 3 measurements.
9. Every sample should be measured by two parallel probes. Good result should be within 10% deviation. If deviation is more than 10%, test must be repeated.

WASTE DISPOSAL PROCEDURES:

Hazardous waste in this clinical laboratory can be represented by chemical waste and biohazard waste.

CHEMICAL WASTE CONSISTS OF TWO TYPES OF WASTE:

1. Wash down drains with excess water:
   a) Concentrated and dilute acids and alkalis.
   b) Harmless soluble inorganic salts (including all drying agents such as CaCl2, MgSO4, Na2SO4, P2O5).
   c) Alcohols containing salts (e.g. from destroying sodium).

2. It should be noted that no material on the "Red List" should ever be washed down a drain. This list includes following elements: antimony, arsenic, barium, beryllium, boron, cadmium, chromium, cobalt, copper, lead, mercury, molybdenum, nickel, selenium, silver, tellurium, thallium, tin, titanium, uranium, vanadium and zinc.

MEDICAL OR BIOHAZARDOUS WASTE:

1. Liquid human and animal waste, including blood and blood products and body fluids as well as used plastic disposables (pipette tips, Eppendorf tubes, 15 ml or 50 ml centrifuge tubes).
2. Sharps (syringes and needles) shall be disposed of by a certified waste hauler. Place discarded needles and syringes into an approved MSU sharps container. An approved sharps container is one that is leak proof, puncture – resistant, closable, bears the biohazard symbol and is manufactured as a sharp's container.

Biohazardous as well as "Red List" chemical waste must be accumulated and stored within laboratory space until removed by Environmental Health and Safety (EHS) and must be:
Collected in a container that is compatible with its contents under all conditions that it might be subjected to during accumulation, storage, and shipment.

Handled only by personnel trained in the requirements of these hazardous waste rules.
Removed from the accumulation area within three days if the quantity of any one waste exceeds fifty-five gallons. (From a safety perspective, no more than five gallons should be accumulated in a laboratory or shop.)

Labeled with the words "hazardous waste," the waste identification number (see below), the chemical description, and concentration(s).

If the waste does not meet the description as listed below, label it with the words "Non RCRA Waste", the chemical description and concentration(s).

GLASS RECYCLING:

For environmental reasons the recycling of glass is encouraged, but only certain items of waste glass produced within laboratories are acceptable for recycling. Each laboratory should have a bin for recyclable glass. Only clean glass bottles such as those in which chemicals are received and broken or waste plate glass are allowed. All broken laboratory glassware, items significantly contaminated by chemicals, sample tubes, droppers and glass wool must be disposed of as controlled waste. The recycling service will refuse to empty a recycling container if any of these prohibited items is discovered in it.

Emergency Procedures:

SPILLS AND SPLASHES:

1. Cover the spill immediately with absorbent material (gauze, cotton mat, paper towel) then flood with 0.5% Sodium Hypochlorite solution.
2. Notify the buddy and safety officer immediately.
3. The buddy should mark the area with warning sign.
4. Allow 10 minutes contact time then gently clean the area of the spill in a circular motion starting from the outer edges towards the center.
5. If there are broken glass or sharps, use forceps to pick-up the pieces and absorbent material.
6. If splashes are suspected, decontaminate the area within 1 meter of the spill, including vertical surfaces (e.g. walls and sides of equipment).

BIOLOGICAL SPILL KIT:

1. Biological spills kits should be available and readily accessible in the laboratory area. Which should contain at least:
   A) Enough absorbent material (Gauze, cotton mat, or paper towel) and decontaminating agent (0.5% Sodium Hypochlorite solution) to cover an area of 1 meter in radius.
   B) Autoclavable, leak-proof bags to contain waste from clean-up procedure.
   C) Long handle forceps.
   D)Warning signs, marker or label.

SAMPLE DELIVERY OVERLOAD:

In case of sample delivery overload, we have to consider two scenarios:

1. Delivery overload at the end of the working day, but at the beginning or at the middle of the week. Samples could be:

a) Placed in the refrigerator till next day.

b) Centrifuged, serum should be separated from clotted blood (all operation takes 20-25 minutes), serum could be left in the refrigerator till next day and blood clot placed into -80oC freezer.

2. Delivery overload at the end of the working week. Somebody should come next day (extra hours), centrifuge sample, serum should be separated from clotted blood (all operation takes 20-25 minutes), serum could be left in the refrigerator till next working day (up to 2-3 days) and blood clot placed into -80oC freezer.

CONTINGENCY PLAN IN THE CASE OF EXTRAORDINARY EVENT:

In any case technician/technologist must separate serum from blood clot within 24 hours from delivery arrival (if it was mailed less than 42 hours ago) and please them separately in the deep freezer (-80°C).

EARTHQUAKE, AND FIRE IN THE IMMEDIATE VICINITY:

1. Secure samples by closing the door of the BSC.
2. Remove outer gloves.
3. Grab a bottle of decontaminating agent.
4. Isolate your team (buddy and safety officer) and decontaminate before removing the PPE.
5. Inform ICC nurse for appropriate post exposure treatment.
6. Inform local emergency responding units of the hazardous material left behind.

HEALTH EMERGENCIES (AMBULATORY):

1. Stop all work and notify immediately the buddy and the safety officer.
2. Leave the area, Doff PPE and perform hand hygiene.
3. Inform ICC and proceed to EHS for management.

HEALTH EMERGENCIES (UNCONSCIOUS):

1. Stop all work and notify immediately the buddy or safety officer.
2. Secure samples.
3. Remove outer gloves.
4. Grab a bottle of decontaminating agent (one per person)
5. Carry unconscious person outside of the laboratory area.
6. Call for emergency health services.
7. Check for breathing.
8. If the person is breathing, Disinfect and doff PPE’s then move to a clean area.
9. If the person is not breathing, perform cardiac massage according to HEMS guidelines without removing PPE’s.

REMINDERS:

1. Eye wash station should be available and readily accessible in the laboratory area.
2. First aid kits should be available and readily accessible near the laboratory area.
3. Fire extinguisher and fire blanket should be available and readily accessible near the laboratory area.

References

- Please refer to section 10

ATTACHMENT 4- SUPPORT LETTER AD

ATTACHMENT 4

Support Letter from Voluntary Association: Friends of Alzheimer ONLUS

Associazione di Volontariato Associazione Amici Alzheimer ONLUS

Sede Operativa: Via delle Baleari, 127 – 00121 Roma
C.F. 97642920587

By Claudia Martucci
President of the Association

Associazione di Volontariato
Associazione Amici Alzheimer
ONLUS
Sede Operativa: Via delle Baleari, 127 – 00121 Roma
C.F. 97642920587

November 25, 2019

Dear FDA-BQP qualification program,

On behalf of Associazione Amici Alzheimer Onlus, I would like to offer our firm support for IGEA Research Corporation's application for the qualification of Free Copper as a risk biomarker for Alzheimer's Disease.

The social, economic, and personal costs of Alzheimer's Disease globally are well documented, and there remain significant scientific challenges that must be met to develop effective treatment and prevention techniques.

We believe that Free Copper's connection to Alzheimer's Disease represents an exciting new avenue for early identification of persons at risk. FDA biomarker qualification would grant further recognition to the compelling literature about Free Copper as an Alzheimer's risk factor and give an advantage to future research that may impact as many as half of all Alzheimer's cases. IGEA Research, with scientific and technical experts standing at the forefront of innovation in this field, is well positioned to support this application and pursue further advances in the fight against Alzheimer's.

We appreciate your careful consideration of IGEA Research's promising application as another step toward worldwide relief from Alzheimer's Disease.

Sincerely

Claudia Martucci
President

Sito: www.associazioneamicialzheimer.it e-mail: associazioneamicialzheimer@gmail.com

ATTACHMENT 5- SELECT PUBLICATIONS

ATTACHMENT 5

Copies of Select Publications and Literature

Scientific Support for Use of Free Copper in Alzheimer's disease (AD)

IGEA Research Corporation
2600 SW 3rd Avenue 350,
Miami, FL 33129

Dr. Rosanna Squitti (Final Review and Approval Authority)