HEP-21-0718

# Supplemental section

## S.1 Hepatic endpoints

### S.1.1. Development of fibrosis. Elastography and biochemical methods

Elastography assesses the stiffness of the liver and is closely correlated to fibrosis on liver biopsy in other liver diseases (1) and to HVPG (2). The most widespread method, transient elastography, by use of Fibroscan®, was evaluated in four studies in WD (3-6). In the largest (3), elastography correlated with histological fibrosis at diagnosis and was able to detect both progression and regression of fibrosis grade, although histological validations were not reported. Transient elastography is a potential surrogate endpoint but prospective studies providing data on the fibrosis progression/regression rate in WD are needed.

More advanced elastographic methods include shear wave elastography, acoustic radiation force impulse imaging, and magnetic resonance elastography (MRE). MRE may be more precise than Fibroscan® for quantifying fibrosis(7).

A new technology, multiparametric MR liver tissue characterization (LiverMultiScan™), may also measure fibro-inflammation, liver fat content and iron overload (8, 9) and should be evaluated in long-term studies in WD patients.

Steatosis is a frequent finding in WD. While transient elastography provides a grading of steatosis (10), MRI can accurately measure the percent hepatic fat. Reversibility of steatosis could be a sign of treatment response, but data are lacking. These measurements should therefore be regarded as exploratory endpoints.

Noninvasive biochemical markers of fibrosis include AST/platelet ~~ratio~~ (APRI), FIB-4 index and Enhanced Liver Fibrosis index (3). In 44 WD patients with measurements and biopsy less than 1 year apart, the areas under the receiver operating curves were 0.96 (0.90-1.0) for elastography, 0.79(0.65-0.93) for APRI and 0.84(0.83-0.96) for FIB-4 (3).

## S.1.2 Markers of liver inflammation

**Aminotransferases.** ALT may be elevated due to hepatic steatosis, inflammation, necrosis and autophagy. In other liver diseases, such as autoimmune hepatitis, the normalization of ALT is well correlated to the clinical success of treatment and ALT can be considered a surrogate endpoint in clinical studies. In WD the relation between normalization of ALT and clinical improvement is less clear.

Many patients will present with elevated ALT. Most studies report ALT to decline towards normal values during treatment particularly within the first 2 years (11-18); but even after several years of treatment ALT remained elevated in a substantial fraction of patients (12, 13, 19).

The fact that ALT generally declines during treatment does not necessarily mean that a normalization of ALT is associated with a better clinical outcome in the individual patient. Attempts to establish such an association have been less conclusive.

Thus, validation against follow-up liver biopsies are inconclusive. In one study (15) with 12-23 years observation time, 7 patients with histological progression had higher ALT (mean±SEM ~~110±9~~ UI/L) than 33 non-progressors (~~55±7~~ IU/L) but the difference was not statistically significant. In another study including 12 patients and 2-12 years follow-up (17), there was also no statistically significant relation between histological progression of fibrosis and ALT.

In a few other studies, ALT was validated against non-histological measures of treatment success. Medici et al (14) followed 23 WD patients with hepatic presentation for 5 years; post-treatment ALT in non-responders was 74±24 (Mean±SD) IU/L and not statistically significantly higher than 43±27 IU/L in responders. In 20% of the patients ALT was elevated throughout the study with no signs of disease progression. Sini et al (15) reported similar observations after 12-23 years of follow up. (15). Arnon et al(16) followed 10 pediatric WD patients for 1-5 years and only 3 had normal ALT at last contact; the data suggested that non-adherence in 4 patients was associated with ALT elevations. Ranucci et al (20) observed a decline in ALT among 48 WD patients during 6 years of follow-up but ALT did not differ between treatment failures and non-failures. Iorio et al (13) reported ALT persistently above upper limit of normal in 25/109 pediatric WD patients with at least one year of follow-up and without worsening of the liver disease. A large retrospective study with long-term follow-up (21) reported that in WD patients on zinc mono-therapy ~~patients~~ ALT was statistically significantly higher in non-responders than responders at 12 month by not at 6 months or ≥24 months. Taken together, these studies did not show a clear relationship between ALT and clinical outcome of treatment.

Two studies including tetrathiomolybdate (TTM) and bis-choline-TTM further illustrates how difficult it may be to use ALT as an outcome measure. In a double-blind comparison of trientine and TTM (22) ALT elevations were more common in the TTM group than in the trientine group but the clinical outcome was better in the TTM group. Similar observations were reported in a single-arm study where bis-choline-TTM treatment of WD patients induced reversible ALT elevations in 39% of patients in a 28- week phase II trial, however overall mean ALT decreased during the study period (18).

Based on these data a recent review advised against the use of ALT as a measure of treatment success (23). AST is less well studied but may be of interest because it is more sensitive to mitochondrial stress.

**Other markers of inflammation**. In two cross sectional studies (24, 25) including 28 WD patients with acute liver failure and 176 with chronic disease the macrophage activation marker CD163 correlated to liver disease severity but longitudinal data are missing. Further studies to identify markers of hepatic inflammation in WD are encouraged.

### S.1.3. Other possible markers of liver disease severity.

Quantitative dynamic liver function tests include the clearance tests methacetin breath test (26) as standardized in the LiMAx test (27, 28) and the lidocaine clearance test (29) both of which depend on hepatic blood flow rate, and the galactose elimination maximal capacity test (30) which is a flow independent measure of hepatic metabolic capacity. Only the latter has been studied in a small group of WD patients (24) and longitudinal studies are missing.

## S.2. Neurological endpoints

### S.2.1 The Unified Wilson's Disease Rating Scale

The Unified Wilson's Disease Rating Scale (UWDRS) was developed as a tool for comprehensive evaluation of neurological signs and symptoms in patients with WD (31-33).

Part I of UWDRS assesses consciousness (0-4 points) while Part II (0-40 points) evaluates disability. Part III (0-142 points) involves a neurological examination and uses clinical rating scales to measure a wide spectrum of neurological items (speech, facial expression, tremor at rest, postural tremor, wing-beating tremor, rigidity, dystonia, posture, gait, chorea, pyramidal signs, and handwriting). In each part, a higher score indicates greater impairment. A fair correlation between

disability (UWDRS II) and neurological impairment ratings (UWDRS III) was observed in a cross-sectional study of 53 newly diagnosed WD patients (34). The inter-observer agreement is good (31, 32) with most items, so single observer assessment can be used in clinical trials. At the same time day-to-day variation that could ensue due to patient stress and fatigue is unknown and may be a significant source of variation.

The UWDRS was used to assess neurological worsening or improvement during treatment with standard therapy with some correlation between change in UWDRS and clinical outcome (35-37). The UWDRS was also used in some recent studies. Thus, in the phase II treatment trial with bis-choline-tetrathiomolybdate (18) improvement in mean UWDRS scores was observed within 28 weeks: From mean 6.6 to 4.1 in UWDRS II and from 22.8 to 16.6 in UWDRS III. In another study, after liver transplantation in highly selected WD patients with severe neurological symptoms, UWDRS (parts I-III) improved in 14 survivors from median 96 (range 75-112) to 38 (18-56) and >20% improvement was seen in 12 out of 14 patients (38).

### S.2.2. MRI

MRI demonstrates the structural cerebral changes in WD with some correlation to neurological scores in cross sectional studies (39-46). Abnormalities are seen even in WD patients without neurological symptoms (47, 48). Different rating scales have been reported (40, 42, 49-51) but with uncertain relation to clinical status. Recently, Dusek et al constructed a MRI rating scale to separately assess acute “toxic“ and potentially reversible disturbances and chronic changes in WD(45). The severity of MRI and UWDRS III scores correlated at baseline and at 24 months; however, a correlation between the changes in MRI and UWDRS scores was not found (personal communication from authors) possibly due to short observation time.

In a few small series with longitudinal observations, MRI abnormalities in WD partly reversed during treatment with trientine (52) or d-penicillamine (40, 45, 49, 53-56) or after transplantation (38, 57). In some (40, 49, 58) but not all (53, 59) studies, MRI changes correlated with clinical regression/progression at the individual level.

Until it is more clear how changes in MRI findings can be objectively correlated to clinical improvement or progression of WD, the use of MRI as an endpoint in clinical trials will be exploratory.

### S.2.3. Other possible neurological endpoints

**Evoked potentials.** A large fraction of WD patients have abnormal sensory evoked potentials including somato-sensory (SEPs), visually (VEPs) and auditory brain stem (BAEPs) (60-66). These

disturbances were related to the severity of neurological disease and findings on MRI (63, 67-69). Abnormalities were also reported in non-neurological WD patients and may reflect preclinical neurologic disease (70). In 6 WD patients on chelating treatment BAEPs and SEPs improved in parallel with symptomatic disease over a 36-month period (63). Further longitudinal studies are needed.

**Cognition.** Measurements of global cognition (71) are relevant to WD treatment trials because cognition relates to quality of life. However, changes during treatment are slow and sometimes minor (42, 72-76) so assessment of cognition will serve as exploratory endpoints in most treatment trials.

## S.3. Copper metabolism

### S.3.1. Measurements of the "free copper" in blood.

In the healthy person, total plasma copper is mainly irreversibly bound in ceruloplasmin. The rest is not really "free" but reversibly bound to albumin, aminoacids and peptides. This non-ceruloplasmin bound fraction is biologically active. In WD it is elevated and presumed to be responsible for organ toxicity.

***~~Nonceruloplasmin~~ bound copper (NCC).*** When NCC is estimated by subtracting ceruloplasmin-bound copper from the total serum copper concentration specific methodological issues must be taken into consideration (77-83). Since the immunologic assay does not discriminate between ceruloplasmin and the copper-free apoceruloplasmin, estimated NCC may be a negative value in up to 20%, a biologically impossible situation (50, 80, 81, 84, 85). The enzymatic method for ceruloplasmin determination (86) provides some improvement, but even then inaccuracies occur (84).

In clinical studies with longitudinal follow-up mean NCC values were above normal at diagnosis and declined with treatment with penicillamine or zinc towards normalization (11, 79, 87, 88) while this was less clear with trientine (79, 87). Reports of whether NCC normalization correlates with clinical outcome at the individual level are conflicting (11, 79, 87, 88) possibly because of the wide variation of results (79). Further longitudinal studies are needed to validate the correlation of NCC with other copper parameters and patient outcome for its use as a surrogate marker or endpoint.

Unique issues arise for NCC determination when copper is complexed in a non-bioavailable form in the circulation, and NCC must be corrected for this complexed copper. In the phase II trial of bis-choline tetrathiomolybdate for WD, NCC was corrected for copper bound in a molybdate-albumin-

Cu complex ($NCC_{corrected)}$ (18). Reduction of mean $NCC_{corrected}$ paralleled median UWDRS score improvements and stabilization of liver function.

***Exchangeable copper (CuEXC).*** Exchangeable copper (CuEXC) is a direct measure of serum non-ceruloplasmin bound copper (89-91) obtained by incubation of serum with EDTA following removal of ceruloplasmin-bound copper by ultrafiltration. CuEXC is the remaining copper stripped by EDTA from albumin, other peptides and amino acids. In two cross sectional studies, CuEXC was elevated and correlated with UWDRS score in newly diagnosed patients (50) and in treated compliant patients (92). In a rodent model of WD progression of hepatic pathology and liver function tests correlated with increasing CuEXC (93). Longitudinal data in patients are needed for further validation.

***Other direct copper assays.*** Solovyev and co-workers (94) developed a mass spectrometry method that separately measured the copper content of ceruloplasmin and total plasma. Their data suggest weaknesses with both the estimation of NCC and CuEXC methodology. Presently, their group and two others are performing clinical studies in WD to try to establish more reliable methods for measuring NCC. These assays will require validation in longitudinal studies prior to use as surrogate markers.

### S.3.2. Other potential Copper measures

**Kayser-Fleischer ring**. The KF ring is formed by copper disposition in the cornea and disappears with treatment. Its intensity can be quantified by anterior segment optical coherence tomography (95-99) and that may be a quantitative marker for the de-coppering of the body. Since Kayser-Fleischer rings are seen only in approximately 50% of patients with hepatic presentation (79, 100) and the rate of loss of rings in comparison to other disease-related symptoms is unknown, this surrogate marker of de-coppering cannot stand alone.

**Cerebrospinal fluid (CSF).** A few small longitudinal studies (101-104), each including from one to five patients, reported that CSF copper concentrations were elevated in neurologic WD patients and decreased in parallel with neurological improvement during decoppering therapy. CSF copper was normal in two WD patients with only hepatic symptoms(103). The CSF copper concentration appears to reflect copper accumulation in the brain and may be useful to monitor the efficacy of the decoppering during therapy. The invasive nature of the procedure is a concern in clinical studies.

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