Reference Limits for Copper and Iron in Liver Biopsies

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Abstract. Using 141 liver biopsy results (103 adults, 38 children) and a rank-order approach, the following reference limits were found: copper 55 µg/g dry weight, iron 1800 µg/g dry weight (adults only), and iron index 1.0. The study was made feasible by the fact that both copper and iron were measured as standard practice in every liver biopsy received for either test. The added analyte tended to contribute more to normal results. Specimens with elevations of both were infrequent (7 of 141) and significant elevations of both (copper >200 µg/g, iron index >2.0) were suggestive of contamination. Advantages of using patient data included studying specimens of limited availability and acquiring information on the distribution of elevated results seen in clinical practice. Disadvantages included increased uncertainty in the reference limits relative to a normal population. Although most of the study population consisted of patients referred for diagnosis of Wilson's disease or hemochromatosis, the reference intervals were similar to those reported from autopsy studies. (received 2 July 2003; accepted 12 July 2003)

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Introduction

Copper and iron are essential trace elements required for normal growth and development in children and homeostasis in adults. Although essential, excessive amounts are also toxic. The liver is an early target for the deposition of excess copper and iron, and liver biopsy can be useful for the evaluation of disorders of copper and iron metabolism. The purpose of the present study was to review the hepatic reference limits for copper and iron used at Children's and Women's Hospital by means of data collected from patient biopsies. Using patient results is an appealing way to examine a specimen type of limited availability, and for the laboratory to generate reference information specific to the population base. Although some trace element reference intervals were available in the literature, it was unclear if they were appropriate for children or the ethnically diverse population served by our laboratory. In addition, it was unclear how the reference limits from a patient-based study would resemble those developed from a population of relatively healthy individuals.

Although copper and iron are infrequently requested on the same biopsy, there are several reasons for the laboratory to consider measuring both together. As with many trace elements, there is a metabolic interplay between copper and iron which raises the question of how these reference limits might be related. Copper and iron share a common method of specimen preparation and analysis, and only a small additional effort is required to measure both. This practice was instituted by the clinical laboratory at Children’s and Women’s Hospital from the outset, with the objective of validating the reference ranges initially adopted from the literature. This also allowed us to gain experience in the analyses and their interpretations, which is particularly helpful for such tests that are requested relatively infrequently.
Excessive copper deposition is a defining feature of hepatolenticular degeneration, an autosomal recessive disorder of copper metabolism commonly known as Wilson’s disease. Normal copper metabolism is a balance between intake and output, with excess copper excreted in the bile. Transport from the hepatocyte into bile is mediated by a membrane-bound copper-binding ATPase [1]. Mutations affecting this protein are responsible for Wilson’s disease and result in copper deposition in sensitive tissues such as the liver, and in the basal ganglia and lenticular formation in the brain.

Although not always required, liver biopsy is the diagnostic standard for Wilson’s disease. Elevated copper levels can also occur in a variety of conditions characterized by chronic biliary obstruction [2-4]. Disorders such as primary biliary cirrhosis and biliary atresia can produce copper elevations in the range sometimes regarded as indicative of Wilson’s disease. Part of the purpose of the present study was to characterize how often elevated hepatic copper was seen from disorders other than Wilson’s disease.

Excessive tissue deposition of iron is characteristic of hereditary hemochromatosis, an autosomal recessive disorder of iron metabolism. Although other definitions may be employed, hemochromatosis is used here as recommended by Bassett [5] to refer specifically to inherited iron overload. In normal individuals, body stores of iron are regulated through absorption [6]; intestinal absorption is increased when iron stores are low and decreased when stores are adequate.

In hemochromatosis, mutations to genes such as HFE induce iron overload by interfering with the normal down-regulation of iron absorption. Aside from bleeding, no mechanism is present to excrete significant amounts of iron and any excess is therefore deposited in the tissues. Decades usually pass before sufficient iron accumulates to induce symptomatic disease. A complicating factor is that iron overload can also occur in a variety of other chronic liver disorders, although usually to a lesser degree. Liver biopsy can be a useful method to characterize iron overload [5], and part of the purpose of the present study was to examine the frequency of elevated results due to hemochromatosis and the secondary disorders of iron overload.

Methods and Materials

Analyses were performed in the Genes, Elements, and Metabolism Program, Department of Pathology and Laboratory Medicine, Children’s and Woman’s Health Centre of British Columbia (4480 Oak Street, Vancouver, BC V6H 3N1, Canada). The study population consisted of all liver biopsies received by the laboratory for patient evaluation of copper or iron from 1989 to 2001. During this period it was routine practice to perform both copper and iron analyses on all biopsies received for either test. This procedure complied with the ethical standards of the institution during the study period.

Only specimens collected from living patients and those with both copper and iron results were included in the study population. Autopsy specimens were excluded, as were 7 specimens of limited sample size that lacked copper results and 6 that lacked iron. One specimen was excluded because it was received in nitric acid. Only the initial biopsy results were included when repeat specimens were taken from the same patient, with one exception noted in the discussion.

The laboratory provided a written protocol for biopsy collection that included warnings about the danger of contamination with exogenous trace elements. The following instructions were included: use a “Kormed Jamshidi” or single-use stainless steel soft tissue biopsy needle; take tissue for histopathology first and for trace element analysis last; collect a 10 mm tissue plug from a 16-gauge/1.5 mm diameter needle for copper and iron analysis; dispense the biopsy plug directly into an acid-washed polypropylene screw-capped vial; decant saline or other liquid if used to expel the tissue plug; and transport the vial frozen or on ice.

Tissue was dried in a vacuum oven, weighed on an analytic balance, and digested in ultra-pure grade concentrated nitric acid in a 100°C oven for approximately 1 hr. Analyses were performed by graphite furnace atomic absorption spectroscopy (Varian SpectrAA-800). To insure consistent results, NIST Standard Reference Material 1577b (bovine liver) was included as part of every analysis and the analytic procedure was not altered during the study period. Final results were expressed as mg/g dry tissue. The
Iron index was calculated as recommended by Bassett [7] by dividing the hepatic iron concentration (µg/g) by the molecular weight of iron (55.8 g/mol) and by the patient’s age. Linear regression analyses of the first kind and the 95% confidence intervals (95% CI) were computed with Microsoft Excel software.

**Results**

A total of 141 liver biopsies comprised the study population. There were 103 specimens from adults (19-78 yr old). The majority of adults were male (69 male, 34 female) and most of the specimens were collected to evaluate iron overload (23 copper, 76 iron, 4 both). There were 38 biopsies from children (3-18 yr old). The sex distribution was about equal (18 male, 20 female), and most of the specimens were for evaluation of copper (20 copper, 13 iron, 5 both). Of the 141 total specimens, the laboratory added copper analysis to 89 and iron to 43.

**Copper.** The range of hepatic copper concentrations in the study population was 3-1600 µg/g (median 26). Copper results were rank-ordered by increasing concentration and then plotted against concentration (Fig. 1). The inflection point at 55 mg/g (rank order 111) was adopted as the reference limit. The adjacent points at rank-order 110 and 112 were 54 and 57 µg/g, respectively (and reflect the uncertainty in the reference limit). No differences were found in terms of age or sex (data not shown). Of 141 specimens, 111 were within the hepatic copper limit as defined above. The majority of these were collected for iron evaluation (28 copper, 79 iron, 4 both). Of the 89 specimens in which copper analysis was added by the laboratory, 79 were within the reference limit.

The study population contained 30 specimens with elevated copper concentrations, the majority of which were requested for copper evaluation (15 copper, 10 iron, 5 both). Of these 30, copper was moderately elevated (<200 µg/g) in 15 and significantly elevated (>200 µg/g) in the remaining 15. Of the significant elevations, 1 was probably elevated secondary to contamination (see discussion), 7 were from patients with chronic obstructive conditions, and 7 were from patients with Wilson’s disease. Patients with Wilson’s disease (5 children and 2 adults) showed a copper range of 246-1600 µg/g (median 865). Those with obstructive disorders (5 children and 2 adults) showed a range of 215-594 µg/g (median 318).
Considering only those specimens requested specifically for copper evaluation, 43 were for copper and 9 for both copper and iron. Of these, 26% (14 of 52) were significantly elevated (>200 µg/g), 12% (6 of 52) were moderately elevated (55-200 µg/g), and 62% (32 of 52) were within the normal limit (55 µg/g).

**Iron.** The range of hepatic iron in adults was 109-61,000 µg/g (median 1410). Employing the rank-order approach in Fig. 1 (data not shown), iron demonstrated an inflection point at 1760 µg/g (rank-order 62). Adjacent rank-orders 61 and 63 were 1720 and 1890 µg/g, which suggests the iron concentration should be limited to 2 significant figures. The range of iron in children (n = 38) was 65-29,500 µg/g (median 472). The associated rank-order graph for children showed a gap in the region of the inflection point, which fell between an iron of 740 and 1150 µg/g (rank-orders 24 and 25). The probable reference limit for children can only be estimated as falling between those concentrations. Selecting all normal adults (1800 µg/g) and children (740 µg/g), the patient’s age was plotted against the iron concentration. The resulting correlation coefficient R² was 0.32 and the regression equation was: y = 14x + 210. This indicates that among those with normal liver iron, the average concentration extrapolated to birth was 210 µg/g (95% CI 27-390), and that iron increased an average of 14 µg/g per year (95% CI 9-18).

**Iron index.** The hepatic iron index was calculated from units of µmol/g dry weight divided by the patients’ age [7,8]. The range of the index in the study population was 0.05-75.6 (median 0.58). Index results were rank-ordered and graphed (Fig 2). An inflection point occurred at an iron index of 1.02 (rank order 92). Adjacent rank-orders 91 and 93 were 0.97 and 1.05, respectively. Results from male patients (n = 87) were subjected to separate rank-order analysis (data not shown), and showed an inflection point at index 0.97 (rank-order 60). Data from females (n = 54) was insufficiently dense, with a gap near the inflection point between an index of 0.79 and 1.02 (rank orders 31 and 32).

Of 141 specimens, 91 showed a normal iron index 1.0 (defined as the normal limit by Basset [7]). Of these, 40 were requested for copper, 44 for iron, and 7 for both. Of the 43 specimens in which iron analysis was added by the laboratory, 40 were within the reference limit. To test how well the iron index calculation adjusted for age, the normal index results were plotted against age. The resulting correlation coefficient R² was 0.0040 and the regression equation was: y = -0.0007x + 0.43. This indicates that among patients with a normal hepatic iron index, the average was 0.43 (95% CI 0.32-0.54) and the association with age was essentially zero (95% CI -0.003 to 0.002).

**Elevated iron index.** Of 50 elevated results >1.0, there were 16 specimens from patients with transfusion-dependent conditions such as thalassemias and dyserythopoietic anemia. Of these, 15 were requested for iron and 1 for both copper and iron. This group consisted of 11 males and 5 females with a median age of 14 yr. Also included were 3 adult males, age 19, 25, and 64 yr. The range of the iron index in transfusion-dependent conditions was 4.07-75.6 (median 19.8).

Excluding the transfusion-dependent patients described in the preceding paragraph, there were 24 specimens with an iron index significantly elevated >2.0, and 10 specimens with an index moderately elevated between 1.0-2.0. In the significantly elevated group, all specimens were requested for iron evaluation and all were from adults (13 males, 11 females) with a range of 35-78 yr old (median 58). The median iron index was 4.09 (range 2.05-20.2). The 10 specimens in the moderately elevated group included 3 requests for copper, 6 for iron, and 1 for both. The 4 requests for copper (including 1 for both) were in children (including 2 with Wilson’s disease), whereas the 6 requests for iron were in adults. The index range was 1.02-1.88 with a median of 1.31 (no cases occurred between 1.9-2.0).

Considering only adults, there were 77 patients who underwent evaluation specifically for hemochromatosis (76 requests for iron, 4 for both iron and copper, excluding 3 adults with transfusion-dependent conditions). Of these, 31% (24 of 77) showed a significantly elevated index >2.0, 7.8% (6
of 77) showed a moderately elevated index between 1.0-2.0, and 61% (47 of 77) were 1.0.

**Both elevated copper and iron index.** Of 141 biopsies, 7 were found with both an elevated copper concentration (>50 µg/g) and an elevated iron index (>1.0). Of these, 4 specimens showed a moderately elevated copper (<200 µg/g), and 2 showed a moderately elevated iron index (<2.0). These last 2 were from patients with Wilson's disease – an 11 yr old female (copper 1170 µg/g, index 1.06), and a 13 yr old female (copper 246 µg/g, index 1.58). Only 1 specimen showed significant elevations of both copper and the iron index (see discussion).

**Discussion**

Using a rank-order approach with patient results gave reference limits of 55 µg/g dry weight for hepatic copper, 1800 µg/g for iron (adults only), and 1.0 for the iron index. Due to the limited number of specimens from children, the reference limit for iron in terms of µg/g could only be determined for adults. In contrast, the iron index can be applied to children because the calculation removes the influence of age. Sufficient data for this study were collected through the routine practice of analyzing both copper and iron on all biopsies requested for either. The additional test, whether copper or iron, contributed more often to normal results than to elevated ones.

The reference limits defined here are similar to those described in a study based on 30 adult autopsy specimens, namely, a copper reference interval of 0-55 µg/g dry weight and iron of 170-2007 µg/g [9]. That study group consisted of apparently healthy individuals coming to autopsy as the result of trauma and similar conditions. This is in contrast to the present study group which comprised patients referred for an invasive procedure, usually on suspicion of Wilson's disease or hemochromatosis. In spite of the population differences, the reference limits were identical for copper and within 11% for iron. The present study also found the copper reference limit applicable to children 3-yr-old, although a significantly higher limit is known to be needed for infants [2].

The reference limit for the iron index of 1.0 found here is also consistent with that generally accepted [7,8]. Published reference limits for iron, however, tend to be more inconsistent, with the upper limit often varying between 900 µg/g [10] and 2,010 µg/g [11]. A factor in this variation is the well-known dependence of iron concentration on age, meaning an older population would tend to have higher normal limits. One way to compensate for this effect would be to divide iron reference intervals into age-specific ranges such as 20-30 yr old, 30-40, and so on. A more convenient method, however, is to use the iron index.

One disadvantage to the rank-order approach used here is that it is based on the visual selection of an inflection point, a process that has a degree of subjectivity. In Fig. 1, for example, there are actually 3 inflection points. The upper limit of normal was selected as the start of the steepest slope, the reasoning being that this corresponded to the point where the degree of copper elevation became appreciably uncommon. When the inflection point in Fig. 1 is examined closely, it can be seen as belonging to a curved region consisting of several points. The choice of the exact point is somewhat arbitrary, and the difference between adjacent points gives an indication of the uncertainty in the choice. In Fig. 2, for example, the inflection point for the iron index is 1.02, and adjacent points are 0.97 and 1.05. This does not translate readily into a standard confidence interval, although the inflection point is consistent with the generally accepted reference limit of 1.0 for the iron index [7].

**Elevated copper.** Of 52 specimens evaluated for hepatic copper, 14 were significantly elevated (>200 µg/g), 6 were moderately elevated (55-200 µg/g), and 32 were within the reference limit (55 µg/g). Published reports suggest that hepatic copper is almost always >200-250 µg/g in Wilson's disease [10,12], even in presymptomatic individuals [1]. This is consistent with the present study, where all 7 patients with Wilson's disease were found to have copper 246 µg/g (median 865). Also in the present series, other disorders contributed to a significantly elevated hepatic copper as frequently as Wilson's disease. Significant elevations can be found in those...
disorders associated with chronic cholestatic conditions, including primary biliary cirrhosis [4], biliary atresia, sarcoidosis [13], and chronic active hepatitis [14]. Distinguishing among these conditions depends on characteristic histologic features [3], clinical history and similar considerations, beyond the scope of this discussion.

In addition to Wilson’s disease, other disorders of copper metabolism are also of interest in the context of hepatic biopsies, although these conditions are rare and not seen in this series of patients. Extreme elevations can be seen in copper-associated childhood cirrhosis [15]. This disorder was originally described in infants from rural India (Indian childhood cirrhosis), and similar conditions have been reported in other countries and ethnic groups, for example, idiopathic copper toxicosis in the United States [4] and endemic Tyrolean infantile cirrhosis in Austria [16]. In Menkes’ kinky hair syndrome, infants exhibit defective absorption, and hepatic copper is low [15]. In contrast, increased hepatic copper is present in normal infants during the first months of life [2].

Iron overload. Significant iron overload is characteristic of the primary genetic disorder hereditary hemochromatosis, whereas moderate elevations are more often seen in secondary conditions associated with many chronic liver disorders [17]. Although other definitions can be employed, hemochromatosis is used here as recommended by Bassett [5] to mean inherited iron overload. The diagnostic dividing line for hemochromatosis has been set at an hepatic iron index >2.0 [7,8] or >1.9 [17,18], depending on the study. Of the 77 specimens in the present series collected from adults to evaluate hemochromatosis, 24 showed an iron index >2.0 consistent with hemochromatosis, 6 specimens showed a moderately elevated index between 1.0-2.0 suggestive of secondary iron overload, and 47 were within the reference limit 1.0. While the iron index can be valuable tool for the diagnosis of hemochromatosis, it is not definitive and is most useful in combination with clinical assessment and genetic studies [17].

Symptomatic hemochromatosis typically develops between 40 and 60 yr of age [6]. Overt disease is uncommon in individuals younger than 30 yr old, and juvenile and neonatal forms are rare. Moderate iron excess can be seen in many chronic liver disorders [17,19] including hepatitis B and C, porphyria cutanea tarda, alcohol-related liver disease, nonalcoholic steatohepatitis, portacaval shunting, and liver cancer. Cirrhosis alone may cause iron accumulation. Some authors maintain that iron overload in these conditions is often unrelated to hereditary hemochromatosis, while others emphasize a subtle relationship [19]. The idea that alcoholism is associated with moderate iron overload is probably inaccurate.

Iron overload can also occur in disorders with defects in hemoglobin synthesis or chronic ineffective erythropoiesis, such as thalassemia and sideroblastic anemia [6]. These patients are often treated with repeated blood transfusions, which can produce massive iron overload. In the present series, patients with transfusion-dependent conditions showed a median iron index of 19.8 (range 4.07-75.6), in contrast to 4.09 (range 2.05-20.2) for those suggestive of hemochromatosis.

Preanalytic contamination and related problems. Trace element analysis has inherently high potential for contamination and false-positive results [20]. Two probable cases of contamination were identified in the study population and are discussed below. False-negative results are also possible, in part because trace elements may not be evenly distributed throughout the liver. An autopsy study found significant differences in iron content at different biopsy sites and these differences became more marked as iron concentrations increased [21]. Uneven distribution of hepatic copper has been suggested as a cause for misdiagnosis of Wilson’s disease [22]. In contrast, copper and iron appear to be homogeneously distributed when concentrations are normal [9].

The first case of contamination involved a specimen collected from a 69 yr old male to evaluate elevated ferritin and liver enzymes. The hepatic iron index of 7.71 was consistent with hemochromatosis (iron 29,700 µg/g, copper 31 µg/g). Histologic examination, however, showed normal liver architecture with no stainable iron. (In contrast, copper staining does not always detect elevated copper
deposits in Wilson's disease [2,3].) A second biopsy was collected and showed markedly different iron index of 0.15 (iron 569 µg/g, copper 17 µg/g). The first biopsy was attributed to contamination with exogenous iron, most likely caused by improper specimen collection. Only the repeat biopsy was included in the study population.

The second case involved probable contamination of a specimen collected to evaluate the degree of iron overload in a 16 yr old male with transfusion-dependent β-thalassemia. The iron index was 6.24 (iron 5570 µg/g) and consistent with the expected result, however, copper had been added by the laboratory and was markedly elevated at 740 µg/g. The present series included 14 other cases of transfusion-dependent iron overload, all of which showed normal liver copper (range 3-43 µg/g, median 22). Since the copper result appeared to be unrelated to the disease process, the most probable cause of the elevated copper concentration was contamination.

This second case also highlights another problem: what happens when an unrequested analyte generates an abnormal result? Whenever the laboratory adds unrequested analytes, such situations are inevitable and have the potential to generate considerable complications. When the purpose is well-defined, the authors believe that the information gained outweighs the problems created. One way to gain institutional support for such studies is to stress the need for precise, up-to-date reference intervals for accurate patient evaluation.

The problem presented by the second case can also be seen from another perspective – the identification of the characteristics of contamination. Significant elevations of both copper and iron were found only in this specimen (ie, 1 of 141 biopsies). This is not to say that this pattern is clearly due to contamination, but rather that contamination should be considered as one likely cause. It is interesting to speculate that superior markers of contamination could be found in other trace elements, for example, zirconium, titanium, and lead. These elements have been suggested as markers of soil ingestion in children [23], which can be considered contamination in a different context. With the development of the ability to measure several trace elements simultaneously, examining the pattern of elevated elements could prove to be a useful tool to detect contamination.

In conclusion, patient biopsy results and a rank-order approach were employed to determine reference limits for hepatic copper and iron. Advantages to rank-order analysis include the ability to establish reference limits on a specimen type of limited availability, and to examine the range and frequency of abnormal results. Disadvantages include an increased level of uncertainty relative to reference studies based on normal populations. The present study required the laboratory routinely to add analytes, either copper or iron, to every biopsy requested for one or the other. This occasionally produced an elevated result in an unrequested analyte, but the associated problems were outweighed by the value of the information gained. The added analyte, whether copper or iron, tended to contribute more to normal results than to elevated ones. Significantly elevated copper levels (>200 µg/g) and the iron index (>2.0) were simultaneously elevated in only 1 of 141 biopsies, a pattern most likely due to contamination.

References