Blood First Edition Paper, prepublished online August 29, 2002; DOI 10.1182/blood-2002-06-1723

Noninvasive Measurement of Iron:

Report of an NIDDK Workshop

Short Title for Running Head: Noninvasive Measurement of Iron

by

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This workshop was supported by the National Institute of Diabetes & Digestive & Kidney Diseases, and by the Office of Rare Diseases, Office of the Director, National Institutes of Health

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Word Counts: Abstract: 228
Text: 3609
Scientific heading: Red Cells

Abstract

An international workshop on the non-invasive measurement of iron was organized by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) on April 17, 2001, to assess the current state of the science and to identify areas needing further investigation. The workshop concluded that a clear clinical need was evident for quantitative means of measuring body storage iron that are non-invasive, safe, accurate and readily available to improve the diagnosis and management of patients with iron overload, including hereditary hemochromatosis, thalassemia major, sickle cell disease, aplastic anemia, myelodysplasia and other disorders. Magnetic resonance imaging potentially provides the best available technique for examining the three-dimensional distribution of excess iron in the body but further research is needed to develop means of making measurements quantitative. Currently, biomagnetic susceptometry provides the only non-invasive method for measurement of tissue iron stores that has been calibrated, validated and used in clinical studies but the complexity, cost and technical demands of the liquid-helium-cooled superconducting instruments required at present have restricted clinical access to the method. The workshop identified basic and clinical research opportunities for deepening our understanding of the physical properties of iron and iron toxicity, for further investigation of magnetic resonance imaging as a method for quantitative determinations of tissue iron, especially in liver, heart and brain, and for development of improved methods and more widely available instrumentation for biomagnetic susceptometry.

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Introduction

The body iron burden is a principal determinant of clinical outcome in all forms of systemic iron overload, whether from transfusion (for thalassemia major, sickle cell disease, aplastic, myelodysplastic, or other refractory anemias), from increased dietary iron absorption (hereditary hemochromatosis and other forms of primary iron overload), or both (refractory anemia with increased ineffective erythropoiesis). Accurate assessment of the body iron is essential for managing iron-chelating therapy in transfused patients to prevent iron toxicity while avoiding the adverse effects of excess chelator administration. In hereditary hemochromatosis, determination of the magnitude of body iron stores permits identification of individuals who would benefit from phlebotomy therapy from among those at genetic risk for the disease .

On April 17, 2001, an international workshop on the non-invasive measurement of iron was organized by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) to assess the current state of the science and to identify areas needing further investigation. The invited participants included representatives from centers worldwide that are active in the development of non-invasive measures of iron, clinicians who care for patients with iron disorders, and experts in the physics and chemistry of iron and in iron metabolism. The proceedings of the workshop are summarized below; more detailed information is available at the NIDDK website:

http://www.niddk.nih.gov/fund/other/iron/index.html

Clinical Needs for Measurement of Iron: Iron is an essential nutrient required by every human cell. Under physiologic conditions, the concentration of iron in the human body is carefully regulated and normally maintained at about 40 mg Fe/kg body weight in women and about 50 mg Fe/kg in men, distributed between functional, transport and storage compartments.

Iron deficiency designates conditions in which the body iron is decreased and arises from a sustained increase in iron requirements (usually due to blood loss, pregnancy or growth) over iron supply[1]. Most cases of iron deficiency can now be detected by measurements of serum ferritin, serum transferrin receptor concentration, or both, determined in a sample of peripheral blood[2]). ***Iron overload*** arises from a sustained increase in iron supply over iron requirements and develops with conditions in which the regulation of intestinal iron absorption is altered (hereditary hemochromatosis, refractory anemia with ineffective erythropoiesis), bypassed (transfusional iron overload), or both[1]. Iron overload results primarily in an increase in storage iron held in ferritin and hemosiderin[3]; functional iron is little affected. Whether derived from increased absorption of dietary iron or from transfused red blood cells, progressive iron accumulation eventually overwhelms the body's capacity for safe sequestration of the excess. Symptomatic patients may present with any of the characteristic manifestations of systemic iron overload: liver disease with the eventual development of cirrhosis and, often, hepatocellular carcinoma, diabetes mellitus, gonadal insufficiency and other endocrine disorders, arthropathy and increased skin pigmentation; iron-induced cardiomyopathy may be lethal. The prognosis in patients with iron overload is influenced by many factors, including the age at which iron loading begins, the rate and route of iron loading, the distribution of iron deposition between macrophage and parenchymal sites[4], the amount and duration of exposure to circulating non-transferrin-bound iron[5]; ascorbate status; and co-existing disorders, especially alcoholism and viral hepatitis. While ferritin and hemosiderin iron are almost surely not the species directly responsible for the adverse effects of iron, the overall magnitude of storage iron accumulation seems to be a principal determinant of clinical outcome in all forms of systemic iron overload.

The reference method for evaluating the extent of body iron excess in systemic iron overload is measurement of the hepatic storage iron concentration[6], recognizing that the exact relationship

between hepatic iron and the total body iron burden depends on the underlying disorder. The liver is the only storage compartment whose iron content is consistently increased with increased body iron stores; excess storage iron is detectable in both reticuloendothelial (in Kupffer cells) and parenchymal (in hepatocytes) sites. Total body iron stores can be measured by quantitative phlebotomy[7] but this approach cannot be used in transfusion-dependent patients with iron overload and is generally acceptable only if the procedure provides therapeutic benefit. The measurement of *plasma ferritin* provides an indirect estimate of body iron stores, but the usefulness of this measure is limited by the many common clinical conditions in which the plasma ferritin is not a reliable indicator of body iron[4]. Inflammation, infection, liver disease, hemolysis, ineffective erythropoiesis and ascorbate deficiency – common complications of hereditary hemochromatosis, transfusional iron overload, or both – all perturb serum ferritin levels independently of changes in total body iron.

While liver biopsy with chemical analysis of tissue iron content provides the most quantitative direct measure of iron status generally available, the discomfort and risk of the procedure limits its acceptability to patients and precludes its frequent use in serial observations. The workshop concluded that physicians have a pressing clinical need for quantitative means of measuring body storage iron that are non-invasive, safe, accurate and readily available to improve the diagnosis and management of patients with iron overload, including hereditary hemochromatosis, thalassemia major, sickle cell disease[4,8], aplastic anemia, myelodysplasia and other disorders. Priorities in clinical research in iron overload were identified as:

-- developing, calibrating and validating new methods or modifications of current methods for quantitative measurement of tissue storage iron that are non-invasive, safe, accurate and readily available for clinical use;

-- examining the relationship between total iron burden and manifestations of iron toxicity

in different forms of iron overload, e.g. hereditary hemochromatosis and transfusional iron overload in thalassemia major, sickle cell disease, aplastic and myelodysplastic anemias;

-- assessing the value of non-invasive measurements of hepatic iron in guiding (i) iron-chelating therapy in transfusional iron overload in patients with thalassemia major, sickle cell disease, aplastic and myelodysplastic anemias and (ii) in identifying patients who would benefit from phlebotomy therapy among those found to be at genetic risk for hereditary hemochromatosis; and

-- determining the relationships between the iron concentration in specific tissues and damage to the heart, liver, pancreas and other endocrine organs, and joints in different forms of iron overload, e.g., in hereditary hemochromatosis and in transfusional iron overload with thalassemia major, sickle cell disease, aplastic and myelodysplastic anemias.

Physical Properties of Iron: Iron in biological systems is found with a variety of oxidation states, reduction potentials, magnetic properties, degrees of aggregation, solubilities, mobilities, and kinetic and thermodynamic proclivities towards free radical generation[3,9]. Each of these variables influences not only the biological activities and toxicities but also the detection and quantification of iron. Reduction-oxidation reactions are involved in the cellular uptake, transmembrane transport and incorporation of iron into essential heme and non-heme enzymes for utilization and into ferritin for storage[10]. Iron is likely to be found in the ferric oxidation state when tightly locked in place for purposes of transport or storage and in the ferrous oxidation state when a change of chemical environment is necessary. Iron can also be a detrimental catalyst in biological free radical oxidations although the exact nature of the reactions involved remain uncertain. Iron-catalyzed free radical production may take place through the Fenton and Haber-

Weiss reactions, producing hydroxyl radical[3], or, perhaps, by reactions of ferrous iron with dioxygen, producing ferryl, perferryl radicals or other reactive oxygen species[11,12]. The recent extraordinary advances in our understanding of the cellular and molecular bases of iron uptake, transport and storage have revealed new intricacies in the complexities of iron metabolism. Nonetheless, the specific toxic forms of iron have not been identified with certainty and methods of measurement of the harmful species are lacking. Priorities in basic research in the physical properties of iron were identified as[3]:

-- characterizing the chemical nature of cytoplasmic iron in transit among different sites and routes of uptake, storage, utilization and release by cells, including the redox reactions of iron storage and intracellular transport, the structure of iron in ferritin and hemosiderin and the mechanisms of iron mobilization from these storage sites;

-- determining the forms and behavior of iron exported by cells for binding to circulating transferrin or appearing as non-transferrin-bound plasma iron; and

-- understanding mitochondrial iron metabolism and homeostasis and their disorders.

Progress in these areas could lead to the identification of the precise species of iron that are responsible for toxicity and eventually to means for their measurement and management.

Detection of Iron Overload by Magnetic Resonance Imagining (MRI): MRI uses the magnetic properties of the body to provide detailed three-dimensional images of any structure or tissue. Magnetic resonance (MR) measures the contribution of hydrogen nuclei (protons) to the net magnetization of the body within defined spatial regions. Hydrogen nuclei are a principal constituent of body tissues, mostly in water molecules and lipids. A spinning, charged hydrogen nucleus produces a dipole moment (magnetic field) that can interact with an external magnetic field. Magnetic resonance imaging (MRI) instruments generate a strong and homogeneous magnetic field by using a large magnet made by passing an electric field through superconducting

coils of wire. Patients are placed in a horizontal cylinder and exposed to the magnetic field. At equilibrium, hydrogen nuclei in the body, which normally have randomly oriented spins, will align in a direction parallel to the magnetic field. The MRI machine then applies short electromagnetic pulses through a coil at a specific radio frequency (RF). The hydrogen nuclei absorb the RF energy and precess away from equilibrium (i.e., alter the orientation of their spins). When the RF pulse is turned off, the precessing nuclei release the absorbed energy and return to equilibrium. An external RF coil detects the electromagnetic signals that are emitted as the nuclei return to equilibrium. The strength of the signals varies depending on the applied RF magnetic fields. A sample returns to equilibrium in the longitudinal plane over a characteristic interval called the T1 relaxation time (the time constant for excited nuclei to dissipate excess energy to the environment). In the transverse plane, the return to equilibrium occurs over a characteristic interval called the T2 relaxation time (the time constant for excited nuclei to go out of phase with each other). Both T1 and T2 depend on the local environment of the hydrogen nuclei. These values may also be expressed as relaxation rates, R1 (=1/T1) or R2 (=1/T2). Rapid progress is being made in the development of improved MRI instrumentation, especially in the development of machines with higher magnetic field strength[13].

With MRI, tissue iron is detected *indirectly* by the effects on relaxation times of ferritin and hemosiderin iron interacting with nearby hydrogen nuclei. Paramagnetic ferritin and hemosiderin iron shorten proton relaxation times, particularly T2, an effect termed "susceptibility-induced relaxation."[14], but a detailed theoretical understanding of these effects is lacking[15]. The interactions are complex, involving factors such as tissue hydration, the water diffusion coefficient within the tissue, the distribution of iron and water within the tissue examined, the number of iron atoms per molecule of ferritin and hemosiderin (called the loading factor) and, because ferritin iron and hemosiderin iron have different effects on both T1 and T2[16],

the relative proportion of these two iron storage materials. The loading factors within a tissue are not uniform, differing with different types and amounts of iron excess[17]. Studies of ferritin in solution *in vitro* show a clear effect of loading factor on relaxation times[18] and, in tissue, clustering of ferritin within cells further complicates analysis[19]. Moreover, conventional MRI measurements are also affected by the instrument used, the applied field strength, the repetition time used in the imaging sequence, the method used to analyze the relaxation curves and other technical aspects of the measurement procedure. Comparison of absolute signal intensities from one MRI unit to another are unreliable because of substantial intermachine variation[20]. In the absence of a theoretical understanding of the effects of iron on MRI, empirical efforts to estimate hepatic iron concentrations have used a variety of instruments, magnetic field strengths, imaging sequences (spin-echo, gradient recalled-echo), and parameters (T1 and T2 relaxation times, and signal intensity ratios as measured in proton, T1-, T2- or T2*-weighted images) but no standard or generally accepted method has been adopted for clinical application. To date, MRI has been more useful as a screening technique for the detection of marked iron overload[20] than as a means for quantitative measurement. In particular, with increasing iron concentrations, the signal intensity of the liver is reduced to such an extent that discrimination between different concentrations becomes impossible[21], at least with current technology.

New methods for using MRI to estimate tissue iron are under development, based on the inverse relationship between the susceptibility-induced relaxation, measured as R2, and iron concentration. One approach, applied in studies of brain iron, involves measuring the tissue relaxation rate (R2) in subjects in both high- and low-field MRI instruments and then calculating the field-dependent R2 increase (FDRI), the difference between the R2 measured with the two MRI instruments. In tissue, only ferritin iron is known to increase R2 in a field-dependent manner, suggesting that the FDRI measure may provide a specific measure of this tissue iron

pool[22,23]. Another approach also involves measurement of the R2, but uses a spin-echo imaging methodology to produce a series of images at different echo times. Using these images, a "map" of the entire liver is generated in which contrast is predominantly dependent on the R2 of the liver at different echo times [24]. This approach has the advantage that the results are independent of the MRI instrument used. Efforts to validate these approaches against chemical measurements of tissue iron are in progress.

At present, MRI provides a means of probing the three-dimensional distribution of excess iron in the body, but further efforts are needed to make measurements quantitative. Priorities in basic and clinical research in the application of MRI to the measurement of tissue iron were identified as:

-- acquiring an improved understanding of the contribution of ferritin and hemosiderin iron to magnetic resonance effects to guide development of optimal methods for measuring relaxation times and susceptibility, including the best techniques for data acquisition, choice of field strength, selection of timing parameters, reduction of noise, identification of region of interest and selection of analytic methods;

-- devising phantoms and other means for calibrating and validating iron concentration detected by magnetic resonance imaging that could enhance standardization between different laboratories; and

-- developing new methods for non-invasive measurements of iron deposition in the heart, in endocrine tissue, and in specific areas of the brain to determine the role of abnormalities of brain iron in the pathogenesis of neurodegeneratve disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, prion diseases, mitochondrial disorders and Parkinson's disease.

Detection of Iron Overload by Magnetic Susceptometry: The "magnetic dipole moment" is the central physical concept underlying both MRI and magnetic susceptometry. Elementary particle magnetic moments are determined by quantum mechanical considerations, so do not vary with time, magnetic field, or any other variable. In an applied magnetic field, all magnetic dipole moments experience a torque tending to align their axes. The disruptive effect of thermal fluctuations prevents all but a minute fraction of the available elementary particle moments in the human body from aligning in this way. Nonetheless, this minute field-induced fraction provides the diagnostic signals utilized by both MRI and magnetic susceptometry. The *magnetic susceptibility* of a tissue is determined by the strength of the magnetic response evoked in the tissue by an applied magnetic field[6,25]. This property is much simpler than the resonance behavior that results from the application of the oscillating magnetic fields used in MRI. While in a steady applied magnetic field, all materials respond with an induced magnetic field of their own. This response may be exploited diagnostically because the magnitude of the induced magnetic field varies greatly in different materials. In most human biological materials this induced field is diametrically opposed to the applied field. This *diamagnetic* response is so weak (about 10^{-6} of the applied field) that its detection requires sensitive instrumentation, described below. By contrast, *ferromagnetic* materials (such as the common bar magnet) respond with an induced field as strong or even stronger than the applied field and in the same direction. No known human tissues are ferromagnetic. Intermediate between the dia- and ferro- magnetic extremes is the *paramagnetic* response of the iron in ferritin and hemosiderin that is in the same direction as the applied field with a strength about 10^{-4} of the applied field. **This paramagnetic response is directly proportional to the number of iron atoms present in iron storage compounds.** In a measurement of hepatic magnetic susceptibility *in vivo*, the opposing diamagnetic (tissue) and augmenting paramagnetic (ferritin and hemosiderin iron) responses are superimposed. By

taking into account the small and nearly constant diamagnetic effect of the liver tissue, the observed resultant magnetic susceptibility may be used, in effect, to count the number of storage iron atoms present. The contributions of other paramagnetic materials (oxygen, deoxyhemoglobin, some trace metals) to the hepatic magnetic susceptibility are so small that magnetic measurements are highly specific for ferritin and hemosiderin iron. Thus, determinations of the magnetic susceptibility of the liver provide a *direct* measure of hepatic iron.

To date, measurements of hepatic magnetic susceptibility have required the use of superconducting magnetometers to measure the small change in magnetic field produced by the presence of storage iron in the liver (about one part in a billion of the applied field)[25]. Superconducting materials lose all electrical resistance below a certain temperature, called the transition temperature, or T_C. Until 1986, all known superconducting materials had transition temperatures near absolute zero and required a bath of liquid helium (4.2°K) to keep their temperature below T_C. The recently developed "high-T_C" materials have T_C values well above that of liquid nitrogen (77°K). The *s*uperconducting *qu*antum *i*nterference *d*evice (SQUID) is a superconducting loop incorporating a "weak link" known as a Josephson junction that can function as the most sensitive and stable magnetic detector known. Since the development of the first SQUID susceptometer[26] with the support of the NIDDK, the design of this instrument has been the basis for all commercially available instruments[27]. In effect, the susceptometer provides an automated magnetic "biopsy" of liver ferritin and hemosiderin iron. In patients with iron overload, the results of susceptometric measurements of hepatic non-heme iron are quantitatively equivalent to those obtained by chemical analysis of tissue obtained by biopsy[6,26]. Despite providing non-invasive, quantitative measurements of hepatic iron stores, clinical adoption of susceptometry has been limited, in part because of the cost and complexity of the instruments.

At present, the only susceptometers in clinical use are at Columbia University[6], the University of Hamburg, Germany[25], and the University of Turin, Italy; a fourth instrument is being installed at the Children's Hospital Oakland Research Institute, California. These instruments have now been used in studies of thousands of patients with iron overload[6,26,28-32].

At present, biomagnetic susceptometry provides the only non-invasive method for measurement of tissue iron stores that has been calibrated, validated and used in clinical studies but the complexity, cost and technical demands of the liquid-helium-cooled superconducting instruments required at present have restricted clinical access to the method. New approaches to measurement of magnetic susceptibility are under investigation. One method uses MR to measure the magnetic susceptibility difference between two homogeneous macroscopic compartments that are in contact by exploiting a resonant frequency discontinuity between the two materials. One material with a known susceptibility (e.g., blood) may then be considered as a reference to obtain the susceptibility of the second material (e.g., liver or heart)[33]. The use of a room-temperature device for susceptibility measurements has been proposed and is being studied[34]. Still another approach is to retain the advantages of superconductivity but to use the new high-high-T_C SQUIDs and materials to develop a device that could operate using liquid nitrogen as the refrigerant[35]. Priorities in basic and clinical research in the further development of biomagnetic susceptometry for the measurement of tissue iron were identified as:

-- developing innovative instrumentation for biomagnetic susceptometry that is suitable for routine clinical use in studies of iron overload, such as liquid-nitrogen cooled superconducting instruments, devices using non-superconducting magnetometers, or means of adapting magnetic resonance imaging instruments for measurement of magnetic susceptibility;

-- improving biomagnetic susceptometry by the development of methods to more precisely

determine the susceptibility of tissue overlying the organ of interest, as for example, the bone, muscle and subcutaneous tissue overlying the liver.

-- Additional research is needed to develop biomagnetic susceptometry as a means of measuring tissue iron concentrations in the heart, endocrine tissue, brain and other organs, by the use of instrumentation with arrays of magnetic sensors or by examining the potential use of magnetic susceptibility tomography[25].

Summary and Recommendations: Physicians have a pressing clinical need for quantitative means of measuring body storage iron that are non-invasive, safe, accurate and readily available to improve the diagnosis and management of patients with iron overload, including hereditary hemochromatosis, thalassemia major, sickle cell disease, aplastic anemia, myelodysplasia and other disorders. Magnetic resonance imaging provides a means of probing the three-dimensional distribution of excess iron in the body but further research is needed to develop means of making measurements quantitative. At present, biomagnetic susceptometry provides the only non-invasive method for measurement of tissue iron stores that has been calibrated, validated and used in clinical studies but the complexity, cost and technical demands of the liquid-helium-cooled superconducting instruments now required have restricted clinical access to the method. The workshop identified basic and clinical research opportunities for deepening our understanding of iron toxicity, for further development of magnetic resonance imaging as a method for quantitative determinations of tissue iron, especially in liver and brain, and for new approaches to methods for biomagnetic susceptometery.

Note: Since the workshop was held, additional publications on both theoretical[36,37] and practical[38-41] efforts to develop non-invasive methods for measurement of tissue iron have appeared and are cited here for ready reference.

Invited Workshop Participants:

Introductory Remarks: David Badman, DKUHD, NIDDK, National Institutes of Health, Bethesda, MD; Allen Spiegel, Director, NIDDK, National Institutes of Health, Bethesda, MD; Frank Somma, National President, Cooley's Anemia Foundation, Inc.

Session I. Clinical Needs for Measurement of Iron: Gary Brittenham, Columbia University, New York, NY (Chair); Alan R. Cohen, Children's Hospital of Philadelphia, Philadelphia, PA; Roland Fischer, Universitatskrankenhaus Eppendorf, Hamburg, Germany; Harriet C. Isom, Pennsylvania State College of Medicine, Hershey, PA; Elliott P. Vichinsky, Children's Hospital of Oakland Research Institute, Oakland, CA.

Session II. Physical Properties of Iron: Philip Aisen, Albert Einstein College of Medicine, Bronx, NY (Chair); Garry Buettner, University of Iowa; Dennis N. Chasteen, University of New Hampshire, Durham, NH; Alvin L. Crumbliss, Duke University, Durham, NC, Roland Frankel, California Polytechnic State University.

Session III. Detection of Iron Overload by MRI: Thomas Mareci, University of Florida, Gainesville, FL (Chair); George Bartzokis, University of Arkansas, Little Rock, AR; Herbert Bonkovsky, University of Massachusetts, Worcester, MA; Jeff Bulte, National Institutes of Health, Bethesda, MD; Peter Van Gelderen, National Institutes of Health, Bethesda, MD; Pierre Gillis, Université de Mons-Hainaut, Mons, Belgium; Tim St. Pierre, University of Western Australia, Nedlands, Australia; Evan S. Siegelman, University of Pennsylvania, ; Zhiyue J. Wang, Children's Hospital of Philadelphia, Philadelphia, PA;

Session IV. Detection of Iron Overload by Magnetic Susceptometry: David E. Farrell, Case Western Reserve University, Cleveland, OH (Chair); Paul N. Arendt, Los Alamos National Laboratory, Los Alamos, NM; William Avrin, Quantum Magnetics, Inc., San Diego, CA; Gary M. Brittenham, Columbia University, New York, NY; Roland Fischer,

Universitatskrankenhaus Eppendorf, Hamburg, Germany; Ronald B. Goldfarb, National Institute of Standards and Technology, Boulder, Colorado; Joseph L. Kirschvink, California Institute Of Technology, Pasadena, CA; Sankaran Kumar, Quantum Magnetics, Inc., San Diego, CA; Douglas Paulson, Tristan Technologies, Inc., San Diego, CA.

Session V. Discussion and Recommendations: Robert Balaban, National Institutes of Health, Bethesda, MD (Chair).

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