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PATHOGENIC MECHANISMS UNDERLYING IRON DEFICIENCY AND IRON OVERLOAD: NEW INSIGHTS FOR CLINICAL APPLICATION

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Abstract
Iron uptake, utilisation, release and storage occur at the gene level. Individuals with variant forms of genes involved in iron metabolism may have different requirements for iron and are likely to respond differently to the same amount of iron in the diet, a concept termed nutrigenetics. Iron deficiency, iron overload and the anemia of inflammation are the commonest iron-related disorders. While at least four types of hereditary iron overload have been identified to date, our knowledge of the genetic basis and consequences of inherited iron deficiency remain limited. The importance of genetic risk factors in relation to iron overload was highlighted with the identification of the HFE gene in 1996. Deleterious mutations in this gene account for 80-90% of inherited iron overload and are associated with loss of iron homeostasis, alterations in inflammatory responses, oxidative stress and in its most severe form, the disorder hereditary haemochromatosis (HH). Elucidation of the genetic basis of HH has led to rapid clinical benefit through drastic reduction in liver biopsies performed as part of the diagnostic work-up of affected patients. Today, detection of a genetic predisposition in the presence of high serum ferritin and transferrin saturation levels is usually sufficient to diagnose HH, thereby addressing the potential danger of inherited iron overload which starts with the same symptoms as iron deficiency, namely chronic fatigue. This review provides the scientific back-up for application of *pathology supported genetic testing,* a new test concept that is well placed for optimizing clinical benefit to patients with regard to iron status.

Key words: iron status, iron deficiency, iron overload, anemia

INTRODUCTION

The importance of iron metabolism now extends beyond the traditional areas of erythropoiesis and nutrition, representing a key factor in pathology, cardiology, oncology, neurological and infectious diseases. The iron status of an individual is determined by a combination of nutritional, environmental and genetic factors. Normal iron status ensures ready availability of metabolic activity for optimal functioning of the immune system, while at the same time impeding uptake by microorganisms and denying growth advantages in tumour cells. The degree of variation on either side of the normal state that can be tolerated without upsetting this fine balance is probably the critical factor in understanding how changes in iron status can predispose to a wide variety of disorders.

THE CONTROL OF IRON LEVELS IN THE BODY

Iron is absorbed from food into enterocytes lining the duodenum via the divalent metal transporter (DMT1). Iron must therefore be in the divalent form for uptake. This may be achieved by reduction of Fe^{3+} to Fe^{2+} by vitamin C, or by duodenal cytochrome b, which is also located on the cell membranes of enterocytes (19). Once the iron is inside the enterocytes, it is either sequestered into ferritin molecules, or made available for transport into the body by a highly regulated system of interacting proteins which are able to sense the iron status of the body. If the body is iron replete, absorbed iron remains inside the ferritin, and is lost when the enterocytes are sloughed off. However, when the body iron status is low, iron is loaded onto transferrin (Tf) by ferroportin, with the help of haephestin, a copper containing protein that oxidises the iron from Fe^{2+} to Fe^{3+}. Ferroportin is regulated by hepcidin, which binds to it, and causes it to be degraded inside the enterocyte cells. The discovery of hepcidin (34), a 25 amino acid protein which is produced in the liver, circulates in the plasma and is excreted in the urine, has revolutionized our understanding of the regulation of iron absorption and storage. Hepcidin plays a key role in ensuring the maintenance of an optimal iron store, in regulating iron delivery to all body cells in concert with the functional requirements and blocking the absorption of unneeded iron through the intestine. It acts as a negative regulator of release from stores and intestinal absorption. High levels reduce the rate of release from stores and absorption from the intestine by binding to the only known cellular iron exporter, ferroportin, causing it to be degraded. Iron may also be absorbed into enterocytes from heme by heme-carrier protein-1 (HCP-1) and released from heme by heme oxygenase-1 (41). The released iron then follows the same route to be bound to Tf.

Once iron is loaded onto Tf, it is transported to cells that need iron for metabolism. One Tf molecule can bind two iron atoms. In order to enter a cell, Tf binds to Tf receptors (TfR) located on the cell membrane. The Tf-TfR complex is then endocytosed. In these vesicles, the pH is lowered, and iron is released, while the Tf and the Tf receptor are re-circulated to the cell membrane, and Tf is released into the blood as iron-depleted Tf, or apo-Tf. Tf carrying two iron atoms (iron loaded Tf) is called holo-Tf.

Although it is clear that hepcidin regulates the entry of iron into the blood from enterocytes, the mechanisms that allow hepcidin to sense iron levels are still in the process of being elucidated. Hepcidin transcription is upregulated by inflammatory cytokines, iron, and bone morphogenetic proteins and is downregulated by iron deficiency, ineffective erythropoiesis, and hypoxia (25). Furthermore, Gao et al (12) have found that a complex of TfR2 and the hereditary hemochromatosis protein (HFE) is involved in the transcriptional regulation of hepcidin by holo-Tf. Patients with the most common form of hereditary iron overload have mutations in the HFE gene and they have lower levels of hepcidin than unaffected individuals.

Once inside cells, further control of iron is provided by the iron-regulatory proteins 1 and 2 (IRP1 and IRP2) which regulate the expression of multiple iron metabolism genes in order to optimise cellular iron availability. In iron-deficient cells, IRPs bind to iron-responsive elements (IREs) found on the mRNAs of ferritin, the Tf receptor and other iron metabolism transcripts, thereby enhancing iron uptake and decreasing iron sequestration (36). When iron levels are low, the synthesis of ferritin decreases and the levels of Tf receptor-1 (TfR1) increase, while the opposite occurs in the presence of high iron status. The physiological role of the IRP-IRE system was illustrated by the following conditions:

1. A relatively rare human disorder, hereditary hyperferritinaemia cataract syndrome, in which ferritin L-chain IRE mutations interfere with IRP binding and translational processes
2. A syndrome described in adult mice lacking IRP2, which is characterised by raised ferritin levels, progressive neurodegeneration and anaemia.

TESTS USED TO INVESTIGATE IRON DISORDERS

The following tests are performed in the laboratory to determine iron status:

HAEMOGLOBIN

Hemoglobin is the protein molecule in red blood cells that carries oxygen from the lungs to the body's tissues and returns carbon dioxide from the tissues to the lungs. Hemoglobin is made up of four protein molecules (globulin chains) that are connected together. The normal adult hemoglobin molecule contains 2 alpha-globulin chains and 2 beta-globulin chains. In fetuses and infants, there are only a few beta chains and the hemoglobin molecule is made up of 2 alpha chains and 2 gamma chains. As the infant grows, the gamma chains are gradually replaced by beta chains. Each globulin chain contains an important central structure called the heme molecule. Embedded within the heme molecule is iron that transports the oxygen and carbon dioxide in our blood. The iron contained in hemoglobin is also responsible for the red color of blood. Hemoglobin also plays an important role in maintaining the shape of the red blood cells. Abnormal hemoglobin structure can, therefore, disrupt the shape of red blood cells and impede its function and its flow through blood vessels. Hemoglobin is usually measured as a part of the complete blood count (CBC) from a blood sample. A low hemoglobin is referred to as anemia. There are many reasons for anemia. Some of the more common causes are: loss of blood (traumatic injury, surgery, bleeding colon cancer or stomach ulcer), nutritional deficiency (iron, vitamin B12, folate), bone marrow problems (replacement of bone marrow by cancer, suppression by chemotherapy drugs, kidney failure), and abnormal hemoglobin (sickle cell anemia). Higher than normal hemoglobin levels can be seen in people living at high altitudes and in people who smoke. Dehydration produces a falsely high hemoglobin which disappears when proper fluid balance is restored. Some other infrequent causes are: advanced lung disease (for example, emphysema), certain tumors, a disorder of the bone marrow known as polycythemia rubra vera, and abuse of the drug erythropoietin.

SERUM FERRITIN CONCENTRATION

Ferritin is currently considered the most important indicator of iron status as even in the first stage of iron deficiency, its concentration decreases (20). It is important to note that ferritin is increased by many factors, including infection and inflammation, thus a high value does not necessarily indicate a good iron status. It is therefore also valuable to measure parameters for acute (CRP) and chronic infection [alpha-1-glycoprotein (AGP)] (2).

A ferritin molecule consists of 24 subunits of L-ferritin or H-ferritin. H-ferritin has ferroxidase activity, which is necessary for oxidising iron before it can be loaded onto ferritin. Each ferritin molecule can store up to 4000 iron atoms. However, serum ferritin does not contain iron, because it consists of L-ferritin, which does not have the ability to load iron into ferritin. Therefore, a high ferritin level may sometimes be found in patients with iron deficiency, and the soluble TfR /Log(10) ferritin ratio (sTfR-F Index) is better able to distinguish iron status (24). Nevertheless, serum ferritin concentration is an indicator of ferritin produced inside cells. Since inflammation causes increased ferritin production by liver cells, ferritin determinations may be accompanied by measurement of CRP, in order to rule out inflammatory causes of increased serum ferritin. Intracellular ferritin is essential to iron homeostasis and is involved in a wide range of physiologic and pathologic processes, making iron available for critical cellular processes while protecting lipids, DNA, and proteins from the potentially toxic effects of iron. Alterations in serum ferritin are seen commonly in clinical practice, often reflecting perturbations in iron homeostasis or metabolism. In clinical medicine, ferritin is predominantly utilized as a serum marker of total body iron stores. In cases of iron deficiency and iron overload, serum ferritin serves a critical role in both diagnosis and management (24). Ferritin is directly implicated in potentially devastating diseases including familial haemochromatosis, sideroblastic anaemias, neurodegenerative diseases, and hematophagocytic syndrome. Elevated serum and tissue ferritin are linked to coronary artery disease, malignancy, inflammatory conditions and poor outcomes following stem cell transplantation. Additionally, recent research describes novel functions of ferritin independent of iron storage (20).

SERUM TRANSFERRIN SATURATION AND TRANSFERRIN CONCENTRATION

Total iron binding capacity (TIBC) is typically measured along with serum iron to evaluate people suspected of having either iron deficiency or iron overload. The iron concentration divided by TIBC gives the Tf saturation, which is a more useful indicator of iron status than iron or TIBC alone. In healthy people, about 20-50% of available sites in Tf are used to transport iron. In iron deficiency, iron is low, but TIBC is increased, and Tf saturation becomes very low. In iron overload states such as hemochromatosis, iron will be high and TIBC will be low or normal, causing the Tf saturation to increase. The test has been found to be lacking in sensitivity, as linearity becomes questionable once saturation levels above 80% are reached, and the test has not yet proven to be useful in iron estimation for patients with transfusional iron. Although it may not be a reliable test for iron overload, elevated serum Tf saturation is a sensitive and specific disease marker for hereditary hemochromatosis. Fasting serum Tf saturation above 50% in women and above 60% in men, has a sensitivity of 0.92, a specificity of 0.93, and a positive predictive value of 86% for the diagnosis of hereditary hemochromatosis.

It is customary to test for Tf (instead of TIBC) when evaluating a patient's nutritional status or liver function. Because Tf is made in the liver, levels will be low in patients with liver disease. Tf levels also drop when there is not enough protein in the diet, so this test can be used to monitor nutrition status. Although serum ferritin measurement is the investigation of choice in iron deficiency, many laboratories continue to offer iron and TIBC/ Tf measurements. In iron deficiency, serum Tf concentrations are elevated while iron concentrations are decreased, resulting in a decreased Tf saturation. However serum iron concentrations follow a diurnal variation and may be increased after meat ingestion. In some cases, iron and TIBC/ Tf are used instead of ferritin due to their lower cost while in other cases, ferritin is requested in addition to iron and TIBC/ Tf because of concerns regarding ferritin interpretation in inflammatory conditions. Tf can be measured either directly or indirectly by summing the results of serum iron and unsaturated iron binding capacity (UIBC) measurements to give total iron binding capacity (TIBC). The latter approach offers the advantage of relatively inexpensive reagents such as ferric chloride but requires mandatory measurement of serum iron.

Soluble transferrin receptor (sTfR)

The soluble or serum transferrin receptor test (sTfR) is based on the fact that erythroblasts in the bone marrow will increase the presentation of membrane Tf receptor in the setting of iron deficiency, some of which come off and will be detectable in the circulation. The sTfR correlates with this membrane expression of the Tf receptor and also tends to be elevated in the presence of increased erythroid activity. When a patient is noted to have an elevated sTfR, the clinician must determine whether it is due to iron deficiency or because the patient has increased erythroblastic activity which is increasing the membrane and ultimately the serum expression of the TfR. It does seem to be a reasonable index of erythropoietic activity (6). Since sTfR is not affected by inflammation (1), it is a more reliable test than serum ferritin. However ambiguous results can occasionally be obtained and the use of sTfR-log ferritin index is recommended (28). A value greater than 2 suggests iron deficiency, while a value less than 1.0 is consistent with anemia of chronic disease (20).

Serum iron concentration

Serum iron determinations have become the “Cinderella” of iron tests. Widely believed to be unreliable as a result of circadian variation, clinicians often think that determinations of Hb, ferritin or direct Tf would provide better estimations of a patient’s iron status. Hawkins (18) advocates the abolishment of serum iron determinations and considers direct Tf measurements cost effective. However, serum iron concentration is a useful measure for investigation of diseases that may involve inadequate delivery of iron to cells due to inhibition of iron uptake. The iron that is bound to Hb is not available to tissues such as the brain for incorporation into enzymes and proteins involved in energy production, e.g. mitochondrial iron-sulphur proteins and aconitases. Ferritin may be increased due to inflammation. The amount of iron reaching the cells can best be estimated by determining serum iron concentrations in combination with the other tests. Determinations should be done in the morning to rule out circadian variations.

THE ROLE OF IRON IN HEALTH AND DISEASE

Worldwide, approximately one billion people suffer from iron deficiency anaemia, which affects 25% of the general population. The anaemia of chronic disease is frequently seen in patients with autoimmune disorders, malignancies and infectious conditions, due to iron retention in cells of the reticuloendothelial system. While iron deficiency is most common in developing countries, the most frequent inherited disorder in people of Northern-Western European origin is hereditary haemochromatosis (HH), a preventable iron overload disorder caused by mutations in the HFE gene. Iron loading in the presence of a genetic predisposition for HH depends on co-inheritance of other iron-related gene mutations and environmental factors. For example, alcohol consumption increases iron uptake significantly, while black tea has strong inhibitory effects due to the tannins it contains. Secondary iron overload may furthermore develop due to repetitive blood transfusion in patients treated for genetic haemoglobinopathies, cancer-related anaemia or myelodysplastic syndromes.

The balance of iron in the body depends on a complex interaction between genetic and environmental factors. Individuals with variant forms of genes involved in iron metabolism may respond differently to the intake of the same amounts of iron in the diet. Iron deficiency, iron overload and the anemia of inflammation are the commonest disorders of iron metabolism (26).

1. Nutritional iron deficiency results from a diet that contains insufficient bioavailable iron to meet individual requirements. In developing countries, traditional foods usually contain large quantities of iron absorption inhibitors, particularly phytates and polyphenols. Conditions that cause blood loss, particularly hookworm infections, have an important contributory role leading to a high prevalence of iron deficiency in many developing countries.

2. The anaemia of inflammation (anaemia of chronic disease) is the result of increased hepcidin expression induced by inflammatory cytokines which is generally considered to be a host response that evolved to make iron less available to pathogens. This condition is characterized by decreased release from iron stores, low plasma iron and transferrin concentrations, restriction of the available iron supply for red blood cell production and mild or moderate anaemia.

3. Primary iron overload is far less prevalent than iron deficiency. Primary systemic iron overload (haemochromatosis) is almost always the result of an inherited abnormality of the regulation of iron transport that affects hepcidin or its receptor ferroportin. The expression of hepcidin is induced independently by the accumulation of storage iron and by inflammation.

CHRONIC FATIGUE

Chronic fatigue is one of the most common presenting symptoms in the consulting room of the primary care physician, and the diagnosis could be quite challenging. It is important to distinguish between normal physiological causes of fatigue, and pathological causes such as organic (physical) and psychological causes of chronic fatigue. The diagnosis may become quite clear from the anamnesis and though physical examination, but special investigations may be essential to make a definitive diagnosis.

Determination of an iron profile is important to distinguish between possible iron deficiency anaemia and iron overload, both of which may cause fatigue. When feeling tired, many people assume iron deficiency and take iron supplements. However, as discussed below, some mutations may lead to increased iron absorption and hemochromatosis.

IRON DEFICIENCY

Iron deficiency, and anemia resulting from iron deficiency, is considered to be one of the top ten contributors to the global burden of disease. The prevalence of anemia in preschool aged children is approximately 47%, in pregnant

women 41%, and in non-pregnant women 30% (29). Globally, more than 800 million women and children suffer from anemia, mostly in Africa, Asia and Latin America. The fact that iron is the most abundant metal in the world, but also involves the most common dietary deficiency worldwide, may relate to a protective cellular mechanism that evolved in malaria endemic areas whereby iron used in the life cycle of parasites is effectively restricted (2).

Iron is essential for many metabolic processes including DNA, RNA and protein synthesis, the formation and maintenance of myelin, and is a co-factor of many heme and non-heme enzymes. Clinical consequences of iron deficiency include central nervous system dysfunction including cognitive impairment, decreased physical capacity, impaired work performance due to chronic fatigue, pregnancy complications, reduced immunity, gastrointestinal disturbances (e.g. glossitis, stomatitis, gastritis), and impaired temperature regulation in a cold environment.

Iron deficiency generally occurs in three sequential stages: depleted iron stores, iron deficiency erythropoesis and iron deficiency anemia. All three stages can be analyzed biochemically with the measurement of Hb, ferritin and sTfR. Although there are some clinical indicators and the evaluation of iron intake might be helpful, the diagnosis relies mainly on these parameters.

The measurement of Hb is essential for the diagnosis of nutritional anaemia and is one of the most common, easiest and least expensive methods. Unfortunately, Hb measurement is not very sensitive and specific for iron deficiency (only the third stage affects Hb synthesis). Thus, to determine whether iron deficiency is responsible for anemia, it is usually necessary to include other indicators.

Iron deficiency may result from inadequate intake, increased iron requirements due to genetic risk factors, increased blood loss or decreased absorption of iron, or a combination of these factors. Nutritional anemia is also characterized by other nutritional deficiencies. Both copper and zinc are essential nutrients and deficiencies of both result in anemia. Resistance to infections depends on a healthy immune function and copper and zinc are both necessary for normal function of the immune system. Copper deficit should be included in the differential diagnosis of anemia unresponsive to iron supplementation.

Although most forms of anemia are due to iron deficiency, a proportion may be due to deficiency of vitamins of the B complex, principally folate and vitamin B12. The anemia is macrocytic but with presence of abnormal red cell precursors in the bone marrow called megaloblasts. Because of the well proven case of increased risk for spina bifida, neural tube defects and other birth defects, folic acid supplementation before, during and after pregnancy is now accepted as being critical regardless of the nutritional status of the woman. However, vitamin B12 should be given as well, to prevent macrocytic anemia due to vitamin B12 deficiency. Vitamin B12 enters the human food chain exclusively through animal sources. Its synthesis is completely absent in plants of all kinds, only being present in such foods by way of bacterial contamination or fermentation. For this reason vegetarians and more particularly vegans, are at high risk of insufficient dietary intake.

Iron deficiency is linked to increased homocysteine levels, which is a marker of vitamin B deficiencies and inflammation. The close relationship between inflammation, iron and folate appears to be of particular relevance in patients with demyelinating diseases such as multiple sclerosis (MS) (7, 21, 44,45). Impaired methylation is implicated by the finding of higher homocysteine levels in MS. Low levels of vitamin B12 conversely lead to low iron uptake, which is associated with gastritis and atrophy of glands producing intrinsic factor in the stomach. Inhibition of vitamin B12 uptake causes pernicious anaemia, a condition that needs to be excluded before a diagnosis of MS can be made. A study performed by Selzer et al. (40) clearly demonstrated that optimal functioning of the folate-vitamin B12-methyl transfer pathway is a prerequisite for myelin production and maintenance. While functional polymorphsisms in genes of the methyl transfer pathway may cause inadequate myelination and serious disability from childhood, supplementation with the chemical substrate following each metabolic block could restore the myelin as well as some of the functional deficiencies. When the substrates or co-factors of the folate pathway (including B vitamins and zinc) are depleted, demyelination may follow (40). In addition, the latter study showed that demyelination might also occur when this metabolic pathway is blocked by nitrous oxide anaesthesia, since nitrous oxide causes irreversible oxidation of the cobalt in vitamin B12.

It is possible that the relevance of iron deficiency in MS has previously been missed because iron is traditionally associated with oxidative damage, and iron chelation would rather be considered appropriate to prevent oxidation during demyelination (45). However, under normal circumstances the presence of iron is stringently controlled. All intracellular iron is either bound up inside proteins such as the haem groups of cytochromes and catalase or sequestered by the iron transporters (Tf and ferritin). Superoxide dismutase and glutathione peroxidase are also continually present to scavenge free radicals as part of a highly functional antioxidant system within oligodendrocytes.

INTERVENTION

Results from intervention trials seem to indicate that daily iron intake together with multiple micronutrients is most effective in improving anemia and iron deficiency (44). Iron supplementation administered to deficient individuals was found to increase oxidative stress, but treatment with a combination of iron and vitamins A, C and E proved effective in protection against oxidative stress (43).

Recent research on iron supplementation in malaria-endemic areas showed disturbing results of an increased incidence of adverse effects and death (38). It has been suggested that the adverse effects of iron supplementation on malaria are due to increased peripheral availability of young red blood cells, increased iron reserves for parasite development and the loss of the inhibitory effect of microcytosis on intraerytrocytic parasites (32). Thus iron supplementation of iron deficient children in malaria endemic areas is not recommended. Mosquito control and avoidance of the insect vector are fundamental (8).

It is vital to consider the safety of interventions when attempting to reduce nutritional anemias:

Supplementation: It is extremely important to do an iron profile before taking or prescribing iron. Supplements available commercially may contain more than the physiological daily requirements for a nutrient, in particular for iron. It is critical to avoid new imbalances that may arise from iron-induced oxidative effects as a result of unintended overdosing. Although the uptake of iron is stringently controlled, any mutations present in the control proteins mentioned above may modulate the iron that results from supplementation. Whereas moderate iron supplementation will usually not cause iron overload, it is important to test iron parameters regularly in subjects who regularly take iron supplements, for example in sports nutrition.

Fortification: When exogenous nutrients are placed in food, the variation across the population consuming the fortified foods becomes important. Fortification levels should provide safe exposure for the upper distribution of consumers.

Dietary diversification: Promoting foods as a source of nutrients are not without potential safety issues and might encourage a dietary pattern that is less than healthy, by increasing the intake of foods promoting chronic disease risk. Distorted intakes of red meat as a natural source of bioavailable iron, for example, could increase the risk of colon cancer and many diseases associated with saturated fat exposure. Neither supplementation nor fortification can be effective on their own. The preferred intervention, leading to sustainable improvements of micronutrient intake, would be moderate intake from animal sources and greater consumption of fruit and vegetables, especially vitamin C-rich foods (42).

Nutritional anaemias are currently the greatest global nutrition problem. A comprehensive, multiple intervention approach is necessary for sustainable success and must include improved social conditions by poverty alleviation measures, as well as the more direct measures of fortification, supplementation and improved health care. Recent research points to functional consequences even before the clinical onset of iron deficiency anaemia. Longitudinal studies caution that chronic iron deficiency in infancy permanently retards cognitive, motor, and socio-emotional development (14).

HEREDITARY IRON OVERLOAD

Our knowledge of iron absorption and regulation has greatly improved due to the discovery of new genes responsible for iron overload. Four genes are responsible for the distinct types of haemochromatosis unrelated to HFE gene mutations: Hepcidin and hemojuvelin are the genes involved in autosomal recessive juvenile haemochromatosis (type 2), while the transferrin receptor-2 (type 3) and ferroportin (type 4) genes underlie relatively rare forms of autosomal recessive and autosomal dominant forms of adult-onset haemochromatosis, respectively.

The predominant feature of iron overload or haemochromatosis is over-absorption of dietary iron. Excess body iron is stored in organs and tissues where it may cause injuries resulting in a variety of disorders, including heart disease, diabetes, arthritis, cancer, cirrhosis, impotence and sterility. The possibility of inherited iron overload should always be considered in patients with unexplained mild changes in liver function, abnormal fatigue, right hypochondrial pain, arthritis, diabetes, impotence (particularly in young adults), and unexplained cardiac complaints; particularly if more than one of these symptoms are present. Determination of Tf saturation and serum ferritin levels should be followed up by genetic testing in cases with high iron stores. Haemochromatosis can be prevented by regular blood donation or phlebotomy and therefore detection of a genetic predisposition at an early age, before irreversible damage to cardiac, hepatic and endocrine tissue occurs, represents an important clinical goal.

The importance of iron within oxygen-binding and respiratory proteins cannot be overemphasised, but it may also lead to the production of free radicals and consequently tissue damage (3). Whilst these free radicals may cause DNA strand breakage and disruption of DNA structure (direct mutagenesis), iron can also interfere with immunologic tumour surveillance and macrophage disposal of transformed cells (indirect mutagenesis) (5). Excess body iron has been linked to colon cancer, while deposition of iron in the liver is associated with liver cirrhosis and hepatocellular carcinoma (HHC). Several lines of evidence suggest that increased production of reactive oxygen species (ROS) in a liver already compromised by another disease, such as alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD), can cause further insult and therefore more severe disease. Iron and alcohol interact synergistically to cause liver damage and may explain the small number of alcoholics that actually develop ALD despite excessive alcohol intake over long periods of time (17). Oxidative stress may lead to inhibition of hepcidin promoter activity and transcription in the liver, which in turn leads to an increase in intestinal iron transport and liver iron storage. Since the synthesis of hepcidin is suppressed by alcohol in parenchymal cells of the liver, hepcidin may act as a secondary risk factor in the progression of ALD due to its crucial role in the regulation of body iron stores.

Iron accumulation in the brain is believed to contribute to neurodegenerative diseases, including Parkinson and Alzheimer's disease. While an imbalance in brain iron status may cause free radical generation and oxidative damage, the possibility that such iron may be insoluble and unavailable for cellular use must also be considered. Haacke et al. (15) used susceptibility weighted MR imaging to characterise iron deposition in multiple sclerosis brain lesions, and stated that whereas the origin of the iron is still unclear, the iron deposition may result from microhemorrhaging and hemosiderin buildup.

In some diseases, the pathology associated with iron accumulation may result from functional iron deficiency (37). Neurological deficit may therefore result from an imbalance in the compartmentalisation of iron rather than a decrease or increase in the absolute amount of body iron (22). This is evident in patients with aceruloplasminemia where, unlike other iron-overload syndromes, neurological manifestations appear to dominate (16). Reduction in iron binding to Tf as a consequence of defective oxidation of ferrous iron to ferric iron results in impaired transport of iron from intracellular stores to plasma, resulting in decreased serum iron and microcytic anaemia. Affected patients eventually die from effects of iron accumulation in the basal ganglia while the initial problem lies with the supplying of iron for key synthesis processes required for cell growth. These may include optimal expression of the ceruloplasmin gene implicated in neuronal survival in the retina and basal ganglia (13) and, although not investigated, interference of iron delivery for haem biosynthesis involved in energy production and neurological integrity (30).

CLINICAL CHARACTERISTICS

The diagnosis of genetic haemochromatosis is complicated by clinical variability and the non-specific symptoms associated with body iron overload (Table 1). Early features of iron overload such as fatigue, joint pain, abdominal pain and loss of libido are non-specific and are commonly not ascribed to haemochromatosis (31). Liver function tests are frequently normal in patients with early iron overload and when found to be slightly abnormal, are commonly ascribed to excessive alcohol use. Excess iron accumulates in the liver, pancreas, heart and other organs eventually causing organ failure, as the body has no regulated way of excreting iron. Symptoms of iron overload could typically appear in middle-age after years of damage, although iron overload may also occur in young persons in their early 20's, as well as children depending on the penetrance of genetic risk factors that may be involved. The clinical presentation of haemochromatosis has changed over recent years due to the incorporation of molecular genetic research findings into clinical practice, from diagnosing patients with advanced disease (e.g. liver cirrhosis, diabetes) to early detection of patients presenting with abnormal liver function tests, elevated ferritin and/or increased Tf saturation levels (23).

Table 1. Symptoms and signs of inherited iron overload.

Symptoms and signs	Medical conditions
Abnormal liver function	Arrhythmias
Abdominal pain (unexplained)	Arthritis, arthralgia
Bronzing of the skin	Cardiomyopathy
Amenorrhoea (no menstrual periods, females)	Chronic fatigue
Anterior pituitary failure	Chronic liver disease
Frequent diarrhoea	Cirrhosis of the liver
Hyperferriteinaemia	Depression
Impotence (males)	Diabetes mellitus Type 1
Insulin resistance	Diabetes mellitus Type 2
Joint pain	Fatty liver disease
Loss of body hair	Hepatocellular carcinoma
Loss of libido	Infertility
Mood swings	Metabolic syndrome
Muscle pain	Porphyria cutanea tarda
Skin pigmentation	Testicular atrophy (males)
Weakness	

PREVALENCE

Genetically predisposed individuals occur with an estimated frequency ranging from 1/100 to 1/300 depending on the population studied. The carrier frequency of the most common mutation underlying hereditary haemochromatosis (HH), C282Y in the HFE gene, is about 1 in 6 in the Caucasian population of South Africa. This means that approximately 1 in 100 (estimated 1/115) individuals of European descent will inherit two copies of the defective gene (9,10).

PENETRANCE

Consideration of factors that affects the penetrance of HH is important for both the diagnosis and treatment of iron overload conditions. It is uncertain what percentage of individuals with a genetic predisposition for HH will develop organ damage, as the disease penetrance varies from less than 1% on the one extreme in some populations, to between 40-60% depending on the genetic background and environmental exposures. Gender is also important as females are usually affected about 10 years later than males due to iron loss through menstruation and childbearing.

Many experts do not recommend population screening for detection of abnormal iron-loading genes, because of the low penetrance of the most common late-onset form of haemochromatosis. However, extending genetic investigations to family members of an affected individual may allow accurate genetic diagnosis in the initial asymptomatic phase and disease prevention. An argument for genetic screening of people known to be from high risk populations (e.g. people originating from North Western Europe) has been made and may in some areas be justified. It is advisable that individuals with a genetic predisposition for inherited iron overload must have their iron status determined to assess possible gene expression and to monitor response to treatment, if indicated.

DIAGNOSIS OF HAEMOCHROMATOSIS

DNA testing provides a definitive diagnosis in the majority of patients with elevated Tf saturation and ferritin levels, without the need to perform a liver biopsy. Determination of fasting Tf saturation can detect cases of iron overload before organ dysfunction has occurred and is usually recommended as a first line screening method for haemochromatosis. Elevated Tf saturation is relatively non-specific and is frequently seen in chronic liver diseases due to secondary iron overload.

The latest classification of hereditary iron overload disorders broadly divides them into HFE-related haemochromatosis, which constitutes about 90% of cases, and non-HFE-related haemochromatosis. Five categories based on different genetic mutations and clinical presentation can be discerned (4):

- Type 1 is classic haemochromatosis, where the affected persons are most often homozygous for the C282Y a mutation in the *HFE* gene causing a substitution of tyrosine for cystine at amino acid 282 in its product.

- Type 2A (with mutations in the *HJV* gene, encoding the hemojuvelin protein) and 2B (mutations in the *HAMP* gene that encodes hepcidin), both presenting at 20-30 years of age

- Type 3 (mutations in the *TFR2* gene, encoding the transferrin receptor 2)

- Type 4 (mutations in the *SLC40A1*gene, encoding ferroportin)

- A(hypo)ceruloplasminemia (a rare autosomal recessive form)

Types 1 to 3 have autosomal recessive inheritance patterns and manifest as parenchymal iron overload with organ failure, targeting mainly the liver, heart and endocrine organs. Type 4 is autosomal dominant and manifests as storage (macrophagal) iron overload. The products of the genes that are associated with the 4 major types of haemochromatosis discussed above, all exert either regulatory effects on the synthesis or affect the function of hepcidin. Most of the clinical, biochemical and pathological features of iron overload disorders can now be explained by hepcidin deficiency or failure.

INHERITANCE PATTERN

Knowledge about the inherited nature of haemochromatosis and the application of genetic testing is important, because the disease goes undetected in many patients especially in the early preventable phase due to the non-specific symptoms of iron overload such as fatigue, joint aches, abdominal pain, loss of libido and depression. Except for one rare form of adult-onset haemochromatosis caused by mutations in the ferroportin gene, inherited iron overload follows an autosomal recessive inheritance pattern. This means that patients with haemochromatosis have inherited a defective copy of the gene from both parents. The most common form is caused by mutations in the HFE gene on chromosome 6 (11). Three mutations (HFE C282Y, H63D and S65C) account for the disease in the majority of affected patients.

People with one copy of a defective HFE gene are called heterozygotes or carriers. Mutation carriers do not necessarily develop clinical symptoms and the gene can be passed on in a family without anyone being aware of it. Children of two mutation carriers have a 25% chance of inheriting two copies of the defective gene. Those who inherit a defective copy of the gene from both parents are homozygous and are likely to develop the disease whereas those who inherit from one parent are carriers who are unaffected or may show a lesser increase in iron absorption.

GENETIC TESTING AND INTERPRETATION

Patients are usually referred for genetic testing to confirm or exclude clinical/biochemical diagnosis, assess carrier status in families or for pre-clinical diagnosis in at-risk family members. Genetic testing is important since it can provide a definitive diagnosis of inherited iron overload without the necessity of an invasive liver biopsy. Several polymerase chain reaction (PCR)-based methods have been developed for detection of mutations underlying haemochromatosis, including a reverse-hybridisation method that allows simultaneous analysis of multiple mutations in a single reaction (33). Today real-time PCR are mostly used for mutation detection in patients at risk of haemochromatosis. An important consideration in the test design is to be aware of the fact that certain gene regions of relevance to PCR-based tests frequently contain non-functional sequence changes that may interfere with the test procedure and data interpretation (9).

To facilitate interpretation of genetic test results, information on clinical symptoms and iron parameters has to be provided when patients are referred for genetic testing. This is equally important for confirmation and exclusion of HH.

TREATMENT AND MONITORING

It may be necessary to treat patients with iron overload according to the genetic subtype: venesection is the treatment of choice in patients with haemochromatosis related to hepcidin deficiency, but is poorly tolerated or contraindicated in patients with iron overload due to ferroportin failure. In patients with low or normal Tf saturation levels in the presence of high ferritin levels Hb levels need to be monitored if phlebotomy is performed, to prevent the possibility of the patient becoming anaemic.

Standard treatment for HH patients with high Tf saturation and ferritin levels involve weekly therapeutic phlebotomy of 500 ml whole blood (equivalent to approximately 250 mg iron) (35). Regular venesection should be continued until ferritin levels are <50 ng/ml and Tf saturation <30%. Although some patients with HH, for reasons that are unclear at this time, do not reaccumulate iron, most patients will require maintenance phlebotomy of 1 unit of blood to be removed every 2-3 months.

Worldwide a paradigm shift is happening in healthcare as it is moving from primarily disease management towards a more prominent role for health management. Haemochromatosis is a good example of a preventable genetic disorder caused by an interaction between genetic and environmental factors. We therefore should not focus only on using genetic testing for diagnosis, but should use it also for risk management.

PATHOLOGY SUPPORTED GENETIC TESTINGTM AND INTERVENTION

Numerous and major advances characterised the evolution of modern medicine during the past centuries and brought about many changes to the practice of clinical and diagnostic health sciences. The rapidly expanding integration and overlapping of traditionally distinctly separate fields of medicine introduced a new era of interdisciplinary- and team-approaches to clinical practice and patient care. In this regard, clinical human genetics including molecular genetics is no exception (39).

In April 2009 the Department of Pathology at Stellenbosch University launched an initiative to develop pathology supported molecular genetic testing based on an integrated service- and research approach. It is considered important to develop innovative approaches to risk management of complex multi-factorial diseases to be applied in a clinical context where the genetic test results are fully integrated with relevant clinical information and other diagnostic pathology data. To determine clinical usefulness, careful review of the literature is performed to prevent the use of single nucleotide polymorphisms (SNPs) of uncertain functional significance in genetic tests, following genome wide association studies that may identify risk alleles in the absence of supporting data on relevant metabolic impairments. The aim is to match the disease diagnosis and therapeutic design with the clinical picture, pathology, environmental risk factors and genetic profile of the patient. In order to address the important ethical and scientific issues pertaining to pathology supported gene-based intervention, the information must be captured in a database preferably as part of properly designed and ethically approved research projects that will advance evidence-based medicine.

Haemochromatosis provides an excellent example of a complex disease that is best addressed by a pathology supported genetic testing approach. What this means, is that genetic testing for haemochromatosis is performed within a specific pathology/biochemical and clinical profile, that both the biochemical and genetic test results are provided in a patient report together, and that gene expression and/or response to treatment is monitored through these accompanying pathology and genetic test parameters.

Until recently, the HFE gene was considered the only major cause of inherited iron overload. Failure to identify mutations in a relatively large number of patients referred by clinicians for genetic testing contributed to the identification of several other genes involved in iron overload. It also highlighted the importance of a step-wise approach in the diagnosis and treatment of iron overload disorders (4). Pathology supported genetic testing in patients at risk of iron overload involves the following steps:

- Consider iron overload based on presenting clinical features and iron status, taking into account the main confounding factors such as alcoholism, inflammatory conditions, acute or chronic hepatitis and polymetabolic syndrome.

- Evaluate hepatic vs splenic iron load in order to direct the diagnosis to the most likely cause of iron excess.

- Rule out acquired iron overload due to external factors such as prolonged iron supplementation (e.g. in the setting of competitive sports) or repeated transfusions in patients with haematological diseases, represented by chronic anaemias such as thalassaemia major and sickle cell disease.

- Identify the genetic origin of iron overload by considering both family and personal information (e.g. plasma ceruloplasmin level when Tf saturation is normal or low).

- Treat patients with iron overload according to genetic subtype: venesection is the treatment of choice in patients with haemochromatosis related to hepcidin deficiency, but is poorly tolerated or contraindicated in patients with iron overload due to ferroportin failure.

- Monitor treatment response by assessment of relevant biochemical parameters and health outcomes.

Factors that need to be taken into account in addition to the clinical indicators summarized in Table 1 and ethnic risk, when genetic testing is considered to confirm or exclude a diagnosis of inherited iron overload, include iron status, inflammation and environmental factors that may modify gene expression (Table 2). Improvement of existing concepts through incorporation of new knowledge is necessary to combat iron-related disorders (27).

Table 2. Biochemical determinations and environmental factors of relevance to iron status

Biochemistry	Environmental factors
Haemoglobin	Alcohol intake
Serum iron	Tea intake
Transferrin	Copper deficiency
Transferrin saturation	Folate deficiency
Ferritin	Zinc deficiency
Liver function tests (ALT, AST, LH)	Iron supplementation
C-reactive protein (CRP) levels	Vegetarian/vegan
Alpha-1-glycoprotein (AGP)	Vitamin C supplementation
Homocysteine	Blood donation

CONCLUSIONS

Sequence variation in a multitude of iron-related genes underlies defective proteins or differences in expression levels of alleles at these loci, which contributes to individual variability in iron metabolism. Loss of iron homeostasis is central to the pathogenic events underlying many medical conditions. In particular, everyone should take notice of the potential danger of inherited iron overload as it starts with the same symptoms as iron deficiency, namely chronic fatigue. Detection of a genetic predisposition in the presence of high serum ferritin and Tf saturation levels is usually sufficient to diagnose inherited iron overload However, care must be taken to prevent misdiagnosis of hereditary haemochromatosis in patients with hyperferriteinaemia caused by the insulin resistance hepatic iron overload (IR-HIO) syndrome, also known as dysmetabolic iron overload.

Our own experience and new insights gained from an interactive workshop on Iron Disorders and Well-being held in May 2009 as part of the *GeneTalk* series (www.genetalk.co.za), formed the basis of this review that provides the scientific back-up for application of *pathology supported genetic testing* to confirm or exclude HH in patients with raised ferritin levels. This new test concept requires that (1) genetic testing is performed within a specific pathology/biochemical and clinical profile that also defines the test selection criteria, (2) the patient report contains both the biochemical and genetic test results for clinical application in the context of relevant documented environmental factors, and (3) gene expression and monitoring of response to treatment are assessed through the accompanying pathology and genetic test parameters. The identification of the spectrum of risk factors underlying iron overload provides a major healthcare opportunity to reduce the burden of heart disease, cancer, diabetes, arthritis, infertility and many other complications of organ damage in the population.

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