REVIEW OF LITERATURE

Thalassemia Epidemiology

Thalassemia is considered the most common genetic disorder worldwide. It occurs with a particularly high frequency in a broad belt extending from the Mediterranean basin through the Middle East, Indian subcontinent, Burma and Southeast Asia, and Melanesia and islands of the Pacific. In Egypt it is considered the most common chronic hemolytic anemia (85.1%).[11]

A carrier rate of 9-10.2% has been estimated in 1000 normal random subjects from different geographical areas of Egypt.[12] It is also relatively common in populations of African descent. The highest incidences are reported in Cyprus (14%), Sardinia (12%), and South East Asia.[12] According to recent data collected through the Hereditary Disease Program of the World Health Organization and based on local surveys and reports by visiting experts, the carriers of hemoglobin disorders in the world are estimated to be 269 million.

The high gene frequency of β-thalassemia in these regions is most likely related to the selective pressure from Plasmodium falciparum malaria, as it is indicated by its distribution quite similar to that of present or past malaria endemia.[13] Carriers of β-thalassemia are indeed relatively protected against the invasion of Plasmodium falciparum. However, because of population migration and, to a limited extent, slave trade, β-thalassemia is, at present, also common in Northern Europe, North and South America, Caribbean, and Australia.

Pathogenesis

β-thalassemia is caused by the reduced (β^+)or absent (β^0) synthesis of the β globin chains of the hemoglobin (Hb) tetramer, which is made up of two α globin and two β globin chains (α2β2). Three clinical and hematological conditions of increasing severity are recognized, i.e., the β-thalassemia carrier state, thalassemia intermedia, and thalassemia major. The β-thalassemia carrier state, which results from heterozygosity for β-thalassemia, is clinically asymptomatic and is defined by specific hematological features. Thalassemia major is a severe transfusion-dependent anemia. Thalassemia intermedia comprehend a clinically and genotypically very heterogeneous group of thalassemia-like disorders, ranging in severity from the asymptomatic carrier state to the severe transfusion-dependent type. The clinical severity of β-thalassemia is related to the extent of imbalance between the α globin and nonα globin chains. The nonα globin chains include, in addition to the β globin chains, also the γ chains, which are a specific component of fetal Hb (HbF; α2γ2) and are present in a small amount in normal adult individuals and in increased but variable amount in the β-thalassemia syndromes. Within the red blood cell precursors, when the β globin chains are reduced or absent, the unassembled α chains precipitate and lead to oxidative damage of the cell membrane, thereby resulting in apoptosis (ineffective erythropoiesis).[14–16]

Clinical Features

Beta-thalassemia major

Homozygotes for β-thalassemia may develop either thalassemia major or thalassemia intermedia. Individuals with thalassemia major usually come to medical attention within the first 2 years and require regular blood transfusion to survive. Those presenting later do not require transfusion and receive a diagnosis of thalassemia intermedia. Differentiation of thalassemia major from thalassemia intermedia at presentation is a difficult and critical issue that should be strongly pursued, because it may avoid unnecessary transfusions in thalassemia intermedia and start early transfusions in thalassemia major. Analysis of the genotype at the α and β loci and testing for the presence of ameliorating genetic factors may be useful in this differentiation. [17]

Affected infants with thalassemia major fail to thrive and become progressively pale. Feeding problems, diarrhea, irritability, recurrent bouts of fever, and enlargement of the abdomen, caused by splenomegaly. If a regular transfusion program that maintains a minimum Hb concentration of 95–105 g/L is initiated, then growth and development are normal until the age of 10–11 years. After the age of 10–11 years, affected individuals are at risk of developing severe complications related to posttransfusional iron overload, depending on their compliance with chelation therapy.

Complications of iron overload include growth retardation and failure of sexual maturation and also those complications observed in adults with HFE-associated hereditary hemochromatosis (HH): involvement of the heart (dilated myocardiopathy and pericarditis), liver (chronic hepatitis, fibrosis, and cirrhosis), and endocrine glands (resulting in diabetes mellitus and insufficiency of the parathyroid, thyroid, pituitary, and, less commonly, adrenal glands). Infectious complications, including hepatitis B and C virus and HIV, relatively common in old patients, are now very rare because of the introduction of vaccination (hepatitis B) and development of blood donor screening methods based on viral nucleic acid enzymatic amplification. Other complications are hypersplenism (usually related to late and irregular transfusions), venous thrombosis (occurring especially after splenectomy), osteoporosis (which is multifactorial, being strongly associated with bone marrow expansion, hypogonadism, diabetes mellitus, hypothyroidism, hypoparathyroidism, low insulin-like growth factor 1, cardiac dysfunction, and may regard specific patients because of the presence of peculiar genetic predisposing factors , and lung hypertension (secondary to chronic hemolysis).[18–19]The risk for hepatocellular carcinoma is increased because of liver viral infection, iron overload, and longer survival.[20]

Survival of individuals who have been well transfused and treated with appropriate chelation extends beyond the age of 30 years. Myocardial disease caused by transfusional siderosis is the most important life-limiting complication of iron overload in β-thalassemia. In fact, cardiac complications are reported to cause 71% of deaths in individuals with β-thalassemia major. [21]

The classic clinical picture of thalassemia major is currently only seen in some developing countries, in which the resources for carrying out long-term transfusion programs are not available. The most relevant features of untreated or poorly transfused

individuals are growth retardation, pallor, jaundice, brown pigmentation of the skin, poor musculature, genu valgum, hepatosplenomegaly, leg ulcers, development of masses from extramedullary hematopoiesis, and skeletal changes that result from expansion of the bone marrow. These skeletal changes include deformities of the long bones of the legs and typical craniofacial changes (bossing of the skull, prominent malar eminence, depression of the bridge of the nose, tendency to a mongoloid slant of the eye, and hypertrophy of the maxillae, which tends to expose the upper teeth). Individuals who have not been regularly transfused usually die before the third decade.

β-thalassemia intermedia

Patients with thalassemia intermedia show a markedly heterogeneous clinical picture. Principle symptoms are pallor, jaundice, cholelithiasis, liver and spleen enlargement, moderate to severe skeletal changes, leg ulcers, extramedullary masses of hyperplastic erythroid marrow, a tendency to develop osteopenia and osteoporosis, and thrombotic complications resulting from a hypercoagulable state because of the lipid membrane composition of the abnormal red blood cells (particularly in splenectomized patients).[11,22] By definition, transfusions are not required or only occasionally required. Iron overload occurs mainly from increased intestinal absorption of iron caused by ineffective erythropoiesis.

The mechanism of iron hyperabsorption in β-thalassemia has been elucidated. Iron absorption is essentially controlled by hepcidin, a small peptide secreted by the hepatocytes, which blocks iron uptake in the intestine and iron release from the reticuloendothelial system. [23] Hepcidin binds ferroportin, an iron transporter present on the surface of absorptive enterocytes, macrophages, and hepatocytes, and the hepcidin-ferroportin complex is internalized and rapidly degraded, thereby explaining the defective intestinal iron absorption.[24] Transcription of hepcidin is controlled by bone morphogenetic proteins (BMP), which activate the SMAD protein complex that translocates to the nucleus and stimulates hepcidin transcription. [25] Hepcidin expression is enhanced by iron overload and inflammation, whereas it is inhibited by anemia and hypoxia.

Serum from untransfused thalassemia patients have high level of growth differentiation factor [25], a member of the transforming growth factor superfamily-like BMPs, whose produc tion is related to the expansion of the erythroid compartment. (Figure 1) Growth differentiation factor [25] inhibits hepcidin expression by opposing the effect of BMP, thereby leading to intestinal iron hyperabsorption and increased iron release from macrophages. [26] Consequently, the secretion of ferritin is reduced and its serum level relatively decreased. Therefore, in thalassemia intermedia, the determination of serum ferritin underestimates the extent of iron accumulation. In contrast, in thalassemia major, red cell transfusions decrease the erythropoietic drive and increase iron overload, resulting in relatively high hepcidin level, which reduces dietary iron absorption and iron release from macrophages. This may contribute to iron accumulation in Kupffer cells. [27–29] The associated complications of iron overload may present later, but it may be as severe as those seen in individuals with thalassemia major who depend on transfusions.

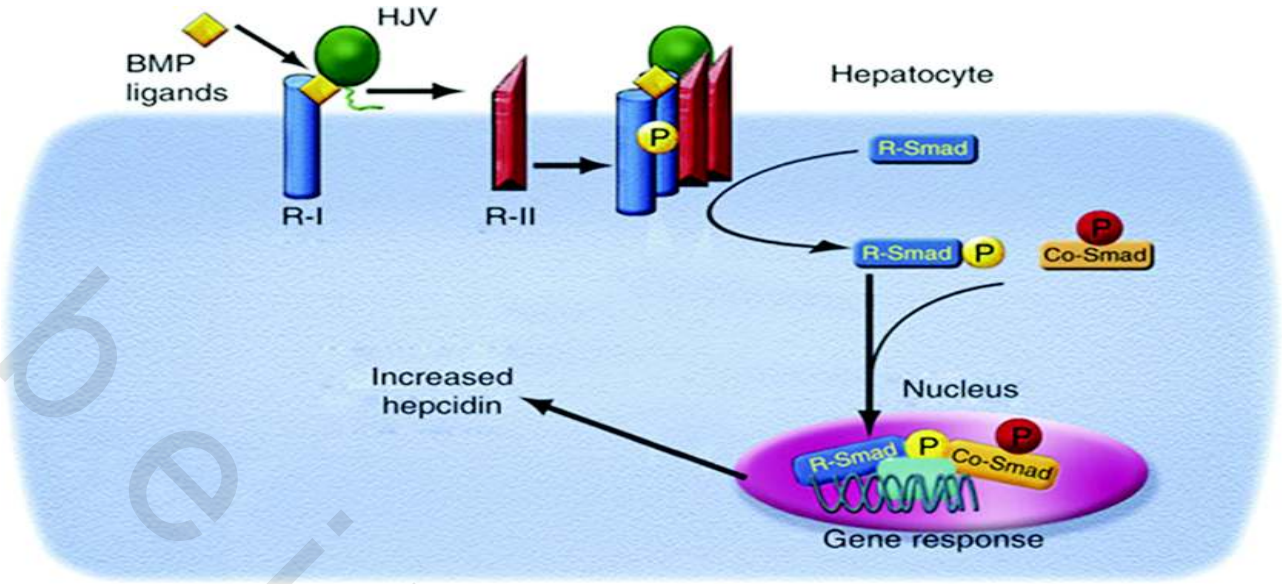

Fig. 1: Mechanism of control of hepcidin synthesis. [25]

Hematological Features

β-thalassemia carrier state

Carriers of β-thalassemia are clinically asymptomatic. The characteristic hematological features are microcytosis (reduced red blood cell volume), hypochromia (reduced red blood cell Hb content), increased HbA2 level (the minor component of the adult Hb, which is made up of two α and two δ chains [α2δ2]), and slightly imbalanced α/β + globin chain synthesis ratio (1.5–2.4).

The Hb pattern of β-thalassemia heterozygotes is characterized by 92–95% HbA, >3.8 HbA2, and variable amount of HbF (0.5–4%). Examination of the blood smear reveals microcytosis, hypochromia, and marked variations in size and shape of the red blood cells.

Thalassemia major

Patients with thalassemia major have a severe microcytic and hypochromic anemia, associated with increased number of red blood cells and low mean corpuscular volume (MCV) and mean corpuscular Hb (MCH). Peripheral blood smear shows, in addition to microcytosis and

Hypochromia, anisocytosis, poikilocytosis (spiculated tear drop and elongated cells), and nucleated red blood cells (i.e., erythroblasts). The number of erythroblasts is related to the degree of anemia and is markedly increased after splenectomy.

Hb pattern (by cellulose acetate electrophoresis or high-performance liquid chromatography [HPLC]) varies according to the type of β-thalassemia. In β^0-thalassemia, characterized by the lack of β globin chain synthesis, HbA is absent, HbF is 95–98%, and HbA2 is 2–5%. In β^+-thalassemia homozygotes with a residual variable β globin synthesis

or β^0/ β^+compound heterozygotes, the Hb pattern shows HbA between 10 and 30%, HbF in the order of 70–90%, and HbA2 of 2–5%.

Bone marrow examination is usually not necessary for diagnosis of affected individuals. Bone marrow is extremely cellular, mainly as a result of marked erythroid hyperplasia, with a myeloid/erythroid ratio reversed from the normal 3 or 4 to 0.1 or less.

Thalassemia intermedia

Patients with thalassemia intermedia have a moderate anemia and show a markedly heterogeneous hematological picture, ranging in severity from that of the β-thalassemia carrier state to that of thalassemia major.

Molecular Genetics

β-thalassemia

The β globin gene (HBB) maps in the short arm of chromosome 11 in a region also containing the δ globin gene, the embryonic epsilon gene, the fetal A-γ and G-γ genes, and a pseudogene (ψB1). The five functional globin genes are arranged in the order of their developmental expression.

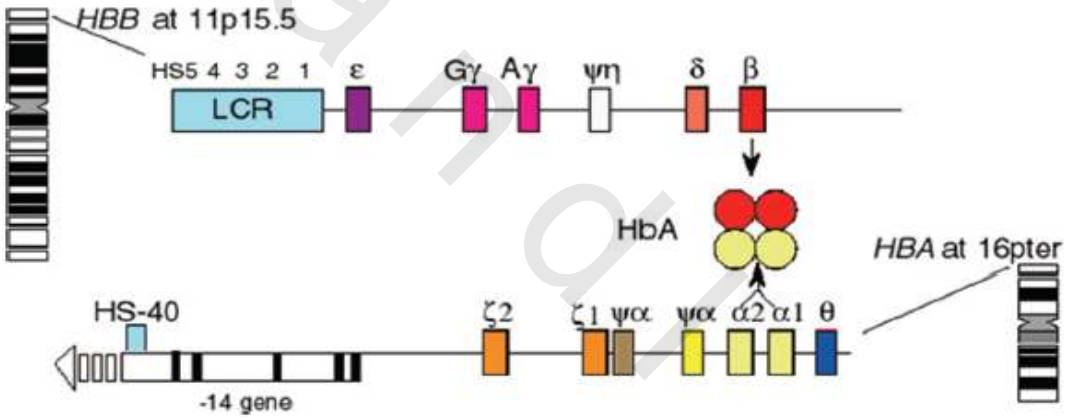

Fig. 2: Chromosome localization and structure of α and β globin gene clusters [30]

The HBB gene, which spans 1.6 Kb, contains three exons and both 5′ and 3′ untranslated regions (UTRs). The HBB is regulated by an adjacent 5′ promoter in which a TATA, CAAT, and duplicated CACCC boxes are located. A major regulatory region, containing also a strong enhancer, maps 50 Kb from the β globin gene. This region, dubbed locus control region (LCR), contains four (HS-1 to HS-4) erythroid specific DNAse hypersensitive sites (HSs), which are a hallmark of DNA-protein interaction. Each HS site is constituted by a combination of several DNA motifs interacting with transcription factors, among which the most important are GATA-1 (GATA indicates the relative recognition motif), nuclear factor erythroid 2, erythroid Kruppel-like factor, and friend of GATA 1.The importance of LCR for the control of the β-like globin gene expression has been discovered by studying a series of naturally occurring deletions that totally or partly remove the HS sites and result in the inactivation of the intact downstream β globin gene. Several transcription factors bind and regulate the function of the HBB gene, the most

important of which is erythroid Kruppel-like factor 1, which binds the proximal CACCC box, and whose knockout in the mouse leads to a β-thalassemia-like clinical picture.

β-thalassemias are heterogeneous at the molecular level. More than 200 disease-causing mutations have been so far identified. The majority of mutations are single nucleotide substitutions or deletions or insertions of oligonucleotides leading to frameshift. Rarely β-thalassemias result from gross gene deletion.

Point mutations affecting the β globin expression belong to three different categories: mutations leading to defective β-gene transcription (promoter and 5′ UTR mutations); mutations affecting messenger RNA (mRNA) processing (splice-junction and consensus sequence mutations, polyadenylation, and other 3′ UTR mutations); and mutations resulting in abnormal mRNA translation (nonsense, frameshift, and initiation codon mutations).

B^0-thalassemias, characterized by the complete absence of β chain production result from deletion, initiation codon, nonsense, frameshift, and splicing mutations, especially at the splice-site junction. On the other hand, β^+-thalassemias, characterized by reduced production of the β chains, are produced by mutations in the promoter area (either the CACCC or TATA box), the polyadenylation signal, and the 5′ or 3′ UTR or by splicing abnormalities. According to the extent of the reduction of the β chain output, the β+-thalassemia mutations may be divided into severe, mild, and silent.

The silent mutations, which are characterized by normal hematological findings and defined only by a mildly imbalanced α/β globin chain synthesis ratio, result from mutations of the distal CACCC box, the 5′ UTR, the polyadenylation signal and some splicing defects. The mild mutations show moderate thalassemia-like hematological features and imbalanced globin chain synthesis and are produced by mutations in the proximal CACCC box, TATA box, 5′ UTR, or exon 1, causing alternative splicing (Hb Malay, HbE, and codon 24 T--A), or in the consensus splicing sequence, 3′ UTR, and poly-A site. Mutations activating a cryptic splicing site in exon 1, at codons 19, 26, and 27, are associated with a mild or silent phenotype, because of the preferential use of the normal splice site, and result in the production of the abnormal Hb Malay, HbE (extremely common in South East Asia), and Hb Knossos, respectively.

A few β^0-thalassemias display a mild phenotype (for instance, cd6-A and cd 8-AA) because of the linkage with the nondeletion promoter mutation of the G-γ gene (−158 G-γ), which is associated with high production of HbF during hematologic stress.

Deletions affecting the β globin gene are very rare, except for a 619-bp deletion removing the 3′ end of the β globin gene, which is relatively common in Sind and Punjab populations of India and Pakistan. Another group of deletions (complex β-thalassemia), in addition to the β globin gene, involve also the δ (δ- β^0-thalassemia), the δ and A-γ genes (G-γ A-γ δβ0-thalassemia), or the whole β globin gene cluster. Finally, partial or total deletions of the LCR, but leaving the β globin gene intact, inactivate the β globin gene.

Despite the marked molecular heterogeneity, the prevalent molecular defects are limited in each at risk population, in which 4–10 mutations usually account for most of the β globin disease-causing allele. (Figure 2)

β-thalassemic hemoglobinopathies

Under this category are included different structurally abnormal Hbs associated with a β-thalassemia phenotype. Three different molecular mechanisms are responsible for these phenotypes: activation of cryptic splice site in exons, leading, for instance, to the production of HbE; formation of a δ-β hybrid gene; or mutation causing hyperunstable β globin.

The δ-β hybrid genes leading to β-thalassemia are dubbed Hb Lepore genes and are made up of N-terminal amino acid sequence of the normal δ chain and the C terminal sequence of the normal β chain. Depending on the codon of transition from δ to β sequences, different Hb Lepore genes have been described. In peripheral blood, Hbs Lepore are present in low percentage of total Hb (around 10% in carriers) because of the low production, which depends on the reduced activity of the δ promoter and on the relative instability of the variant Hb. These characteristics explain why Hbs Lepore are considered a β-thalassemia-like mutation.

Hyperunstable β globins comprehend a group of β globin mutants, which result in the production of Hb variants that are extremely unstable and precipitate before assembling with the α chains to produce the Hb tetramer. This results in ineffective erythropoiesis, which is exacerbated by the concomitant relative excess of the α chains, thereby leading to phenotypic thalassemia-like manifestations (more frequently in thalassemia intermedia than in the heterozygous state).

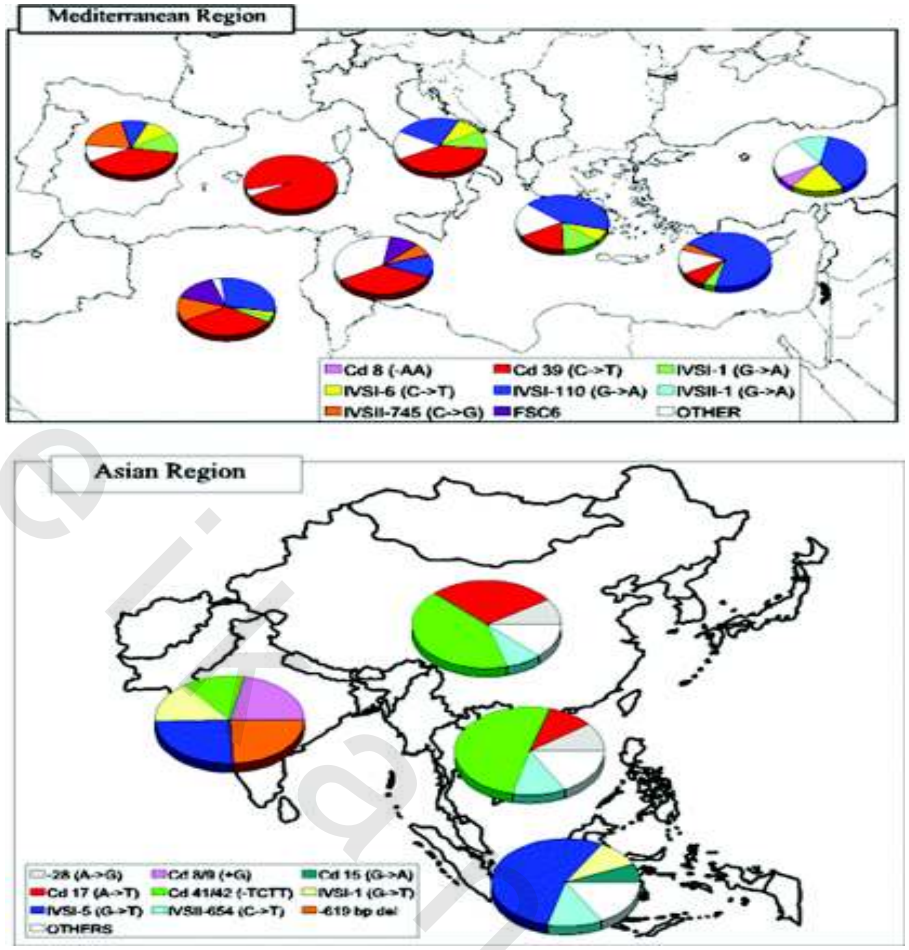

Fig 3: Most common β-thalassemia mutations in different countries. [31]

β-thalassemia-like mutations mapping outside the β globin gene cluster

In rare instances, the β-thalassemia defect does not lie in the β globin gene cluster. Some mutations in the X-linked transcription factor GATA-1 may produce thrombocytopenia associated with thalassemia trait, whereas molecular lesions affecting the general transcription factor TFIIH result, in addition to thalassemia-like hematological features, in xeroderma pigmentosum and trichothiodystrophy. (Figure 3) [30,31]

Molecular diagnosis of β -thalassemia

Commonly occurring mutations of the HBB gene are detected by a number of polymerase chain reaction (PCR)-based procedures. The most commonly used methods are reverse dot blot analysis or primer-specific amplification with a set of probes or primers complementary to the most common mutations in the population from which the affected individual originated. [32] Other methods based on real-time PCR or microarray technology because of their reproducibility, rapidity, and easy handling are potentially suitable for the routine clinical laboratory. [33,34]

If targeted mutation analysis fails to detect the mutation, scanning or sequence analysis can be used. Sensitivity of both mutation scanning and sequence analysis is 99%.

In the meantime, the presence of an extended deletion should be investigated by using multiplex ligation-dependent probe amplification (MPLA).

Phenotype-genotype correlation

Homozygous β -thalassemia

Homozygosity or compound heterozygosity for β-thalassemia most commonly result in the clinical phenotype of transfusion-dependent thalassemia major. However, a consistent proportion of homozygotes develop milder forms, called thalassemia intermedia, which range in severity from thalassemia major to the β-thalassemia carrier state. [35–39]

To understand the clinical-molecular relationships, we should remember that the main pathophysiological determinant of the severity of the β-thalassemia syndromes is the extent of α/nonα globin chain imbalance. Therefore, any factor capable of reducing the α/nonα chain imbalance may have an ameliorating effect on the clinical picture.

The most clinically important mechanism consistently resulting in thalassemia intermedia is the coinheritance of homozygosity or compound heterozygosity for mild β -thalassemia alleles, namely a β -thalassemia defect associated with a consistent residual output of β chains from the affected β globin locus. The most common mild mutations are β+IVS-I nt 6 (T⇒C)—found in the Mediterranean area, and β codon 26 (G⇒A), which gives rise to HbE, prevailing in South-East Asia. By contrast, compound heterozygotes for a mild and a severe defect result in a spectrum of phenotypes ranging from severe to mild forms. Therefore, from the clinical point of view, in presence of a mild β-thalassemia/severe β-thalassemia genotype, we cannot predict the development of mild clinical picture.

The mildest β -thalassemia alleles are the silent alleles , which show normal hematological features and can be identified solely by a slight imbalance of α/nonα globin chain synthesis ratio.[40–42] Homozygosity for silent alleles produces a very mild form of thalassemia intermedia. Mild β-thalassemia also results from the β-silent/β-mild or β-silent/β-severe genotypes.

It should be pointed out that, even the homozygous β0 or severe β^+-thalassemia may develop an attenuated form resulting from coinherited modifying ameliorating genetic factors.

The second mechanism leading to mild β-thalassemia is the coinheritance of homozygous β-thalassemia and an α-thalassemia determinant that, by reducing the α chain output, decreases the α/nonα chain imbalance. A single α globin gene deletion is sufficient to improve the clinical phenotype of homozygous β^+-thalassemia, whereas in β^0-thalassemia, the deletion of two α globin genes or the presence of an inactivating mutation of the major α2 globin gene is necessary. [36,43,44]

The third mechanism is the coinheritance of a genetic determinant that is able to sustain a continuous production of γ chains in adult life, thereby reducing the extent of the α/nonα chain imbalance. The nature of the β-thalassemia mutation per se may affect the ability to produce γ chains. This mechanism occurs in δ β^0-thalassemia, which is because of deletions of variable extent within the β globin cluster, and in those more limited

deletions involving only the 5′ region of the β globin promoter. In other cases, the reason for the high γ chain output depends on the co-transmission of a nondeletion form of hereditary persistence of HbF (HPFH), caused by point mutations at G-γ or A-γ promoters (−196 C→T A-γ ; −158 C→T G-γ). A C→T mutation at position −196 A-γ has been found to be associated in cis with the codon 39 nonsense mutation in some Sardinian β-thalassemia chromosomes (Sardinian $\delta\beta^0$-thalassemia).[45] The increase in γ chain production from the −196 A-γ gene probably compensates for the absence of β chain production from the affected β locus. Compound heterozygosity for this determinant and for typical β–thalassemia, thus, develops thalassemia intermedia. Homozygotes for the Sardinian δβ0-thalassemia are clinically normal and can be detected solely by hematological analysis. [46] Heterozygotes for Sardinian δβ-thalassemia have thalassemia-like hematological features and high HbF levels. [47] In contrast, C→T −158 G-γ is silent both in normal subjects and β-thalassemia heterozygotes but leads to a high HbF production rate during hematopoietic stress, as occurs in homozygous β-thalassemia or sickle cell anemia. [48] The −158 G-γ mutation may be associated with IVS II nt 1 (G→A), frameshift 8 (AA), frameshift 6 (-A), and someone with codon 39 nonsense mutations, thereby explaining the mild phenotype possibly associated with these mutations.

Coinheritance of genetic determinants capable of sustaining a continuous production of HbF in adult life and mapping outside the β globin cluster may also determine a mild phenotype. It should be noted that the residual amount of HbF in normal adults is unevenly distributed and mainly contained in a subpopulation of red blood cells, denominated “F cells.” Both HbF and F-cells percentage, in normal adults and in β-thalassemia heterozygotes, show a wide variation quantitative trait loci (QTLs)with a continuous distribution.[38] Those individuals with a moderate increase of HbF in the order of 0.8–5%, with an heterocellular distribution, are indicated as carriers of heterocellular HPFH. So far, two HPFH have been mapped on chromosome 2p16 and chromosome 6q23.[49–51]The locus on chromosome 2p16 has been located by genome-wide association study on the BCL11A gene, and the single nucleotide polymorphism (SNP) rs11886868 in its intron 2 was found strongly associated with HbF levels. Furthermore, the C allele of this SNP was significantly higher in Sardinian individuals with elevated HbF levels (HPFH). The BCL11A variants were shown to influence HbF levels also in nonanemic Caucasians from a European twin study.[50] The same C variant was significantly higher in β0-thalassemia homozygotes for the codon 39 nonsense mutation with a mild phenotype (thalassemia intermedia), as compared with those with the same β globin genotype, but with a severe phenotype, compensating for the imbalance of Hb production through the augmentation of HbF levels.[49] Furthermore, the BCL11A variant C allele by increasing HbF levels was associated with a mild phenotype also in sickle cell disease.[49,52] In conclusion, the BCL11A gene is able to modify the phenotype of homozygous β-thalassemia and sickle cell anemia by increasing HbF levels. The BCL11A gene encodes a zinc-finger transcription factor, which regulates the globin switching during ontogeny by interacting with specific sequence in the β globin cluster and repressing the HbF expression. [53,54]

According to these results, the identification of BCL11A polymorphism in young homozygous βthalassemia and sickle cell anemia patients may serve as a prognostic indication for the severity of the disease. Furthermore, targeted down-regulation of BCL11A in patients could elevate HbF levels, thereby ameliorating the severity of these inherited anemias.

Another HPFH locus has been mapped many years ago on chromosome 6q23 by linkage analysis in a large Indian family with segregating β-thalassemia[55] and confirmed more recently by twin studies.[48] Further studies have shown that the chromosome 6 genetic variant responsible for HbF variation maps in the HBS1L-MYB region. Specifically, the G allele of SNP rs9389268 is associated with high HbF levels. The causal variant in this region and its mechanism for increasing HbF is not yet known. HBS1L is a putative member of the "GTPase superfamily," whereas MYB has a crucial role in normal erythropoiesis. Recent studies have shown that the HBS1L-MYB intergenic polymorphisms contain regulatory sequences controlling MYB expression.[56] However, further studies are necessary to elucidate the biological role of these two genes in the modulation of HbF. The HBS1L-MYB locus contributes 3–7% of trait variance,[49,52] whereas the BCL11A variant explain 7–12%.[52,57]

Two QTL loci mapping on chromosome 8([58,59] and chromosome X,[60,61] respectively, have not been validated in subsequent genome-wide linkage and association studies. [49,50]

The loci-modulating HbF levels explain only partially its variation. This consideration clearly indicates the existence of other QTL traits for HbF in the human genome. Taken together, the loci are able to correctly predict 75% of the phenotypes of homozygous β^0-thalassemia. [62]

Heterozygous β –thalassemia

From the clinical point of view, heterozygous β-thalassemia, whether β^0 or β^+ , is completely asymptomatic and is characterized hematologically by high red blood cell count, microcytosis, hypochromia, increased HbA2 levels, and unbalanced α/non-α globin chain synthesis. However, several environmental or genetic factors may modify this phenotype, leading either to thalassemia intermedia, despite the presence of a single β globin gene affected, or to hematologically atypical carrier states.

To date, two mechanisms have been identified, which may increase the clinical and hematological severity of β-thalassemia heterozygotes. The first is related to the coinheritance of both heterozygous β-thalassemia and triple or quadruple α globin gene arrangement, which, by increasing the magnitude of imbalance of α/non α globin chain synthesis, may cause an excess of unassembled α chains, thereby resulting in premature destruction of red blood cell precursors. β-thalassemia carriers who are heterozygous or homozygous for the triplicated α globin gene arrangement may indeed develop a clinical phenotype of intermediate severity.[63–66] Similarly, phenotype of thalassemia intermedia has also been seen in β-thalassemia carriers who have inherited a chromosome containing the quadruplicated α globin gene arrangement.[67–69] However, normal people carrying the triplicated or quadruplicated α globin gene arrangement have a normal phenotype most likely, because the small excess of α chains that is synthesized can be eliminated by proteolysis.

The other mechanism increasing the severity in β-thalassemia heterozygotes depends on the presence of a mutation in the β globin gene, which causes an extreme instability of the β globin chains.[70,71] Heterozygotes for this condition are more severely affected than subjects with the β-thalassemia carrier state, because in addition to producing an excess of α chains, they synthesize highly unstable β chains that bind heme and precipitate in red

blood cell precursors before assembling with the α chains. This leads to the production of inclusion bodies made up of α and unstable β chains. β-thalassemia resulting from hyperunstable β chains is transmitted in a dominant fashion or may result from a de novo mutation.

Mutations leading to hyperunstable β chains include missense mutations, minor deletions leading to loss of intact codon, and frameshifts. Most mutations in the phase termination codons that result in dominant β-thalassemia lie in exon 3, whereas those producing the typical recessively inherited forms are located in exons 1 or 2. In exons 1 or 2 mutations, very little β globin mRNA is found in the cytoplasm of red blood cell precursors, whereas exon 3 mutations are associated with a substantial amount of abnormal cytoplasmic mRNA. This leads to the synthesis of truncated β chains products, which are unstable and thereby act in a dominant-negative fashion, causing premature destruction of red blood cells. This difference depends on the fact that premature termination codon mutations lying in exons 1 or 2 activate the process of nonsense-mediated mRNA decay, therefore precluding the accumulation of mRNA encoding for truncated peptides. [72] The diagnosis of dominant β-thalassemia is difficult, because the unstable β globin chains in peripheral blood are not easily detected, not even by very accurate electrophoretic or chromatographic procedures. The suspicion for such conditions should derive from the presence of β-thalassemia-like disorders of intermediate severity arising de novo or transmitted according to a dominant pattern.

Exceptionally, β-thalassemia intermedia may develop in subjects heterozygous for β-thalassemia because of a mosaic somatic deletion of the in trans β globin gene in a subpopulation of hematopoietic cells. [73,74]

Compound heterozygosity for β-thalassemia and some β chain structural variant (HbD-Los Angeles β 121 Glu→Gln; HbC β 6 Glu→Lys; HbO-Arab β 121 Glu→Lys) may produce thalassemia intermedia as a result of globin chain imbalance in combination with the modified structural and functional characteristic of the variants. [75]

The proposed role, if any, of the α-Hb stabilizing protein (AHSP) as a modulating factor of the phenotype has not yet clarified. [76] AHSP forms a stable complex with free α-Hb and protects free α chain from precipitation, thereby acting as a specific α-Hb molecular chaperone. In the mouse system, knockout of AHSP leads to reduced lifespan of circulating red blood cells, causing increased apoptosis of erythroid precursors and exacerbating the severity of heterozygous β-thalassemia, which usually displays a thalassemia intermedia phenotype. [76] The studies of AHSP in humans led to inconstant results.It seems, however, that variation of the AHSP level may be able to aggravate the phenotype of simple heterozygotes for β-thalassemia. [77,78]

Other clinical genetic modifiers

In addition to the variation of the phenotype resulting from allelic heterogeneity at the β globin locus, from the effect of α or γ globin gene mutation or from coinheritance of heterocellular HPFH delineated above, the phenotype of β-thalassemia could also be modified by the action of genetic factors mapping outside the globin gene cluster and not influencing the HbF. Among these factors, the ones best delineated so far are those affecting bilirubin, iron, and bone metabolisms. [79]

Because of the rapid turnover of red cell precursors and the resulting breakdown of the heme products, both homozygotes and heterozygotes for β-thalassemia may develop mild jaundice and have the propensity to gallstone formation. In β-thalassemia, the level of indirect bilirubin and gallstone formation are related to a polymorphic motif in the promoter of the gene involved in the hepatic glucuronidation of bilirubin, namely bilirubin UDP-glucuronosyltransferase (UGT1A). In normal individuals, the promoter has six TA repeats in the TATA box (TA)6. Homozygotes for an additional repeat (TA)7 develop mild unconjugated hyperbilirubinemia (Gilbert syndrome) because of less efficient activity of UGT1. Studies performed in the last few years in thalassemia major, thalassemia intermedia, and β-thalassemia carrier state have shown that patients developing hyperbilirubinemia, jaundice, and gallstones usually have the more common and less efficient TA7 motif. [80–85] It should be pointed out that the same phenomenon has been observed in many varieties of hemolytic anemia. [86–89] These findings have led us to conclude that the Gilbert syndrome mutation acts as a modifying gene in β-thalassemia by determining or promoting the development of jaundice or gallstones.

Patients with β-thalassemia, especially when not well chelated, develop extensive iron accumulation in many tissues including liver, heart, and endocrine glands, in part, because of the destruction of transfused red blood cells and, in part, particularly in thalassemia intermedia, because of increased iron absorption. Some studies seem to indicate that the common mutation of the HFE gene (C282Y), which causes the common type of HH, might be involved in determining the variability of iron overload in patients with thalassemia intermedia. [90] Longo et al, by studying a large group of thalassemia major patients found that the presence of a single mutation in HFE gene (C282Y and H63D) does not influence the severity of iron loading, assessed by serum ferritin and liver iron concentration, likely because the effect of the mutations on iron overload is hidden as a result of treatment (i.e., posttransfusional iron overload and iron chelation). [91] Furthermore, homozygosity for the H63D mutation, whose functional significance in HH is still being evaluated, when coinherited with heterozygous β-thalassemia seems to determine an increase in iron overload. [92] Conversely, coinherited heterozygosity for β-thalassemia seems to increase the rate of iron accumulation in C282Y homozygotes. [93]

Beta-Thalassemia Carrier Identification

The typical phenotype of the β-thalassemia trait, essentially characterized by reduced MCV, MCH, and increased HbA2, may be modified by several coinherited genetic factor, which may cause problems in carrier identification.

It should be pointed out that there is marked evidence indicating that the high frequency of β-thalassemia in certain areas of the world is related to heterozygote advantage vis-a-vis Plasmodium falciparum malaria.[94] Exposure to malaria, however, resulted also in the expansion of polymorphisms at many other genetic loci, including human leukocyte antigen (HLA), tumor necrosis factor α, and intercellular adhesion molecule-1, which have an important role in the defense mechanisms against infectious diseases.[95-97] This consideration may indicate that children with β-thalassemia may respond to infections differently than normal children.

Coinheritance of heterozygous β-thalassemia and α-thalassemia may raise the MCV and the MCH, high enough to determine normal values at least in some of these double

heterozygotes. This may occur as a result of either a deletion of two α globin structural genes or as a nondeletion lesion affecting the major α globin gene (the two functional α genes, denominated as αl and α2, have a relative expression of 1:3). Fortunately, these carriers may be easily identified for their high HbA2 levels. [98,99]

Elevation of HbA2 is the most important feature in the detection of heterozygous β-thalassemia, but a substantial group of β-thalassemia heterozygotes may have normal HbA2. The first mechanism to account for the abnormally low HbA2 levels in a β-thalassemia carrier is the presence of a specific mild β-thalassemia mutation, such as the β+ IVS-I nt 6 mutation. [100]

A second common mechanism is the coinheritance of heterozygous β-thalassemia and δ-thalassemia. The decreased output of the δ globin chains may result in normalization of HbA2 levels. [101,102] Also, γδ- and δβ-thalassemia carriers have normal HbA2. However, all these normal-HbA2 atypical heterozygotes have low MCV and MCH. Because of this phenotype, normal HbA2 β-thalassemia heterozygotes should be differentiated from α-thalassemia heterozygotes by globin chain synthesis analysis and/or by α, β, and δ globin gene analysis. Δβ thalassemia, in addition, may easily be defined by the variable but markedly increased HbF.

Another major problem in carrier screening is the identification of silent β-thalassemia or the triple or quadruple α globin gene arrangement, both of which may lead to the production of intermediate forms of β-thalassemia by interacting with typical heterozygous β-thalassemia. Silent β-thalassemias are characterized by normal MCV and MCH values and normal HbA2 and by the fact that they are defined only by the slight imbalance in the α/nonα globin synthesis.

Nevertheless, on examining the hematological features of these carriers, one may find borderline HbA2 or MCV and MCH values, which may alert for the presence of atypical β-thalassemia, thus requiring further studies (globin chain synthesis or gene analysis). The most common silent β-thalassemia is the β+ −101 C→T mutation; others are very rare. [103]The triple-quadruple α globin gene arrangement may show a slight imbalance of α/nonα chain synthesis or, more commonly, may be completely silent. An extreme, although rare, instance of thalassemia gene combination, which may result in carrier identification, is the coinheritance of α, δ, and β-thalassemia, which may lead to a completely silent phenotype.[104]

Carrier detection procedure

Several procedures have been proposed for β-thalassemia carrier screening. [105]The cheapest and simplest is based on MCV and MCH determination, followed by HbA2 quantitation for subjects showing microcytosis (low MCV) and reduced Hb content per red blood cell (low MCH). However, because with this procedure a considerable proportion of double heterozygotes for β- and α-thalassemia may be missed (these are found in many populations, such as Sardinians, where both disorders are common), it can only be used in populations with a low frequency of α-thalassemia. It should be stated that HPLC is also capable of detecting Hb Knossos, a mild β-thalassemia allele, which is not identified by using common procedures for Hb analysis. In the presence of low MCV and MCH and elevated HbA2 levels, a diagnosis of heterozygous β-thalassemia is made. A phenotype characterized by microcytosis, hypochromia, normal-borderline HbA2, and normal HbF

may result from iron deficiency, α-thalassemia, γδ-thalassemia, β^{+}δ-thalassemia, or mild β-thalassemia. After excluding iron deficiency through appropriate studies (red blood cell, zinc protoporphyrin determination, and transferrin saturation), the different thalassemia determinants leading to this phenotype are discriminated by globin chain synthesis analysis and eventually by α, β, and δ globin gene analysis. (105)

In the presence of normal MCV and borderline HbA2 levels, we are inclined to suspect the presence of a silent mutation or the triple or quadruple α globin gene arrangement and, therefore, proceed directly to α- and β globin gene analysis, because the α/β globin chain synthesis ratio could also be normal. (106) Definition of the type of thalassemias in these carriers is solely recommended when they mate with a carrier of a typically high HbA2 β-thalassemia or an undetermined type of thalassemia. In those rare cases showing normal or low MCV-MCH, normal or reduced HbA2 levels, and high HbF, we suspect the presence of δβ-thalassemia, which should be differentiated from HPFH. (Figure 4) This distinction is performed by globin chain synthesis analysis (normal in HPFH and unbalanced in δβ-thalassemia) or β-cluster gene analysis or both.

Molecular diagnosis of modifying genes

Molecular diagnosis is carried out in patients affected by homozygous β-thalassemia for defining the genotype, which may be useful for predicting the severity of the disease, and in carriers identified by hematological analysis.

Delta globin gene analysis may be necessary to define double heterozygotes for δ- and β-thalassemia that may be mistaken for α-thalassemia trait. The suspicion of interacting δ-thalassemia may arise when borderline HbA2 levels are found or when family studies show segregating δ-thalassemia (characterized by normal MCV-MCH and low HbA2) and β-thalassemia. However, identification of δ and β double heterozygotes may be accomplished by globin chain synthesis analysis and/or α, β, and δ globin gene analysis.(106,107)

Definition of the δ-thalassemia mutation may be carried out using PCR-based methods. As in β-thalassemia, also in δ-thalassemia, each population at risk has its own spectrum of common δ-thalassemia mutations that may be defined through a limited number of specific primers/probes. In Sardinians, for instance, few δ-thalassemia mutations have been so far detected. The list of δ-thalassemia mutations is available at the repository of the human β and δ globin gene mutation. Although most of the δ-thalassemia determinants are in trans (on opposite chromosomes) to β-thalassemia, some have also been detected in cis (on the same chromosome). (108)

Definition of the α globin gene arrangement may be performed to discriminate between heterozygosity for α-thalassemia and double heterozygosity for δ- and β-thalassemia or γδ-thalassemia. This analysis could also be useful in defining coinherited α-thalassemia in homozygous β-thalassemia, which may lead to the prediction of a mild clinical condition. Deletion α^{0}- or α^{+}-thalassemias are detected by PCR using two primers flanking the deletion breakpoint, which amplify a DNA segment only in presence of specific deletions. (109) Nondeletion α-thalassemia may be detected by restriction endonuclease analysis or allelic oligonucleotide specific probes on selectively amplified α1 and α2 globin genes. α globin gene triplication and quadruplication may be detected by the MPLA procedure.

Definition of coinherited HPFH determinants can be useful in predicting the severity of the phenotype of an affected fetus. In fact, on increasing the γ chain output, coinherited HPFH with homozygous β-thalassemia may lead to a milder phenotype. The presence of high HbF in the parents may lead to the suspicion of double heterozygosity for β-thalassemia and HPFH. The 196 C→T in the A-γ gene and −158 C→T in the G-γ gene mutations have been proved to be capable of ameliorating the clinical phenotype of homozygous β-thalassemia. The HPFH determinants may easily be detected through restriction endonuclease or dot blot analysis with oligonucleotide-specific probes on PCR-amplified DNA. Furthermore, definition of the polymorphisms at the BCL11A and HBS1L-MYB region may lead to predict the development of a specific mild phenotype.

If targeted mutation analysis fails to detect the mutation, mutation scanning or sequence analysis can be used to detect mutations in the HBB coding region (mutations in the noncoding region would not be detected by this analysis). Sensitivity of both mutation scanning and sequence analysis is 99%. Deletions of variable extent of the β gene or of the HBB cluster that result in β-thalassemia or in the complex β-thalassemias, called γδ-thalassemia and δβ-thalassemia, are rare causes of β-thalassemia and testing that deletions is available clinically by using MPLA.

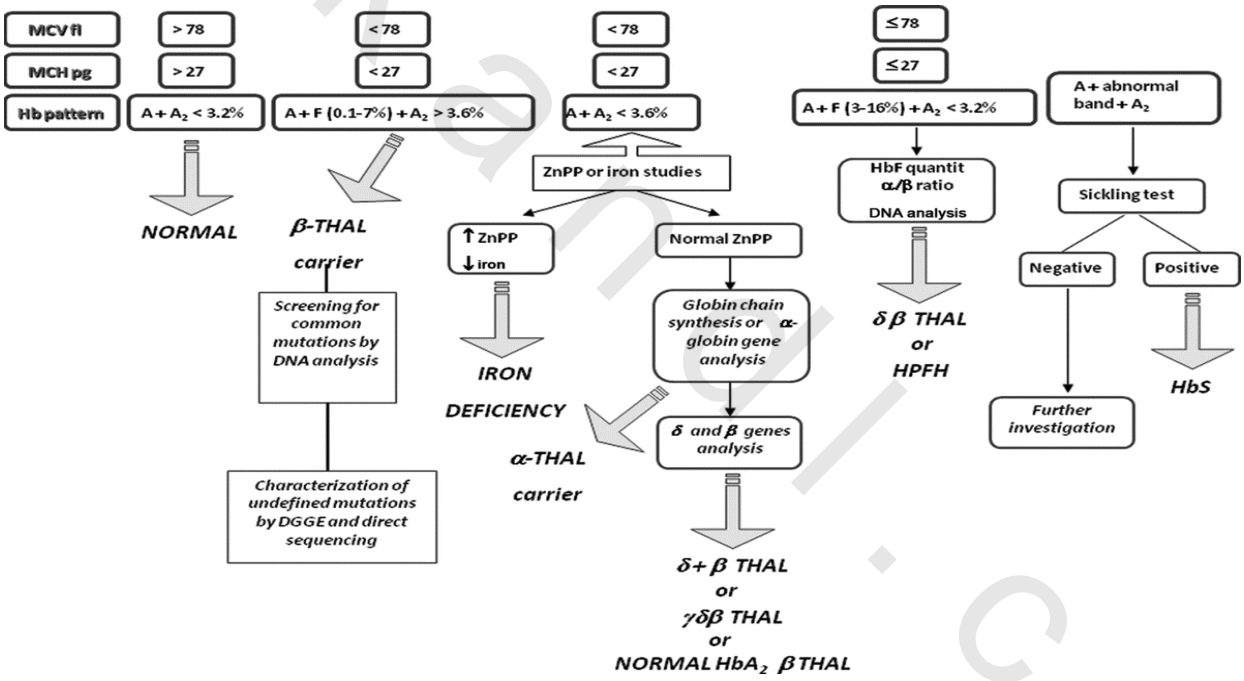

Fig. 4: Flowchart for thalassemia carrier identification. [105]

Population Screening

Because of the high carrier rate for HBB mutations in certain populations and the availability of genetic counseling and prenatal diagnosis, population screening is ongoing in several at-risk populations in the Mediterranean. [110] Carrier testing relies on hematological analysis. When the hematological analysis indicates a β-thalassemia carrier state, molecular genetic testing of HBB can be performed to identify a disease-causing mutation. If both partners of a couple have the HBB disease-causing mutation, each of their offspring has a ¼ risk of being affected. The optimal time for the assessment of

genetic risk, definition of carrier status, and genetic counseling is before pregnancy. It is appropriate to offer genetic counseling (including discussion on the availability of prenatal diagnosis, potential risks to offspring, and reproductive options) to young adults who are carriers.

Population screening associated with genetic counseling is extremely useful by allowing couples at risk to make informed decision on their reproductive choices.

Prenatal diagnosis

In high-risk pregnancies in which both members are defined carriers for β-thalassemia, prenatal diagnosis is possible by analysis of DNA extracted from fetal cells obtained by amniocentesis, usually performed at approximately 15–18 weeks' gestation, or chorionic villus sampling at approximately 10–12 weeks' gestation. Both disease-causing alleles must be identified before prenatal testing can be performed.Alternatively, following an accurate genetic counseling explaining the pros and cons of the procedure preimplantation genetic diagnosis may be considered.

Prenatal diagnosis by analysis of fetal cells in maternal blood is not yet available but is being investigated on a research basis. [111,112] Analysis of fetal DNA in maternal plasma for the presence of the father's mutation may lead to prenatal exclusion of homozygous β-thalassemia. This testing is not yet clinically available but under investigation on a research basis with promising results. [113,114]

In indeterminate-risk pregnancies, either one parent is a definite heterozygote and the other parent has a β-thalassemia-like hematologic picture, but no HBB mutation has been identified by sequence analysis, or a mother is a known heterozygote and the father is unknown or unavailable for testing, especially if the father belongs to a population at risk. In indeterminate-risk pregnancies, the prenatal testing strategy is the analysis for the known HBB mutation. If the known HBB mutation is present, analysis of globin chain synthesis is performed on a fetal blood sample obtained by percutaneous umbilical blood sampling at approximately 18–21 weeks' gestation.

Management of thalassemia

A comprehensive review of the management of thalassemia major and thalassemia intermedia has been published by Thalassemia International Federation. [115]

Transfusion program

In thalassemia major, regular transfusions correct the anemia, suppress erythropoiesis, and inhibit increased gastrointestinal absorption of iron. Before starting the transfusions, it is absolutely necessary to carry out hepatitis B vaccination and perform extensive red blood cell antigen typing, including Rh, Kell, Kidd, and Duffy and serum immunoglobulin determination, the latter of which detects individuals with IgA deficiency who need special (repeatedly washed) blood unit preparation before each transfusion. The transfusion regimen is designed to obtain a pretransfusion Hb concentration of 95–100 g/L. Transfusions are usually given every 2–3 weeks.

Treatment of individuals with thalassemia intermedia is symptomatic and based on splenectomy and folic acid supplementation. Treatment of extramedullary erythropoietic masses is based on radiotherapy, transfusions, or, in selected cases, hydroxyurea (with a protocol similar to that used for sickle cell disease). Hydroxyurea also increases globin γ chains and may have other undefined mechanisms. Because individuals with thalassemia intermedia may develop iron overload from increased gastrointestinal absorption of iron or from occasional transfusions, chelation therapy is started when the serum ferritin concentration exceeds 300 μg/L.[116]

Iron overload

The most common secondary complications are those related to transfusional iron overload, which can be prevented by adequate iron chelation. After 10–12 transfusions, chelation therapy is initiated with desferrioxamine B (DFO), administered 5–7 days a week by 12-hour continuous subcutaneous infusion via a portable pump. Recommended dosage depends on the individual's age and the serum ferritin concentration. Young children start with 20–30 mg/kg/day, increasing up to 40 mg/kg/day after the age of 5–6 years. The maximum dose is 50 mg/kg/day after growth is completed. The dose may be reduced if serum ferritin concentration is low. By maintaining the total body iron stores below critical values (i.e., hepatic iron concentration <7.0 mg per gram of dry weight liver tissue), DFO therapy prevents the secondary effects of iron overload, resulting in a consistent decrease in morbidity and mortality. Ascorbate repletion (daily dose not to exceed 100–150 mg) increases the amount of iron removed after DFO administration. Side effects of DFO are more common in the presence of relatively low iron burden and include ocular and auditory toxicity, growth retardation, and, rarely, renal impairment and interstitial pneumonitis. DFO administration also increases susceptibility to Yersinia infections. The major drawback of DFO chelation therapy is low compliance resulting from complications of administration.

In clinical practice, the effectiveness of DFO chelation therapy is monitored by routine determination of serum ferritin concentration. However, serum ferritin concentration is not always reliable for evaluating iron burden, because it is influenced by other factors, the most important being the extent of liver damage.

Determination of liver iron concentration in a liver biopsy specimen shows a high correlation with total body iron accumulation and is the gold standard for evaluation of iron overload. However, liver biopsy is an invasive technique involving the possibility (though low) of complications; liver iron content can be affected by hepatic fibrosis, which commonly occurs in individuals with iron overload and hepatitis C virus infection; and irregular iron distribution in the liver can lead to possible false-negative results.[117]

Magnetic resonance imaging (MRI) techniques for assessing iron loading in the liver and heart have improved. [118-120]T2 and T2* parameters have been validated for liver iron concentration. Cardiac T2* is reproducible, is applicable between different scanners, correlates with cardiac function, and relates to tissue iron concentration.[123,124] Clinical utility of T2* in monitoring individuals with siderotic cardiomyopathy has been demonstrated.[121] Calibration of T2* in the heart will be available in the near future. Magnetic biosusceptometry (SQUID), which gives a reliable measurement of hepatic iron concentration, is another option. [122]

Two other chelators have been introduced into clinical use: deferiprone and deferasirox.

Deferiprone, a bidentated oral chelator, available for several years in many countries, is administered in a dose of 75–100 mg/kg/day. The main side effects of deferiprone therapy include neutropenia, agranulocytosis, arthropathy, and gastrointestinal symptoms that demand close monitoring. [123]No correlation was found between deferiprone treatment and progression of liver fibrosis. [124]The effect of deferiprone on liver iron concentration may vary among the individuals treated. However, results from independent studies suggest that deferiprone may be more cardioprotective than DFO. Compared with those being treated with DFO, individuals on treatment with deferiprone have better myocardial MRI pattern and less probability of developing (or worsening preexisting) cardiac disease. [125-128]These retrospective observations have been confirmed in a prospective study. [129]After many years of controversy, deferiprone is emerging as a useful iron chelator equivalent/alternative to DFO. [130,131]

Deferasirox became available for clinical use in patients with thalassemia. It is effective in adults and children and has a defined safety profile that is clinically manageable with appropriate monitoring. The most common treatment-related adverse events are gastrointestinal disorders, skin rash, and a mild, nonprogressive increase in serum creatinine concentration. [132-134]

New strategies of chelation using a combination of DFO and deferiprone have been effective in individuals with severe iron overload; toxicity was manageable.[135-140] In the past few years, particular attention has been directed to the early diagnosis and treatment of cardiac disease because of its critical role in determining the prognosis of individuals with β-thalassemia. Assessment of myocardial siderosis and monitoring of cardiac function combined with intensification of iron chelation result in excellent long-term prognoses. [141]

Monitoring the effectiveness of transfusion therapy and chelation therapy

For individuals with thalassemia major, follow-up to monitor the effectiveness of transfusion therapy and chelation therapy and their side effects includes the following:

- Physical examination every month by a physician familiar with the affected individual and the disease.
- Assessment of liver function tests (serum concentration of alanine transaminase) every 2 months.
- Determination of serum ferritin concentration every 3 months.
- Assessment of growth and development every 6 months (for pediatric patients).
- Annual assessment includes the following:
    1. Ophthalmologic and audiologic examinations.
    2. Complete cardiac evaluation and evaluation of thyroid, endocrine pancreas, parathyroid, adrenal, and pituitary function (usually after 10 years).

3. Liver ultrasound evaluation, determination of serum α-fetoprotein concentration in adults with hepatitis C and iron overload for early detection of hepatic carcinoma.

4. Bone densitometry to assess for osteoporosis in the adult.

5. Assessment for liver and heart iron with MRI should be in general recommended after 10 years of age and repeated according to the severity of iron overload, transfusion, and chelation regimes. (142)

6. Regular gallbladder echography for early detection of cholelithiasis, particularly in individuals with the Gilbert syndrome genotype (i.e., presence of the [TA]7/[TA]7 motif in the promoter of the UGT1A gene).

Bone marrow transplantation (BMT) for β-thalassemia major(BTM)

Bone marrow transplantation (BMT) from an HLA-identical sibling represents an alternative to traditional transfusion and chelation therapy for BTM. If BMT is successful, iron overload may be reduced by repeated phlebotomy, thus eliminating the need for iron chelation. The outcome of BMT is related to the pretransplantation clinical conditions, specifically the presence of hepatomegaly, extent of liver fibrosis, and magnitude of iron accumulation. In children who lack the above risk factors, disease-free survival is over 90%. (143)A lower survival rate of approximately 60% is reported in individuals with all three risk factors. Chronic graft-versus-host disease of variable severity may occur in 5–8% of individuals.

BMT from unrelated donors has been performed on a limited number of individuals with β-thalassemia. Provided that selection of the donor is based on stringent criteria of HLA compatibility and that individuals have limited iron overload, results are comparable with those obtained when the donor is a compatible sib. (144) Cord blood transplantation from a related donor offers a good probability of a successful cure and is associated with a low risk of graft-versus-host disease. (145,146)

For couples who have already had a child with thalassemia and who undertake prenatal diagnosis in a subsequent pregnancy, prenatal identification of HLA compatibility between the affected child and an unaffected fetus allows collection of placental blood at delivery and the option of cord blood transplantation to cure the affected child.(147) On the other hand, in case of an affected fetus and a previous normal child, the couple may decide to continue the pregnancy and pursue BMT later, using the normal child as the donor.

Therapies under investigation

New chelation strategies, including the combination or alternate treatment with the available chelators, are under investigation. Induction of HbF synthesis can reduce the severity of β-thalassemia by improving the imbalance between α and nonα globin chains. Several pharmacologic compounds including 5-azacytidine, decytabine, and butyrate derivatives have had disappointing results in clinical trials. (148)These agents induce HbF by different mechanisms that are not yet well defined. Their potential in the management of β-thalassemia syndromes is still under investigation.

The studies on BCL11 pave the way to develop a therapy based on down-regulation of this gene, which may lead to an increase of HbF level, thereby ameliorating the clinical severity of the disease. Interesting possibility of a new therapy are open by the finding of association of low cMYB level (one of the gene mapping on 6q23) and high HbF level.[149]

The efficacy of hydroxyurea treatment in individuals with thalassemia is still unclear. Hydroxyurea is used in persons with thalassemia intermedia to reduce extramedullary masses, to increase Hb levels, and, in some cases, to improve leg ulcers. A good response, correlated with particular polymorphisms in the β globin cluster (i.e., C→T at −158 G-γ) has been reported in individuals with transfusion dependence.[150,151]

The possibility of correction of the molecular defect in hematopoietic stem cells by transfer of a normal gene via a suitable vector or by homologous recombination is being actively investigated. [152]The most promising results in the mouse model have been obtained with lentiviral vectors. [153,154] Specifically, the vector developed by Sadelain et al. containing HS2, 3, and 4 from LCR associated with an extended β globin gene led to partial correction of anemia in a β-thalassemia mouse.([152] Regarding the alternative approach, in a mouse model of sickle cell anemia, Chang et al. [155]were able to correct the molecular defect by homologous recombination by transfecting the embryonic stem cells from the affected mice with a DNA fragment containing the normal β globin gene sequences. Hematopoietic stem cells, derived from the corrected embryonic stem cells ex vivo, were able to produce HbA and HbS, thereby leading to a phenotype similar to human sickle cell trait. This and other similar experiments indicate the possibility of curing inherited hemoglobinopathies by homologous recombination in embryonic stem cells.

A new opportunity in the field of stem cell research has been recently determined by the discovery that transfection of four transcription factors (Oct 3–4, Sox 2, cMyc, and Klf4) in several types of adult cells (e.g., fibroblasts) may produce a cell with very similar characteristics to embryonic stem cells (induced pluripotent stem cell [iPS]). [156,157] Further research in this field demonstrated that cMyc may not be necessary, thereby reducing the risk of oncogenic transformation. iPS from patients affected by a number of monogenic diseases, such as spinal muscular atrophy or amyotrophic lateral sclerosis, have been already produced. Based on this technology, Hanna et al . [158] have obtained iPS from fibroblasts of a mouse affected by sickle cell anemia. The iPSs were corrected ex vivo by homologous recombination. Hematopoietic stem cells derived from ex vivo-corrected iPS were able to cure the affected mouse. This experiment offers proof of principle that genetically corrected iPS cells may lead to a cure for the inherited hemoglobinopathies.

Hematopoietic Stem Cells Transplantation for β-Thalassemia Major

Anemia and blood transfusion leads to iron overload that is fatal in a few years if not removed by iron chelation. Recent advances in understanding the pathophysiology of iron overload and iron chelation as well as the development of safe and efficient chelators [158] have transformed thalassaemia from a severe disease fatal in infancy to a chronic disease in which survival to 40 years and overis possible, at least in the industrialised world.

Rationale of transplantation in hemoglobinopathies

The basis of HSCT in thalassaemia consists in substituting the unhealthy haemopoietic stem cells generating ineffective erythropoiesis with allogeneic stem cells capable of normal erythropoiesis. Of course this replacement is not limited to the diseased erythropoietic component but involves the entire haemopoietic system.

The same concept is applicable to Sickle cell disease (SCD) in which erythropoiesis is effective but the haemoglobin produced is abnormal. HSCT in haemoglobinopathies is therefore a cellular replacement therapy. The transplantation approach for a non-malignant disease is different from transplantation in malignancies. In this setting, the detrimental immunologic properties of the engrafted HSC (i.e. GVHD) are not balanced by any anti-malignancy effect.

History of HSCT in thalassaemia HSCT in thalassaemia was developed and grew into accepted routine clinical practice. Thirty years ago ,the Pesaro group developed a prognostic scheme to predict transplant outcome in patients younger than 17 years. [159]

This prognostic scheme included three variables, all related to iron burden:

1. Quality of chelation received during the entire life before transplantation (regular versus not regular).
2. Hepatomegaly (more than 2 centimetres below the costal margin).
3. Presence of any degree of liver fibrosis on pre-transplant hepatic biopsy examination.

These variables stratified patients aged less than 17 years in 3 groups, having either none, one/two, or all three of the risk factors. These three factors identified as low risk patients who had optimal control of iron overload for their entire life and as high-risk patients who had no iron control and no prevention of iron-related tissue damage. Results were impressively different in the three groups. [159]

Even if this classification may not remain accurate today, it includes several important concepts which are still important for current clinical practice:

a. Optimal medical therapy (transfusion chelation therapy) is the key to a successful transplant. Patients who had optimal life-long control of iron overload and no iron-related tissue damage (liver fibrosis) had an outstanding survival and thalassaemia-free survival exceeding 90% and 80%, respectively. Conversely, patients who did not have this degree of control of iron overload had an unacceptable transplant-related mortality approaching 50%.
b. In the high-risk group, after reduction of the intensity of conditioning by reducing the cyclophosphamide dose from 200 mg/kg to 120 mg/kg, transplant related mortality persisted at 18% but the risk of thalassaemia recurrence was increased by up to 30%.
c. Adult patients had high transplant-related mortality (35%) and very limited risk of thalassaemia recurrence.

Alternative sources of hematopoietic stem cells

The large majority of transplant centres continue to use bone marrow-derived HSC rather than peripheral blood-derived HSC. In 2003 Locarelli first reported the feasibility of using HLA identical sibling, cord blood-derived haemopoietic stem cell for HSCT in haemoglobinopathies.

Alternative donors

The clinical development of HSCT from alternative donors has been more challenging and includes three possible approaches: 1. matched unrelated donors; 2. Mismatched related donors; 3. unrelated cord blood.

1. Matched unrelated donors

During the last decade, the use of unrelated donors for HSCT in malignancies has considerably expanded with improving results. Crucial determinants of this success have been the technological improvement of molecular HLA typing and the improved capability of selecting the appropriate unrelated donor.

2. Unrelated cord blood

Unrelated cord blood haematopoietic cells are a very promising source of stem cells for transplantation in non-malignant diseases.However unrelated cord blood transplant in thalassaemia has not been explored in systematic studies. [160]

Conditioning regimens

The Pesaro experience demonstrating a significant increase of thalassaemia recurrence rate with the reduction of cyclophosphamide dose from 200 mg/kg to 120 mg/kg confirmed the critical importance of the myeloablative capability of the conditioning regimen in this disease, which is characterised by an expanded erythroid system.

Non-myeloablative HSCT has the theoretical advantage, based on the experience with malignancies, of obtaining allogeneic engraftment with a very low early mortality rate. However, the increased reliance on immunological effects which is required to sustain engraftment requires a prudent approach to the wide use of such regimens and very few cases have been reported, with unsatisfactory results.

Ineffective erythropoiesis and chronic transfusion lead to iron overload. Thus, for thalassaemia, a conditioning regimen capable of eradicating an expanded bone marrow and providing adequate immunosuppression to sustain engraftment with acceptable toxicity on iron-damaged tissues is required. These challenges are not present in SCD, where chronic transfusion is not universal practice, the eventual transfusion burden is less relevant, and the erythroid bone marrow, at least relatively, is less expanded.

Mixed chimerism

It is generally observed that a significant group of patients (approximately 10%) develop long-term stable mixed chimerism after transplantation. Mixed chimera patients,

despite a limited (even 20%) engraftment, achieve a functioning graft status characterised by normal haemoglobin level, no red cell transfusion requirement, no increase in iron stores and a limited, not clinically relevant, erythroid hyperplasia. Thus, in chimera patients, the genetic disease is substantially under complete clinical control, without achieving a complete eradication of the thalassaemia haemopoietic clones . [161]

Supportive care

Supportive care comprises all treatments given to prevent, control, or relieve complications and side effects in the HSCT process. Supportive care in HSCT is essential in optimizing the outcome of the treatment.

Protective issues

Patients with HSCT are at great risk of developing infective complications because of marked immunosuppression and prolonged pancytopenia, together with mucosal injury. In addition to antimicrobial prophylaxis, there are other important strategies to prevent infections, together building up a network of infection control measures. Key points of these are: protective environment, protective clothing and equipment, hand hygiene, low microbial diet, vaccination and exclusion policy and monitoring infectious complications.

Protective environment (isolation) and cleaning

A variety of practices exist regarding the use of isolation for immunocompromised patients; however, the effectiveness of protective isolation has not been established. Numerous studies have assessed the effect of laminar airflow or HEPA filtration with conflicting results.

Personal and hand hygiene, protective clothing and equipment

Because a large proportion of infections in patients with neutropenia is associated with the patient's own microbial flora, the patient's personal hygiene is of outstanding importance. Hand washing and hand disinfection of nursing and other personnel is also important and has been proven by multiple, well-designed studies to be one of the most effective ways to prevent the transmission of infection. There have also been studies of the benefit of antiseptic baths, but the evidence for an association between antiseptic bathing and reduced risk of infection is contradictory.

Low bacterial diet

It has been argued that a diet containing food with low levels of bacteria can possibly help to reduce the number and/or severity of infections in transplanted patients. However, there is no clear evidence that the use of a low bacterial diet (LBD) actually decreases the number of infections.

Vaccination and exclusion policy

It is advisable that health care workers and visitors in contact with transplant patients should be vaccinated for influenza, especially during the flu season. Personnel should also possess protective titers for *Varicella-zoster virus* (VZV). Individuals showing signs and

symptoms of a respiratory, gastrointestinal or muco-cutaneous infection should be excluded from work or should not be allowed to visit the patient.

Monitoring infection rate and antimicrobial resistance

Every center should have a policy for monitoring the incidence of infections. At the same time trends of antimicrobial resistance must carefully be followed. The incidence of invasive fungal infections must be monitored, and a twofold increase within a six month period should prompt examination of possible environmental and logistic factors.

Nutritional and metabolic support

Nutritional and metabolic support prevent loss of lean body mass, fluid and electrolyte imbalance, increase patient comfort and improve survival for patients who are unable to eat or absorb nutrients for a prolonged period of time. The goal is to enable the patient to recover the ability to take in and absorb food orally as quickly as possible following transplantation.

Caloric and metabolic alterations

Most HSCT patients develop significant mucositis and have difficulties in maintaining adequate oral nutrition. Decreased oral intake caused by nausea, vomiting and diarrhoea, decreased nutrient absorption and loss of nutrients from the gut result in a negative nitrogen balance. This is further complicated by the catabolic effects on skeletal muscle exerted by the underlying disease, the conditioning regimen and subsequently by transplant complications such as GVHD and sepsis.

Nutritional support

Impaired nutritional status before transplantation is a negative prognostic factor for outcome after HSCT and better nourished patients have a shorter time to engraftment. Irrespective of nutritional status, however, parenteral nutritional support is commonly administered prophylactically after HSCT until patients are able to maintain an adequate oral nutritional intake, usually following bone marrow recovery. Although hypothetically enteral nutrition is possible, total parenteral nutrition (TPN) is largely favoured in HSCT patients because nausea, vomiting and oro-oesophageal mucositis prevent the insertion and subsequent tolerability of nasogastric tubes.

Evaluation of nutritional status and monitoring nutritional support

Nitrogen balance is considered the most accurate way of assessing nutritional status in HSCT recipients as it is the direct expression of the imbalance existing between protein breakdown and synthesis. From studies published of TPN in HSCT some kind of consensus can be derived concerning nutritional status/support monitoring parameters. Daily monitoring of weight (primarily to judge hydration status) is essential, together with electrolytes, BUN, creatinine, and glucose. Liver function tests, serum albumin, transferrin, triglyceride and nitrogen balance are also helpful (Table 1).

Table 1: Monitoring of nutritional support during the in-patient stay

Daily	Two times a week	Once a week
- weight (fluid balance) - blood glucose - serum electrolytes - BUN - serum creatinine - calorie and protein intake	- liver function tests - serum calcium - serum magnesium - serum phosphorus	- nitrogen balance - serum transferrin - serum albumin - serum triglyceride - serum zinc

Central venous devices

Health-care institutions purchase millions of intravascular catheters each year. Central venous catheters (CVC) are the devices most frequently used for vascular access in HSCT. Although CVC are indispensable in HSCT, they also represent a significant source of complications including catheter-related bloodstream infections, complications of insertion, venous thromboembolism, mechanical obstruction, dislodgment and leakage. The most important of these complications are bloodstream infections with an estimated incidence of 5/1000 patient days and a mortality rate between 3–25%.

There are four main possible mechanisms for developing a CVC related infection:

1. Migration of skin organisms at the insertion site into the cutaneous catheter tract with colonisation of the catheter tip
2. Contamination of the catheter hub leading to intraluminal colonisation
3. Occasionally, catheters may be haematogenously seeded from another focus of infection and rarely
4. Infusate contamination.

The most important recommendations concerning the prevention of CVC related blood stream infections are listed in Table 2, based on the guidelines developed in the USA by a working group led by the Infectious Disease Society. [162]

Table 2: Recommendations for prevention of Central venous catheter (CVC)related blood stream infections [162]

Education	Health-care worker education and training for the insertion and maintenance of CVCs is essential. Moreover, periodic assessment of their knowledge of and adherence to guidelines is strongly recommended. Trained personnel for the insertion and maintenance of CVCs should be designated.
Catheters and materials	An important pathogenetic determinant is the material from which the device is made. *In vitro* studies demonstrate that CVCs made of polyethylene or polyvinyl chloride are less resistant to adhesion of micro-organisms than are CVCs made of teflon, silicone or polyurethane. The number of ports must be kept to the minimum required for the patient's management. Cuffed and tunnelled CVCs should be employed if their use is to be prolonged (e.g. allogeneic transplant).
Site of catheter insertion	In adults, a subclavian site is preferred as lower extremity sites are associated with a higher risk of infection (and deep venous thrombosis). Subclavian sites also reduce the risk of infection compared to jugular sites.
Maximal sterile barrier precautions during insertion	Full aseptic techniques should be used at the time of insertion. 2% aqueous chlorhexidine gluconate (preferably), tincture of iodine, or 70% alcohol can be used to prepare the skin before CVC insertion. Organic solvents (e.g. acetone and ether) should not be applied.
Catheter and catheter site care	One port should be designated exclusively for hyperalimentation if a multilumen catheter is used. The routine use of prophylactic intranasal or systemic antibiotics before insertion or during the use of the CVC and antibiotic lock solutions are not recommended. The catheter site can be covered by sterile gauze or a sterile, transparent semi-permeable dressing and the dressings should be replaced whenever they become damp or loosened. Gauze dressings should be replaced at least every two days and transparent dressings every 7 days. The catheter sites must be monitored visually or by palpation regularly and if patients have tenderness at the insertion site and/or fever without obvious source, the dressing should be removed for thorough examination.
Replacement of catheter	Catheters that are no longer essential should be removed promptly. However routine replacement of CVCs to prevent catheter related infections is not advised. CVCs must not be removed on the basis of fever alone. Clinical judgement is required to assess the appropriateness of catheter removal if infection is evidenced elsewhere or if a non-infectious cause of fever is suspected.
Administration set replacement	• Following administration of blood, blood products immediately • Following total parenteral nutrition – after 24 hours • Other fluid sets – after a maximum of 72 hours

Haematopoietic growth factors

G-CSF

Prolonged neutropenia and subsequent infections are the most frequent causes of morbidity and mortality following HSCT. The administration of G-CSF posttransplantation results in a clear clinical benefit by shortening time to engraftment and hence reducing complications associated with neutropenia. However, the optimal way of administration is still debated. (163)

Erythropoietin

Erythropoietin (EPO) has been used with the aim to accelerate the recovery of red blood cells following stem cell transplantation. There may be some benefit in the reduced intensity transplant setting where EPO responsive erythroid precursors may persist following conditioning, but prospective studies are lacking. Because of the modest and mixed results of the studies, most centres do not use EPO in the early post-transplant setting. A few randomised trials have addressed the efficacy of EPO following autologous transplantation. None found a significant reduction of transfusion requirements. The lack of benefit is particularly evident with peripheral blood stem cell transplantation. (164)

Oral mucositis

Oral mucositis (OM) occurs in most patients treated with high-dose therapy and stem cell transplantation. It has been associated with an increased need for total parenteral nutrition and opioid analgesics, prolonged hospitalisation, and increased risk of infection.

Despite the benefits of TPN, mucositis *per se* remains an important clinical problem.It is characterised by mucosal damage ranging from mild inflammation to extensive ulceration, which may affect the oral cavity and other parts of the alimentary tract. Typically, oral mucositis peaks between day 6 and 12, and resolution coincides with engraftment. Mucositis is associated with an increased risk of systemic infection resulting from bacteraemia associated with the breakdown of mucosal barriers. Management of oral mucositis requires a multidisciplinary approach. Basic oral care consists of a pre-transplant oral/dental examination aimed at decreasing the oral infectious and inflammatory burden.

Chemotherapy (CT) induced nausea and vomiting

The objective of antiemetic treatment is the perfect prevention of nausea and vomiting during the course of the transplant (tables 3).

Table 3: Classification of Chemotherapy induced emesis [165]

Acute emesis	**Delayed emesis**
Occurs during the first 24 h following CT	Occurs later than 24 h

Prophylaxis and treatment

The main principles of emesis control are:

- Nausea and vomiting are far easier to prevent than to treat
- Antiemetic therapy should be adjusted for the drug with the highest emetic risk
- The risk for emesis following highly emetogenic chemotherapy lasts approximately4 days
- Patients must be protected throughout the full period of risk
- Oral and IV formulations have equivalent efficacy.

Infections after HSCT

Infections remain a main cause of morbidity and mortality in patients undergoing HSCT. In recent years, improvement in supportive care measures, better understanding of the mechanism of immunosuppression, the introduction of reduced intensity conditioning (RIC) regimens and new anti-infectious agents and prophylactic strategies have decreased infectious morbidity and mortality; however, there is still room for improvement. [166]

The principal risk factors for infections after HSCT are the status of the haematological disease at HSCT, the co-morbidities of the patient, the degree of neutropenia, the disruption of anatomical barriers (mucositis and indwelling catheters), depressed T- and B-cell function and immunosuppressive therapy. The reconstitution of immune status after HSCT depends on the type of transplantation (autologous or allogeneic), the source of progenitor cells (bone marrow, peripheral blood or cord blood), the conditioning regimen (myeloablative, RIC, or non-myeloablative), the degree of histocompatibility between the donor and the recipient (sibling, unrelated or mismatch), the type of GVHD prophylaxis (calcineurin or mTOR inhibitors, monoor polyclonal antibodies or T-cell depletion) and the presence and grade of GVHD and its treatment. Depending on these factors, the patient can be immunodeficient for months or years after HSCT. There is a clear relationship between the type of immunodeficiency after HSCT and the incidence of certain infections. According to this, three different periods can be distinguished, with a predominance of specific pathogens in each phase. [167]

Chronology of infections after HSCT

Early or neutropenic phase (days 0 to +30)

This first period spans from conditioning up to engraftment. During this period, all risk factors for infections are present. Although neutropenia and disruption of anatomical barriers (mucosal damage and vascular devices) are the most relevant risk factors in this phase, cellular and humoral immunodeficiency and, in patients receiving TBI, functional asplenia are also present. The principal pathogens observed in this phase are Gram-positive and -negative bacteria, *Candida* spp. and the *Herpes simplex virus* (HSV) and the most frequent type of infections are bacteraemia/sepsis, pneumonia, oropharingitis, sinusitis, proctitis and cellulitis.

Intermediate phase (days +30 to +100)

The second phase starts at marrow engraftment. At this time, neutropenia and mucositis have disappeared but central lines, immunodeficiency and functional asplenia, which may be worsened and maintained by GVHD and its treatment, persist. This favours the development of viral and fungal infections. *Cytomegalovirus* (CMV), adenovirus, BK polyomavirus, respiratory viruses, *Pneumocystis jiroveci* (Pj), *Candida* spp., *Aspergillus* spp. and other moulds are responsible for infections in this phase.

For decades, CMV disease was the principal infectious complication in this phase; however, with the introduction of good surveillance techniques that allow anticipated diagnosis and pre-emptive treatment, the mortality due to CMV has decreased notably .[168] Currently, invasive aspergillosis (IA) is observed in 5–15% of allogeneic HSCT recipients, 60% of whom will die because of this infection despite the efficacy of the new antifungal agents . [169]

Late post-transplantation phase (days > +100)

Infections occurring during this period are associated with the presence and severity of chronic GVHD, which prevents the normal recovery of cellular and humoral immunity. In addition, functional asplenia persists in patients with GVHD and in those receiving TBI. For this reason, in this phase, infections are generally secondary to encapsulated bacteria (*Streptococcus pneumoniae* and *Haemophilus influenzae*), *Aspergillus* spp. and other moulds, *Pj* and *Varicella zoster virus* (VZV).

Bacterial infection

Early phase after HSCT

The main sources of bacterial infections in neutropenic patients are the normal endogenous flora in the gastrointestinal tract, which is responsible for infections by Gram-negative bacteria, and the exogenous acquisition of organisms from vascular devices, which is the main cause of infections by Gram-positive microorganisms. [170]

Prophylaxis

Anti-bacterial prophylaxis has been general practice in this phase of HSCT. It is based on the elimination of endogenous gastrointestinal flora and on the prevention of the

acquisition of exogenous organisms. The most important measures are handwashing, oral hygiene, low-bacterial diets and gastrointestinal decontamination (GID) using oral antibiotics. Hand-washing is the only environmental measure with proven efficacy (level of recommendation AI) and should be used systematically in all cases. In this phase, the use of masks is indicated for all visitors and health care personnel in close contact with the patient, or if they present symptoms of upper respiratory tract infection and contact with the patients cannot be avoided. The effectiveness of other protective measures (gowns, caps and leggings) has not been proven in this context. [171]

Table 4: Vaccinations recommended after autologous and allogeneic HSCT [167]

Vaccine	Recommended after HSCT	Time to vaccine	Doses
Pneumococcal (conjugated)*	Yes (BI)	3–6 months	3*
Tetanus	Yes (BII)	6–12 months	3
Diphtheria	Yes (BII)	6–12 months	3
Pertussis (acellular)	Yes (CIII)	6–12 months	3
Haemophilus (conjugated)	Yes (BII)	6–12 months	3
Meningococcal (conjugate)	National recommendations (BII)	6–12 months	1
Polio (inactivated)	Yes (BII)	6–12 months	3
Hepatitis B (recombinant)	National recommendations (BII)	6–12 months	3
Influenza (inactivated)	Yearly (AII)	4–6 months	1–2
Measles	Yes (BII)	24 months	1–2
Mumps	Yes (CIII)	24 months	1–2
Rubella	Yes (BIII)	24 months	1–2

Fungal infections

Pathogenesis and epidemiology

Fungal infections follow a chronological pattern after HSCT. In the first period, with neutropenia as the main risk factor, infections due to Candida spp. are the most common. The most relevant fungal infections occur in the intermediate/late phase and are favoured by the presence of GVHD and its treatment. Approximately 5–15% of HSCT patients develop invasive aspergillosis (IA) and 60% of them will die because of this, rendering it the main cause of infectious mortality after HSCT. [172]

Prophylaxis

Because mould infections are acquired mainly by inhalation, protective environment measures can be extremely relevant. The use of isolation rooms equipped with HEPA filters (filters that retain 99.97% of air particles with more than 0.3 mm, including bacteria, fungi and even viruses adherent to dust) permit the reduction of the risk of mould acquisition. Several studies have demonstrated their usefulness in reducing *Aspergillus* in the air and the incidence of IA. Unfortunately, these isolation measures are only applicable when patients are hospitalised, and the risk of infection by moulds lasts for several months after HSCT, when patients are no longer hospitalised. Only one registry study has shown a survival benefit of HEPA/LAF rooms for patients receiving an allogeneic HSCT for leukaemia. (173)

Table 5: Main criteria for proven, probable and possible invasive fungal Infection (174)

Proven IFI	**Probable IF**	**Possible IFI**
Histological Host factors or culture evidence (in sterile material)	Host factors (neutropenia, immunosuppressants) + **Mycological criteria** (direct - cytology, culture of non sterile material - or indirect tests - GM or βDG) + Clinical criteria (+CT/MRI, FBS, retinal)	Host factors + **Clinical criteria**

GM: galactomannan; bDG: β-D-glucan; FBS: fibrobronchoscopy. Retinal: retinal images suggestive of IFI.

Viral infections

The more relevant viral infections in the allogeneic setting are (CMV, EBV and HHV-6). (175)

Cytomegalovirus

Around 40–80% of people (depending on the country) are infected by CMV (herpes virus type 5) in childhood. After infection (positive IgM serology), CMV becomes latent for life (positive IgG serology). Under certain circumstances, as in immunosuppression following HSCT, latent CMV can reactivate.

Epstein-Barr virus

Another herpes virus, Epstein-Barr virus (EBV), is associated with post-HSCT lymphoproliferative disease (EBV-PTLD), which is a life-threatening complication. The following risk factors increase the risk of PTLD: unrelated and/or mismatched HSCT; use of T-cell depletion, ATG or OKT3; EBV serology mismatch between the donor and the

recipient (increased risk for sero-negative patients with a sero-positive donor); primary EBV infection and splenectomy .[176]

Human herpes virus 6

Human herpes virus 6 (HHV-6) in the setting of allogeneic HSCT reactivates in 50–70% of patients (typically earlier than CMV) and has distinctive clinical manifestations that can help its diagnosis: encephalitis, with characteristic limbic- and hippocampusderived symptoms. [177]

Graft-versus-host disease

In the human setting traditionally recognised two forms of GVHD, acute (AGVHD) and chronic (CGVHD). The original distinction of acute from chronic GVHD, namely the occurrence before or after day 100 post stem cell infusion, has become blurred recently due to the development of an AGVHD-like illness beyond day 100 after reduced-intensity conditioning (RIC) regimens and/or after donor lymphocyte infusions (usually given after day 100). Nevertheless the underlying combination of symptoms and signs affecting the skin, liver and gastrointestinal tract form a classical clinical syndrome enabling the diagnosis and a helpful guide to the appropriate terminology is provided in (Table 6).

Table 6: Distinguishing acute and chronic graft-versus-host disease [178]

Category	Time of symptoms	Acute GVHD features	Chronic GVHD features
Acute GVHD			
Classic acute	≤100 days	Yes	No
Persistent, recurrent or late-onset acute	>100 days	Yes	No
Chronic GVHD			
Classic chronic	No time limit	No	Yes
Overlap syndrome	No time limit	Yes	Yes

Acute graft-versus-host disease

AGVHD remains, directly or indirectly, the major cause of short-term (day 100) mortality after allogeneic HSCT. The pathology of AGVHD has been attributed to a three phase process comprising initial tissue damage from the conditioning regimen which in turn leads to activation of host antigen-presenting cells and activation proliferation of donor T-cells (afferent phase) and finally to the release of inflammatory cytokines such as interleukin-1 and tissue necrosis factor (TNF)-a that eventually produce tissue necrosis (efferent phase). The action of this pathogenetic process in the induction of AGVHD is modulated in part by the presence of cells capable of inhibiting immune responses, most notably T-regulatory cells (T-regs). [178]

Diagnosis and scoring

AGVHD is manifested by one or more of the following features: an erythematous skin reaction, cholestatic liver disease and gastrointestinal dysfunction. The variety of presentations in each organ is provided in more detail in Table 7; the syndrome ranges from a mild self-limiting condition to a serious and potentially fatal disorder.

The first classification of AGVHD was developed by Glucksberg et al. in 1974. Each organ was staged from 0 to 4 (Table 8) and the resultant stages were combined to provide an overall grade. (Table 9) [179]

Table 7: Clinical manifestations of acute graft-versus-host disease [179]

Organ	Clinical manifestations
Skin	Erythematous maculopapular rash, often initially involving the palms and soles May progress to involve entire body surface May be pruritic and/or painful In severe cases, bullae may form leading to desquamation
Liver	Cholestasis with or without frank jaundice Cholestatic enzymes comparatively more deranged than transaminases
Gastrointestinal tract	Anorexia, nausea and vomiting Diarrhoea, typically green and watery In severe cases diarrhoea contains fresh blood and mucosa and is accompanied by abdominal pain and on occasions followed by paralytic ileus

Table 8: Staging of acute graft-versus-host disease [180]

Stage	Skin based on maculopapular rash	Liver based on serum bilirubin	Gastrointestinal tract based on quantity of diarrhea
+	<25% of body surface	34–50 μmol/L	>500 <1000 mL
++	25–50% of body surface	51–102 μmol/L	>1000 <1500 mL
+++	Generalised erythroderma	103–255 μmol/L	>1500 mL
++++	Generalised erythroderma with bullae and desquamation	>255 μmol/L	Severe abdominal pain with or without ileus

Table 9: Overall grading of acute graft-versus-host disease [181]

Grade	Organ and stage of involvement
I	Skin + to ++
II	Skin + to +++ Gastrointestinal tract and/or liver + Mild decrease in clinical performance
III	Skin ++ to +++ Gastrointestinal tract and/or liver ++ to +++ Marked decrease in clinical performance
IV	Skin ++ to ++++ Gastrointestinal tract and/or liver ++ to ++++ Extreme decrease in clinical performance

Treatment

Grade I AGVHD, by definition affecting only the skin, can often be effectively treated with topical steroids alone. More advanced grades require systemic therapy and the mainstay of treatment remains high dose methylprednisolone, usually at a dose of 2 mg/kg/day, continued for 7–14 days and followed by a gradual reduction in dose. [181]

Chronic graft-versus-host disease

Definition and pathology

CGVHD is an immunoregulatory disorder occurring after allogeneic HSCT and shares features of autoimmunity and immunodeficiency. Features of CGVHD resemble other autoimmune diseases such as Sjögren syndrome, scleroderma, primary biliary cirrhosis and immuncytopenias. Similarly to AGVHD, CGVHD is also thought to be induced by the immune cells of the donor but the pathophysiology is even less well understood. Although autoreactive T-lymphocytes are considered to play the key role, recent data revealed the importance of B-cells . CGVHD is the main cause of late non-relapse mortality and morbidity after allogeneic HSCT. Mortality is primarily caused by infections either due to the immunodeficiency of CGVHD or its treatment.

Risk factors

The major risk factors for the development of CGVHD are prior acute GVHD, higher degree of HLA mismatch, older patient age, previous splenectomy, CMV seropositivity, female donor to male recipient and mobilised peripheral blood stem cell graft (Table 10). [182]

Table 10: Major risk factors for the development of chronic graft-versus-host disease[182]

Given factors	Variable factors
Older age of recipient	Higher degree of HLA mismatch
CMV seropositivity of recipient	Older age of donor
Previous splenectomy	CMV seropositivity of donor
Prior acute GVHD	Female donor to male recipient
	Mobilised blood stem cell graft

Diagnosis and scoring

The diagnosis of CGVHD is based on its clinical manifestations. The signs and symptoms of CGVHD may occur in any organ but the most frequently affected organs/sites are the skin, nails, mouth, eyes, female genitalia, gastrointestinal tract, liver, lungs, muscles, fascia and joints. The disease may be mono-symptomatic, but can also be widespread and leading to debilitating consequences such as end stage lung disease or joint contractures. Since the NIH consensus development project on CGVHD in 2005 new diagnostic and staging criteria has been established (Table 11). [183]

Recently, a much more detailed risk score has been proposed by the CIBMTR following a review of 5343 patients with CGVHD. Ten variables (age, prior acute GVHD, time from transplant to CGVHD, donor type, disease status at transplant, GVHD prophylaxis, gender mismatch, serum bilirubin, Karnofsky score and platelet count) were identified resulting in 6 risk groups withsignificantly different non-relapse mortality and overall survival. This may be a useful tool to precisely identify different risk category patients at the diagnosis of CGVHD and making appropriate decisions regarding further therapy and possible enrolment in clinical trials (Table 12) .[184]

Table 11: Signs and symptoms of chronic graft-versus-host disease [183]

Organ/Site	Diagnostic	Distinctive	Other features*	Common (both acute and chronic)
Skin	Poikiloderma Lichen planus-like features Sclerotic features	Depigmentation	Sweat impairment Hypopigmentation Hyperpigmentation	Erythema Maculopapular rash Pruritus
Nails		Dystrophy Longitudinal ridging splitting, or brittle features		
Scalp/hair		Alopecia	Premature gray hair	
Mouth	Lichen planus type features	Xerostomia		Mucositis
Eyes		Dry eyes Keratoconjunctivitis sicca	Photophobia Blepharitis	
Genitalia	Lichen planus type features	Erosions, fissures, ulcers		
Gastrointestinal tract	Esophageal web Stricture or stenosis of the esophagus		Exocrine pancreatic insufficiency	Nausea Vomiting Anorexia, weight loss
Liver				Bilirubin or alkaline phosphatase, ALT or AST >2 x upper limit of normal
Lung	Bronchiolitis obliterans			Bronchiolitis oblit-erans organising pneumonia (BOOP)
Muscles, fascia, joints	Fasciitis, joint stiffness secondary to sclerosis	Myositis Polymyositis	Oedema Muscle cramps Arthralgia, arthritis	
Haematopoietic and immune			Thrombocytopenia Eosinophilia Lymphopenia Hypo- or hyper- γ globulinemia Autoantibodies	
Other			Peripheral neuropathy Myasthenia gravis Ascites, pericardial or pleural effusion	

Table 12: NIH consensus for global grading of chronic graft-versus-host disease [184]

Number of organs/sites	Mild	Moderate	Severe
1 site	Score 1	Score 2	Score 3
2 sites	Score 1	Score 2	Score 3
3 or more sites		Score 1	Score 3
Lung involvement		Score 1	Score 2

Chronic viral hepatitis in thalassemia

In the last 4 decades, regular blood transfusions and chelation therapy have improved the survival of patients with thalassemia major. [185-187] Despite the progress on chelation therapy, cardiac complications remain the main cause of death among transfusion-dependent thalassemia patients related to the susceptibility of cardiac cells to iron overload toxicity. [188,189] The interest in the clinical management of chronic liver diseases has been increasing, however, because of the high prevalence of viral infections in adult transfusion-dependent thalassemia patients and the central role of the liver in regulating the iron metabolism. [189,190]

The assessment of heart and liver iron overload is required to tailor iron chelation therapy. Furthermore, the diagnosis of hepatitis B virus (HBV)– or hepatitis C virus (HCV)–related chronic hepatitis is required to identify patients who have a high risk of developing liver complications and who may obtain a benefit by antiviral therapy.

Epidemiology of hepatitis C

According to recent estimates, more than 185 million people around the world have been infected with HCV, of whom 350 000 die each year.[191,192] Most people infected with the virus are unaware of their infection and, for many who have been diagnosed, treatment remains unavailable.[193] Treatment is successful in the majority of persons treated, and treatment success rates among persons treated in low- and middle-income countries are similar to those in high-income countries. [194] One third of those who become chronically infected are predicted to develop liver cirrhosis or hepatocellular carcinoma. [195]

The prevalence of hepatitis C infection varies substantially around the world when countries are grouped into Global Burden of Disease regions, the estimated prevalence of HCV infection is highest in Central and East Asia and in the North Africa/Middle East regions. In view of the larger populations in Asia, the South Asia and East Asia regions have by far the largest number of persons living with HCV infection.

The relative importance of riskfactors for HCV infection varies substantially, depending on the geographical region and population studied. Greater access to HCV testing and better surveillance are important steps to both increase the number of persons diagnosed with HCV and to improve understanding of the distribution of HCV infection in the general population and groups at increased risk.

Hepatitis C virus structure

The hepatitis C virus is a small, positive-stranded RNA-enveloped virus that is approximately 9.6 kb in length. The genetic sequence was first characterized in 1989, [196] placing the virus in the Hepacivirus genus within the Flaviviridae family. (Figure 5) [197,198] It has a highly variable genome and multiple genotypes and subgenotypes.(Figure 6)[199]The distribution of HCV genotypes and subgenotypes varies substantially in different parts of the world .Some genotypes are easier to treat and, thus, the duration of and recommended medicines for therapy vary by genotype. For this reason, determining a patient's genotype is important to appropriately tailor therapy.

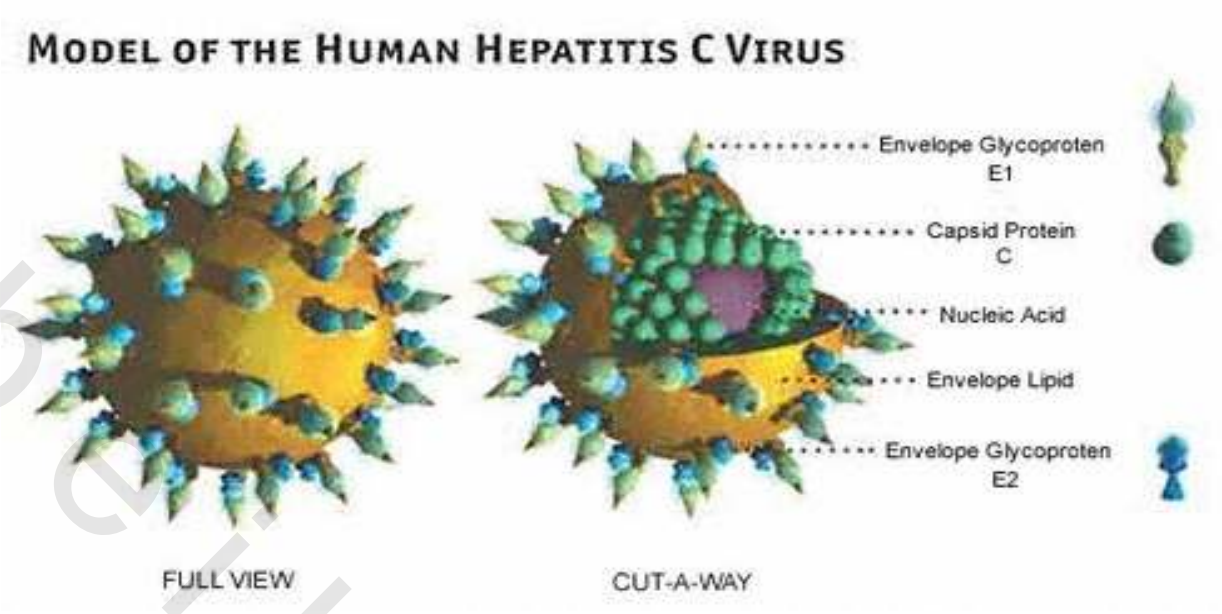

Fig.5: Model of Hepatitis C virus [197]

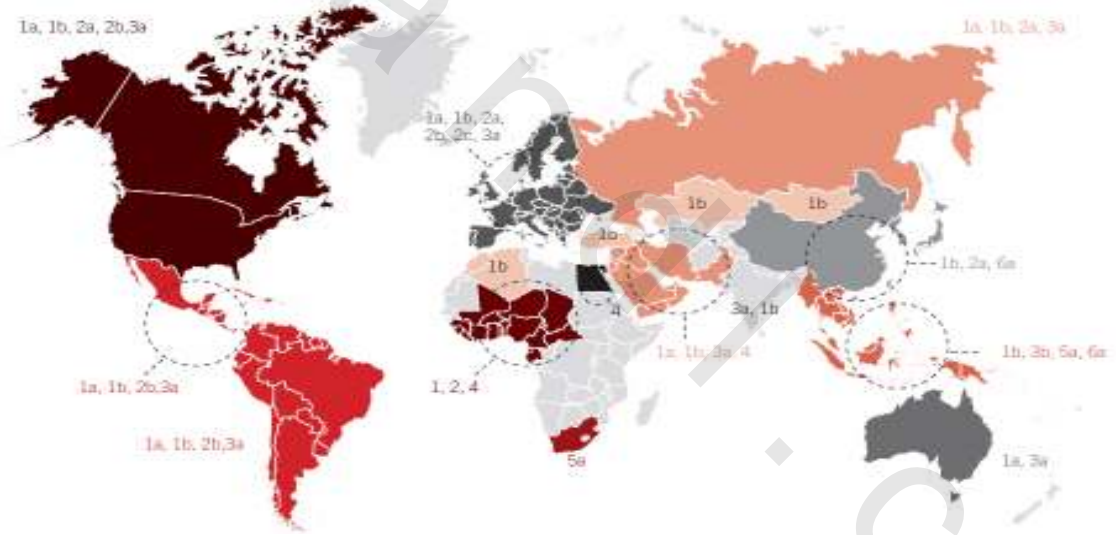

Fig.6: Global distribution of genotypes of HCV [199]

Natural history of HCV infection

Hepatitis C virus causes both acute and chronic infection. Acute HCV infection is defined as the presence of HCV within six months of exposure to and infection with HCV. It is usually clinically silent, and is only very rarely associated with life-threatening disease. Spontaneous clearance of acute HCV infection occurs within six months of infection in 15–45% of infected individuals in the absence of treatment. Almost all the remaining 55–85% of persons will harbour HCV for the rest of their lives (if not treated) and are considered to have chronic HCV infection.

Anti-HCV antibodies develop as part of acute infection and persist throughout life. In persons who have anti-HCV antibodies, a nucleic acid test (NAT) for HCV RNA, which detects the presence of virus, is needed to confirm the diagnosis of chronic HCV infection.[200,201] Left untreated, chronic HCV infection can cause liver cirrhosis, liver failure and hepatocellular carcinoma. Of those with chronic HCV infection, the risk of cirrhosis of the liver is 15–30% within 20 years. [202-204] The risk of HCC in persons with cirrhosis is approximately 2–4% per year. The risk of cirrhosis and HCC varies depending upon certain patient characteristics or behaviours

Screening for HCV infection

Screening for HCV infection is done using HCV serological testing. If positive, a NAT for HCV RNA assay is needed to confirm chronic HCV infection. Several screening assays have been evaluated by WHO, and sensitivity, specificity, and positive and negative predictive value results are available.[205] It is important to consider the possibility of infection with other blood borne viruses in persons with HCV, and to offer screening for HBV and HIV in addition to HCV.

Treatment of patients with HCV infection

HCV is a now a curable disease, and advances in HCV therapy have resulted in steadily higher cure rates. Identification and treatment of chronic HCV infection has a prevention benefit, as persons who are cured of HCV cannot transmit the virus to others. HCV cure is also beneficial for the patient's health, as it reduces the risk of development of HCC among persons at all stages of fibrosis by >75%.[206,207] Six drugs are licensed for the treatment of HCV – standard interferon (IFN) or pegylated interferon α (PEG-IFN), ribavirin (RBV), the protease inhibitors (PIs) boceprevir, simeprevir and telaprevir, and the nucleotide analog polymerase inhibitor sofosbuvir. The limitations of treatment include high cost, the need for sophisticated laboratory tests and trained clinicians, as well as the limited efficacy and high toxicity of some of the medicines. It is anticipated that the number of medicines for the treatment of HCV will expand rapidly over the coming years, and WHO plans to periodically update these guidelines to include newly licensed drugs. Before treatment for HCV can be commenced, it is necessary to genotype the virus as different genotypes require different types and durations of treatment, and the protease inhibitors boceprevir, simeprevir and telaprevir are licensed only for genotype 1 infection. Current therapy for genotype 1 infection is a combination of PEG-IFN, RBV and a PI or nucleotide polymerase inhibitor, which results in high rates of sustained virological response (SVR; a negative HCV RNA test three or six months after the end of treatment).[208-211] Dual therapy with PEGIFN and RBV or sofosbuvir with RBV is used for genotypes 2 and 3 infections. [212,213]

Patients with genotype 4 infection treated with treated with sofosbuvir, PEG-IFN and RBV have similar response rates when compared with genotype 1-infected individuals. Small studies of genotypes 5- and 6-infected patients have shown similar SVR rates to genotypes 2- and 3-infected ones. [214,215] Larger studies in these groups are required to confirm these results and to identify predictors of response or non-response to treatment. Treatment with some HCV medicines may result in marked side-effects and therefore careful patient assessment and close monitoring is required.[216-218]

Recommendations for counseling and prevention of transmission of HCV infection

1. All thalassemia patients should receive information regarding the risk of viral infections associated with blood transfusion and other routes, and chronically infected patients with HCV require counseling on prevention of transmission to other persons (high quality of evidence in the general population and in thalassemia patients).
2. Steady-sexual partners of HBV-infected thalassemia patients are at increased risk of infection and should be vaccinated (high quality of evidence in the general population).
3. Transmission of HCV infection to household members is possible but occurs at low rates (moderate quality of evidence in thalassemia patients).
4. Vertical transmission of HCV infection is possible but is limited to women who have detectable HCV RNA (high quality of evidence in the general population).
5. The existing evidence does not support the choice of planned cesarean section delivery for the prevention of HCV infection (low quality of evidence for general population).
6. Infants born to HCV-positive mothers should be tested for anti-HCV at 18 months of age (moderate quality of evidence for general population).

Diagnosis and management of viral infections

Diagnosis of HCV infections.

Enzyme immunoassay tests to identify HCV antibodies (anti-HCV) are currently used to detect HBV and HCV infection, respectively. A positive qualitative test for HCV RNA, using amplification techniques, such as the polymerase chain reaction (PCR) or transcription-mediated amplification, confirms the presence of viremia in the blood. Quantitative assays measure the quantity of HCV RNA in blood using either target amplification (PCR, transcription-mediated amplification) or signal amplification techniques (branched DNA assay). [(219)] A positive qualitative or quantitative HCV RNA test in blood identifies patients with active HCV replication. In contrast, anti-HCV-positive, but HCV-RNA-negative patients have had a previous HCV infection from which they are cured. This is an important issue because the rate of HCV RNA positivity among anti-HCV-positive thalassemia patients is approximately 50% and is lower than in other HCV-infected populations. [(220)] The determination of HCV genotypes is very useful to predict the efficacy of antiviral therapy, and quantitation of HCV RNA during treatment can be used to monitor response to therapy.

Assessment of chronic liver disease.

The main issues in the evaluation of chronic liver disease in thalassemia patients are assessment of liver inflammation and fibrosis and measurement of iron overload. Until a few years ago, the liver biopsy was the only method available to assess the severity of liver inflammation, the stage of fibrosis, and to measure the liver iron concentration by atomic absorption spectrometry.([221-224)] However, liver biopsy is an invasive procedure associated with some discomfort, and its accuracy for the evaluation of liver fibrosis is questionable in relation to inadequate tissue sampling and intraobserver and interobserver

variability.[225] Finally, severe fibrosis or cirrhosis is responsible for significant variability in iron distribution in thalassemia patients.[226]

Liver biopsy is still considered the "gold standard" for the evaluation of liver damage and is recommended for the assessment of HCV chronic hepatitis by international guidelines. The histologic analysis includes grading of necro-inflammatory damage and staging of liver fibrosis, the evaluation of steatosis, and the diagnosis of cirrhosis, according to standardized scores.[227,228] In recent years, transient elastography (TE), a technique that uses both ultrasound and low-frequency elastic waves whose propagation velocity is directly related to elasticity of the liver tissue, has been proposed as a noninvasive method for assessment of liver fibrosis. [229] Liver stiffness measurement by TE has been shown to correlate well with the diagnosis of cirrhosis assessed by liver biopsy. This technique has been extensively studied in chronic hepatitis C and appears to be a reasonably accurate method for detection of cirrhosis. [229,230] A recent study demonstrated that TE is a reliable noninvasive method for diagnosing of cirrhosis in thalassemia patients regardless of the degree of iron overload. [230]

To date, liver biopsy remains the "gold standard" to evaluate inflammation and fibrosis in thalassemia patients with clinical evidence of liver disease. Alternatively, TE could also be used to define the presence of cirrhosis in centers with expertise on this field.[231]

Recommendations for virologic and clinical evaluation of Thalassemia patients with chronic HCV infection

1. Thalassemia patients who received blood transfusion before 1992 should be tested for anti-HCV antibodies (high quality of evidence in thalassemia patients).

2. HBsAg and anti-HCV tests are recommended in thalassemia patients with elevated serum aminotransferase levels for more than 6 months (high quality of evidence in the general population).

3. Qualitative serum HCV-RNA by PCR methods is recommended to confirm the replication of HCV and HBV, respectively (high quality of evidence in thalassemia patients).

4. HCV genotyping should be performed in thalassemia patients with HCV chronic hepatitis before starting antiviral therapy to plan dose and duration of therapy and to estimate the likelihood of response (high quality of evidence in thalassemia patients).

5. MRI using R2 methodology is the recommended noninvasive method for the assessment of liver iron concentration (moderate quality of evidence in thalassemia patients).

6. The liver biopsy is not mandatory before starting antiviral treatment. However, it should be considered to obtain a more accurate assessment of HCV or HBV chronic hepatitis or further information regarding fibrosis stage for prognostic or other therapeutic purposes (moderate quality of evidence in thalassemia patients).

7. Noninvasive methods, such as TE, may be useful in defining the presence or absence of cirrhosis in thalassemia patients with HCV infection (low quality of evidence in thalassemia patients).

The prevalence of cirrhosis in thalassemia patients ranges from 10% to 20%, as reported in several studies performed in the United States, China, Iran, Italy, and Greece.[232-233] Male sex, high serum alanine transaminase values, positive serum HCV-RNA, and high liver iron concentration were all significantly associated with severe fibrosis or cirrhosis.

Ferritin levels averaged 2000 ng/mL, suggesting a limited role for iron overload in carcinogenesis. [234] Prevalence was calculated to be approximately 6 times the expected value for the Italian male population, but age-specific comparisons, which would be more appropriate, are not possible because such data are not available. A prospective study identified a 2% incidence in HCC during a one-year period of observation in a cohort of 105 adults with thalassemia major.[235] in a recent prospective survival analysis, the hazard ratio for death was significantly higher in thalassemia patients with cirrhosis. In light of these reports, thalassemia patients with HBV or HCV and cirrhosis are at high risk of the development of HCC. The international guidelines suggest that all patients with chronic HBV hepatitis and patients with HCV and cirrhosis should receive liver ultrasound every 6 months for the surveillance of HCC.

Iron Overload and Hematopoietic Stem Cell Transplantation

Iron overload (IO) is a relatively common condition in patients with hematological malignacies and HSCT recipients. Free iron which accompanies IO might contribute to the already existing prooxidant state in HSCT recipients by inducing the formation of reactive oxygen species (ROS). Tissue peroxidation and organ damage, as a consequence, contribute to the development of some early transplant complications. [236-240] Increasing number of transplants performed each year and improved transplant techniques result in a rise in the number of long term survivors. The primary goal of HSCT is to cure the primary disease.

However long term transplant related morbidity might be very challenging and might significantly impair the quality of life. Late effects might be the consequence of the direct toxicity of chemoradiotherapy and/or the immunologic complications mainly consisting of GVHD. Besides the secondary late effects including osteoporosis and dental caries, very late effects, namely cardiovascular toxicity considered as tertiary late effect may also occur.

Among this wide spectrum of complications, IO has a substantial role as a contributor to liver toxicity, infections and SOS and as a predictor of transplant outcome. Hematopoietic SCT recipients have been demonstrated to have a high degree of liver iron content (LIC) almost in the range of hereditary hemochromatosis (HH) and IO was shown to cause liver fibrosis, heart failure, hypogonadism, diabetes and endocrinopathy in HSCT recipients in the long run. [238,241] Iron is an essential element which plays a key role in several biochemical reactions including oxygen transport and electron transfer. It mediates the conversion of hydrogen peroxide (H2O2) to highly toxic free radicals leading to tissue damage by oxidation of proteins, peroxidation of membrane lipids and modification of nucleic acids. [242] Under normal circumstances, an appreciable concentration of free iron does not exist outside physiological sinks.

Any released ferrous iron (Fe+2) is immediately chelated in cells by compounds such as citrate or adenosine diphosphate. Thus, labile iron could not participate in the Haber–Weiss reaction, which catalyses the formation of ROS. Free iron may directly initiate lipid peroxidation which destroys membrane structure resulting in increased oxidative stress and cellular damage. Excess iron accumulation causes chronic free radical induced tissue damage in multiple organs and leads to progressive organ dysfunction, which results in significant morbidity and mortality. In this respect, IO should be prevented in order to preclude the adverse impact of free iron on natural homeostasis. [240-242]

Iron homeostasis

Iron is vital for all living organisms and takes part in several metabolic processes, including DNA synthesis, oxygen and electron transport. Although iron is a critical element in cell growth and multiplication, it is potentially toxic in excess amounts by generating ROS. [243] Reactive oxygen species have a potential to damage DNA and proteins by lipid peroxidation. Labile iron participates in free radical formation via Fenton reaction. There are no physiological mechanisms in humans to excrete excess iron and iron homeostasis is primarily regulated at the level of absorbtion. [244-246] The majority of iron absorbtion occurs via enterocytes in the proximal small intestine. The conversion of dietary inorganic non–heme iron to Fe+2 is facilitated by the brush border ferri reductases. Iron is transported across the cellular membrane by the divalent metal transporter 1 (DMT1) which transfers Fe+2 across the apical membrane and into the cell through a proton coupled process. (Figure 7) [245,246]

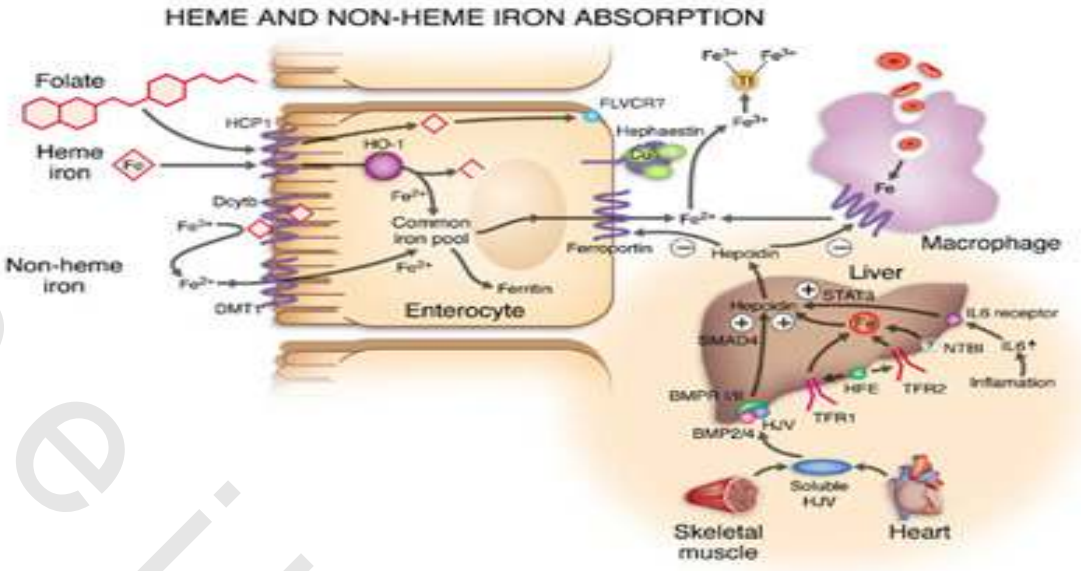

Fig.7: Heme and Non-heme iron absorption [245]

Ferroportin is an iron efflux pump that mediates the export of Fe+3 from the enterocyte. Prior to transport, Fe+2 is converted to Fe+3 by either hephaestin or ceruloplasmin both of which have ferroxidase activity. Subsequently, iron is uploaded to transferrin which is the primary iron transporter in the circulation. Ferric iron bound to transferrin is soluble and non reactive. The majority of iron (60–70%) is incorporated into hemoglobin while the rest is stored in hepatocytes, myoglobin and reticuloendothelial macrophages. [240] Hepcidin, the main regulator of iron absorbtion, inhibits intestinal absorbtion and release of storage iron in iron-overloaded states, whereas its expression is markedly decreased in iron deficiency states. Hepcidin interacts directly with ferroportin, causing its internalization, degradation and blocking iron release from cells to plasma. Hepcidin acts as an acute phase reactant which is responsible for the anemia of inflammation. Its production is upregulated by body iron excess and inflammation whereas downregulated by anemia and hypoxia. [244-246]

Cell survival depends on the balance between the destructive and beneficial effects of iron. [240] Natural iron homeostasis comprises regulation mechanisms to control iron excess. The primary protective pathway is the sequestration of iron in ferritin or transferrin. Ferritin is the chief storage molecule while transferrin is functionary for the transport of iron. Ferritin captures and buffers the intracellular iron pool, thus it makes iron available for critical cellular processes while protecting lipids, DNA and proteins from potentially toxic effects of iron. Iron stored in ferritin is not capable of catalyzing radical reactions and is considered as safe. It is well known that serum ferritin concentration closely parallels body iron reserves.

However, as free iron is the main form of iron which can precipitate in oxidative stress, any measure of unbound iron will result in deleterious effects. The balance of free iron to bound iron changes and free iron becomes available to catalyze free radical reactions in iron overloaded states. [241] Large amounts of excess iron in the circulation are likely to exceed the serum iron binding capacity (SIBC) and non transferrin bound iron

(NTBI) will emerge eventually. Non transferrin bound iron bypasses the normal regulatory mechanism of receptor mediated iron uptake and is able to stimulate the peroxidation of membrane lipids and the formation of ROS. The intracellular counterpart of NTBI is considered as labile iron pool (LIP) which is bound mainly to low molecular weight compounds. Labile iron pool is catalytically active and capable of initiating free radical reactions. The expansion of the LIP and simultaneously increased NTBI may trigger cell toxicity. Generation of LIP leads to unregulated iron uptake and subsequent intracellular storage either within ferritin molecules or as hemosiderin. The adverse effects of IO can arise from the elevation of NTBI and LIP in plasma and might as well cause organ damage mediated by the accumulation of tissue iron in target organs. The equilibrium between the LIP and iron locked in the ferritin shell is critical to maintain the normal function of cellular iron enzymes. Imbalance in this equilibrium results in the uncontrolled loading of organs, such as the liver, heart and endocrine glands, with free iron which generates free radicals and causes cell damage. [247] Eventually, NTBI and LIP may be more relevant iron markers than serum ferritin and transferrin as a predictor of IO induced tissue damage. Alterations in ferritin levels are seen commonly in clinical practice often reflecting perturbations in iron homeostasis or metabolism. Serum ferritin differs markedly from tissue ferritin in molecular weight, iron and carbonhydrate content, subunit size and amino acid sequence. The extracellular form of ferritin, termed as serum ferritin, is used as a clinical marker of iron status. Tissue ferritin is the more efficient storage form of iron than is serum ferritin and the function of serum ferritin has to be clarified in these circumstances. Serum ferritin is usually correlated with NTBI, whereas inflammation, acute and chronic liver diseases and malignancies may also cause elevated serum ferritin levels regardless of the iron stores.

Iron overload and stem cell transplantation

Iron overload is a significant problem in autologous (auto) and allogeneic (allo) HSCT recipients and may adversely affect transplant outcome. [248] the diagnosis of IO has been reported in up to 88% of long term survivors of HSCT on the basis of serum ferritin levels.

The main causes of IO in HSCT are prolonged dyserythropoiesis, increased intestinal iron absorbtion due to anemia and chemotherapy associated mucositis which leads to increased iron absorbtion, transfusion burden and release of iron from injured tissues. [249] Iron overload is particularly common in HSCT recipients with hemoglobinopathies and hematological malignancies which require frequent transfusions and is associated with ineffective erythropoesis such as acute leukemia and myelodysplastic syndrome (MDS). Transfusion load is considered to be the principal cause of IO in this group, as each unit of packed red blood cells (PRBC) contains approximately 200–250 mg iron. Since there is no physiological mechanism for excreting excess iron, iron accumulation is inevitable after 10–20 transfusions. [249-251] Ineffective erythropoiesis might be a contributing factor leading to excessive iron absorbtion particularly in MDS and thalassemia which is mediated by erythroid regulators of iron metabolism which suppress hepcidin and result in increased iron absorbtion. Elevated growth differentiation factor 15 (GDF–15) levels are considered to be the initiating event in this context. Ineffective erythropoiesis either as a feature of the underlying disease or a consequence of intensive treatment leads to inhibition of hepcidin possibly due to overexpression of GDF–15 and thus increases iron absorbtion and toxicity. Hematopoietic SCT recipients are at risk of IO due to prior

transfusion load, increased iron absorbtion related to elevated GDF–15 levels and peri–tansplant transfusions. [251,252]

Conditioning treatment with chemo/radiotherapy during HSCT causes toxicity and immunosuppression leading to organ damage and infectious complications mainly in the first 3 months of the procedure. [240] Free iron, which acts as a free radical catalyser, might increase the toxicity of the conditioning regimen during HSCT.

Hepatic toxicity due to chemotherapy and radiation might lead to hepatocellular damage with subsequent further release of hepatic iron stores. Liver damage may also disturb transferrin synthesis. [253] A decrease in transferrin due to hepatic toxicity, stored iron leaking from injured liver to blood and a suppression of erythropoietic activity during treatment may causes elevated TS levels. Thus, increasing TS succeeds and contributes to the appearance of potentially toxic NTBI in the circulation. Iron in its NTBI form is a potent catalyst in Fenton's reaction which produces ROS capable of causing cellular damage through various mechanisms. Tissue damage such as mucositis and liver injury is common after HSCT and may be partly mediated by NTBI during cytotoxic chemoradiotherapy.[254] It is indicated that increased NTBI levels may contribute to organ toxicity and infectious complications in the early post–transplant period . Iron toxicity may play an important role in the pathogenesis of transplant related complications. In a series of 25 patients who underwent HSCT, very high levels of ferritin (>3000 ng/ml) and TS (>100%) dramatically increased transplant related mortality (TRM) and decreased overall survival (OS) which was particularly attributed to infections. [254] As iron is an essential element for all pathological microorganisms, excess amounts of free iron might increase microbial growth and the probability of severe infections. [257] the coexistence of excess plasma iron with the damage to the mucosal barrier may also predispose to infectious events with bacterial translocation. Hypoferraemia is a normal response to infection and appears to be a part of a natural resistance mechanism whereas hyperferremia can predispose to bacterial and fungal infections. In this context, elevated TS and ferritin levels are proven risk factors for the development of systemic fungal infections in patients with hematological malignancies. [255,256] Furthermore, an increase in late fungal infections, especially mucormycosis, has been reported in iron loaded patients after HSCT. [257]

The adverse impact of IO on transplant outcome has been demonstrated most convincingly in patients with thalassemia where class III patients with extensive liver damage had higher TRM.[258] Besides increased TRM, other complications attributed to IO includes fungal infections, hepatic dysfunction and hepatic SOS/Veno occlusive disease (VOD). [259] In fact, thalassemia is a benign disorder and ferritin is directly a marker of excess iron and elevated levels could not be attributed to the biology of an underlying malignant pathology.

Iron overload and transplant complications

Liver complications

Liver disease is a frequent cause of morbidity and mortality following allo–HSCT and affects 90% of recipients and up to 5–10% of toxic deaths are liver related. Liver injury in the early post–transplant period may be secondary to drug toxicity, SOS, acute

GVHD, opportunistic infections, total parenteral nutrition, tumor invasion and cholestatic disorders. [260]

Accurate diagnosis of the etiology of liver dysfunction is generally problematic even though the patterns of biochemical, clinical and histological abnormalities can aid diagnosis. Liver biopsy in patients following HSCT is not without risks, particularly due to thrombocytopenia during the early post–transplant period. The most common indication for liver biopsy is to assess the possibility of GVHD in allo–HSCT in the late post–transplant period with persistently abnormal LFTs and no evidence of GVHD on other sites. In this clinical setting, the sensitivity and specifity of serum ferritin as a marker of IO is not well defined due to its concomitant role as an acute phase reactant. [260-262] Liver biopsy may be performed when atypical clinical features are present or multiple disease processes are likely to occur simultaneously or when there is poor response to therapy that has been instituted. [263]The management of liver dysfunction under these conditions may be complicated as overlapping features often complicate the diagnosis and establishing the correct diagnosis is crucial to institute disease specific therapy. Autopsies performed in 10 patients who died early after HSCT showed iron accumulation in a range equivalent to that of patients suffering from HH. A cumulative cirrhosis incidence of 3, 8% by 20 years after HSCT has been reported previously. This rate seems to be an underestimation as the majority of long term survivors have not been subjected to liver biopsy. In a retrospective study by Sucak et al, severe IO was demonstrated in 75% of 24 liver biopsies which were performed with the presumptive diagnosis of hepatic GVHD in 20 patients with persistent elevation of liver enzymes in the post–transplant setting. The initial clinical diagnosis of GVHD was refuted in 43, 5% of the patients. Median number of post–transplant transfusions, TS and ferritin levels were found to be significantly higher in patients who had histologically proven hepatic IO. A significant correlation between serum ferritin levels and histological grade of iron in the hepatocytes was also demonstrated. In another study by Iqbal et al, the diagnosis obtained at laparoscopic liver biopsies altered targeted therapy in 31% of patients. Iron overload was found in 81, 25% of a total of 32 biopsies. [264]A diagnosis of IO after HSCT was demonstrated based on histological evidence of siderosis found in 52, 4% of liver biopsies performed at 15–110 days post-transplant in another study. Liver biopsies were performed for diagnostic purposes in patients with chronic liver dysfunction. An improvement in LFT was observed in 21 of the 23 patients (91%) with IO who underwent phlebotomy .[260] Namely, IO seems to be underestimated as a cause of liver dysfunction in HSCT setting and liver biopsy which allows disease specific therapy could be life saving.

Hepatic IO may also worsen the natural course of chronic viral hepatitis and the response to antiviral therapy. Fujita et al demonstrated that liver iron deposition was more common in chronic hepatitis C compared to hepatitis B and was associated with liver disease progression.

Increased hepatic iron stores in chronic hepatitis C were related to resistance to Interferon/ Ribavirin treatment. [265] Thalassemic patients with liver fibrosis and hepatomegaly who undergo HSCT, have a markedly reduced OS and event free survival compared to patients without evidence of liver disease. The liver disease in these patients is due to a combination of severe IO and chronic viral hepatitis both of which improve with effective iron chelation therapy. [266] Iron is also deposited in other tissues such as myocardium or BM. Slow and spontaneous decrease in iron stores has been reported in

thalassemic children in the years following HSCT. This natural iron depletion could normalize iron stores in individuals with mild siderosis. However, in patients with moderate to severe IO this slow depletion could not prevent the development of liver dysfunction. For this reason, iron depletion protocols have been developed for patients with severe IO. [266]

Sinusoidal obstruction syndrome (SOS) (veno occlusive disease)

Sinosoidal obstruction syndrome is a treatment related toxicity associated with auto and allo– HSCT which is seen in 6–54 % of the recipients. The severity of SOS ranges from a mild reversible to a progressive course with a mortality rate close to 100%. [251]

The role of pre–transplant hyperferritinemia in the development of SOS was first demonstrated by Morado et al in a cohort of 180 auto–HSCT recipients. In this prospective study, SOS was defined in 12, 2% of patients based on McDonald criteria. Patients with pre–transplant ferritin levels above 300 mg/dl were shown to have a higher risk of developing SOS. [267] In a recent report by Maradei et al, a pre–transplant serum ferritin level above 1000 ng/dl was identified as an independent risk factor for the development of SOS. [268,269] Serum ferritin may be increased in conditions other than IO in this particular group of patients, including chronic inflammation and infection. Nevertheless, values higher than 1000 ng/ml were rarely reported in these inflammatory conditions. [267-270]

Iron induced hepatotoxicity is multifactorial which involves oxidative stress and modulation of gene expression of Kuppfer cells. Cellular injury is induced by iron generated ROS and peroxidation of lipid membranes. Risk factors associated with the development of SOS are defined as preexisting liver dysfunction, previous abdominal irradiation, and high dose total body irradiation, high dose preperative regimens, advanced disease and HLA mismatch or unrelated HSCT. The typical hepatocellular lesion of SOS mainly occurs in zone 3 of hepatic acines including a characteristic endothelial lesion which is shown to be associated with hypercoagulability. The oxidant effect of iron on endothelial and and hepatocyte membranes mediated by ROS contributes to the development of these typical lesions of SOS. [267-269]The risk of SOS is higher in carriers of at least one allele of the hemochromatosis gene, HFE, which predisposes to iron deposition in the liver. [247]

Infection

Patients with HH and other diseases with IO are considered to be more susceptible to infections, as iron adversely affects the phagocytic, chemotactic and bactericidal capacity of granulocytes and monocytes and inhibits the activity of natural killer cells and macrophages. [271] A number of studies have demonstrated the adverse impact of IO on the development infections in HSCT recipients. A direct correlation between hepatic IO and BSI was demonstrated in a retrospective cohort of 154 allo – HSCT recipients, as patients with hepatic IO tended to experience more frequent and prolonged episodes of lethal BSI.[272] Altes et al reported a ferritin level above 1500 µg/l was associated with the occurence of bacteremia and febrile days in first 3 months after auto–HSCT. A prospective study investigated the risk factors for 140 early infection episodes which occured in 367 multiple myeloma (MM) patients undergoing auto–HSCT. Bone marrow iron stores were identified as significant risk factors for early severe infections. [273] Pre–transplant serum

ferritin levels were demonstrated to be associated with fungal infections after allo–HSCT in several studies.[274,275]Tunçcan et al identified the predictive role of pre–transplant serum ferritin level in the development of hepatosplenic candidiasis among 255 HSCT recipients. Hepatosplenic candidiasis was diagnosed in 6 (2, 3%) patients. Pre–transplant serum ferritin levels were significantly higher in patients with hepatosplenic candidiasis.[274]

Idiopathic Pneumonia Syndrome (IPS)

Idiopathic pneumonia syndrome comprises a group of disorders that result in interstitial pneumonitis and/or widespread alveolar injury with an incidence of 2–8 % and a mortality of up to 70% in the HSCT setting. There is increasing evidence implicating ROS and pro– inflammatory events as major contributing factors to IPS. [251]The mechanism of iron induced IPS probably involves endothelial injury by catalytically active iron released from heme groups, which can trigger a cascade of events leading to acute lung injury and pulmonary fibrosis. [251] Currently,there are no studies regarding the direct association of IO and IPS, except the oxidative milieu, which is partly a consequence of IO.

Graft-versus-host disease (GVHD)

The role of IO in the pathogenesis of GVHD has been evaluated in a number of studies. There are conflicting results regarding the relationship between IO and GVHD in HSCT recipients. Apart from hepatocellular, cardiac and other organ dysfunction, IO may worsen the natural course of liver GVHD, similar to the status with chronic hepatitis and its response to therapy. [276]It is speculated that intestinal iron absorbtion is increased as a result of epithelial injury related to chemotherapy or GVHD. Suggesting that IO might be the consequence rather than being the cause of intestinal GVHD.

The liver and the intestinal mucosa, which express essential iron regulatory genes including hepatic antimicrobial protein (HAMP), the gene that encodes hepcidin and ferroportin 1, are targets of conditioning related toxicity as well as GVHD, initiated by donor derived T lymphocytes. The ensuing release of cytokines including IL-6, might directly affect the expression of hepcidin as IL-6 is a potent inducer of hepcidin via STAT3. [277]