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[1]Passed away on October 20, 2007
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EDITORIAL

Involvement of interleukin-15 and interleukin-21, two γ-chain-related cytokines, in celiac disease

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Abstract

Celiac disease (CD), an enteropathy caused by dietary gluten in genetically susceptible individuals, is histologically characterized by villous atrophy, crypt cell hyperplasia, and increased number of intra-epithelial lymphocytes. The nature of CD pathogenesis remains unclear, but recent evidence indicates that both innate and adaptive immune responses are necessary for the phenotypic expression and pathologic changes characteristic of CD. Extensive studies of molecules produced by immune cells in the gut of CD patients have led to identification of two cytokines, namely interleukin (IL)-15 and IL-21, which are thought to play a major role in orchestrating the mucosal inflammatory response in CD. Here we review the current knowledge of the expression and function of IL-15 and IL-21 in CD.

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Key words: Interleukin-21; Interleukin-15; Celiac disease

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INTRODUCTION

Celiac disease (CD) is a chronic gastrointestinal disorder caused, in genetically predisposed individuals, by ingestion of the gluten proteins of wheat, rye, and barley. The prevalence of CD is approximately 1% in the Western world[1-3]. The disease is strongly associated with *HLA-DQ* genes. Most patients carry a variant of DQ2 (alleles DQA1*05/DQB1*02) and others carry a variant of DQ8 (alleles DQA1*03/DQB1*0302)[4,5]. These haplotypes are relatively common in the healthy population, and only 1:20 individuals who express HLA-DQ2/DQ8 have CD[4,5]. These observations and the fact that the concordance rate between HLA-identical siblings is much lower than between monozygotic twins suggest that most probably other non-HLA genes are involved in the pathogenesis of CD[6-8].

In CD patients, ingestion of gluten triggers a mucosal inflammatory response, which leads to the tissue damage. The histological features of CD are villous atrophy, crypt cell hyperplasia, and increased number of intra-epithelial lymphocytes (IELs). The clinical manifestations can range from asymptomatic to severe malabsorption. Patients can also manifest symptoms of other immune-mediated diseases, which can associate with CD. The only treatment is a lifelong gluten-free diet, which results, in the vast majority of patients, in complete remission of symptoms and recovery of the normal mucosal histology[9].

Although the pathogenesis of CD is not fully understood, it is known that gluten peptides are deamidated by tissue transglutaminase and presented by $DQ2^+$ or $DQ8^+$ antigen-presenting cells to lamina propria $CD4^+$ T cells[9,10]. Upon activation, $CD4^+$ T cells polarize along the T helper (Th)1-type pathway, as substantiated by their ability to produce large amounts of interferon (IFN)-γ, the signature cytokine of Th1 responses[11,12]. In CD patients on a gluten-free diet, IFN-γ production is as low as in healthy controls but it can be stimulated *in vitro* by gluten to reach levels of untreated CD patients. In these mucosal cultures, neutralization of IFN-γ prevents gliadin-mediated

morphological changes thus supporting the role of the adaptive immune response and IFN-γ in CD immunopathology[11,12]. More recently it has become clear that some gluten peptides can induce mucosal damage by directly activating innate immune mechanisms[13]. These observations collectively underline the complexity of the pathogenic mechanism in CD and suggest that the CD-associated mucosal damage relies on the activation of multiple rather than single cell pathways.

GLUTEN PEPTIDES STIMULATE INNATE IMMUNITY AND CAUSE EPITHELIAL DAMAGE *VIA* AN INTERLEUKIN (IL)-15-DEPENDENT MECHANISM

It has long been known that gluten peptides cause epithelial damage when added to *ex vivo* organ cultures of biopsies taken from CD patients on a gluten-free diet, but not from controls[14]. As previously mentioned, this pathogenic response was initially thought to be secondary to the activation of lamina propria $CD4^+$ T cells and production of inflammatory cytokines, such as IFN-γ[11]. However, more recently Maiuri *et al*[13] showed that the gluten p31-43 peptide is able to induce mucosal damage by directly activating innate immune cells. In particular, it was shown that the p31-43 peptide elicits the production of IL-15 by lamina propria macrophages and dendritic cells in *ex vivo* organ cultures of CD biopsies, thus triggering a sequence of events that culminates in epithelial damage (Figure 1). These findings correlate well with the demonstration that IL-15 is over-expressed in both the lamina propria and intestinal epithelium of patients with active, untreated CD as compared with normal controls or inactive, gluten-free diet treated CD patients[15,16].

The majority of intestinal IELs are T cell receptor (TcR)$\alpha\beta^+$ $CD8^+CD4^-$ and a significant proportion are TcR$\gamma\delta^+$ $CD8^-CD4^-$[14]. IELs express a variety of natural killer (NK) lineage receptors, supporting their involvement in epithelial cell damage[14]. Corroborating Maiuri's results, Hüe *et al*[17] showed that treatment of biopsies taken from inactive CD patients with the gluten p31-49 peptide enhances the enterocyte expression of the non-conventional HLA molecules MICA/B. MIC molecules are known to be induced on enterocytes by stress and are up-regulated in active CD mucosa[18]. MICA/B are ligands of the activating NKG2D receptor, which signals through the adaptor protein DAP10 and is expressed on most NK cells, $CD8^+$ TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$, but normally not on $CD4^+$ T cells[19,20]. Expression of NKG2D in IELs is increased in active CD mucosa, and IELs lyse epithelial cells *via* NKG2D[17]. Up-regulation of MICA/B molecules also occurs in CD biopsies treated with exogenous IL-15, and a neutralizing IL-15 antibody blocks the gluten-mediated MIC-inducing effect in organ cultures of CD biopsies[17,21]. Meresse *et al*[22] showed that IL-15 also enhances the expression of both NKG2D and DAP10 in IELs, and that the cytolytic attack of the epithelium by IELs may be perpetuated *via* NKG2D independently of TcR specificity (Figure 1). Taken together these findings emphasize the critical role of IL-15 in intestinal epithelial cell death induced by NKG2D-expressing IELs.

IL-15 AND COUNTER-REGULATORY MECHANISMS IN CD

In the gut, mucosal homeostasis arises from a highly dynamic balance between host protective immunity and regulatory mechanisms[23]. One such counter-regulatory mechanism involves transforming growth factor-β1 (TGF-β1), a cytokine that is able to exert a number of negative effects on immune cells, including inhibition of T cell proliferation and differentiation, as well as down-regulation of macrophage activation and dendritic cell maturation[24]. Consistently, mice with global TGF-β1 defects, such as TGF-β1-deficient mice or transgenic mice expressing a dominant-negative TGF-βRⅡ chain that are unresponsive to TGF-β1 signaling, develop intestinal mucosal inflammation[25,26]. On the other hand, studies in mouse models of gut inflammation have shown that production of TGF-β1 is consistently associated with greatly diminished severity of inflammation[27]. Because TGF-β1 regulates both lymphoid and myeloid cells[24], several researchers have examined the expression and activity of TGF-β1 in chronic gastrointestinal inflammatory diseases, including CD. Initial studies conducted by Lahat *et al*[28] showed that in active CD mucosa there was enhanced expression of transcripts for TGF-β1 as compared to controls. In contrast, Lionetti *et al*[29] reported that the total RNA expression of TGF-β1 did not differ between active CD and controls. However, in active CD, TGF-β1 production was mostly confined to lamina propria T cells and macrophages, while in controls it was mostly produced by epithelial cells[29].

TGF-β1 initiates signaling through the ligand-dependent activation of a complex of heterodimeric transmembrane serine/threonine kinases, consisting of type Ⅰ (TGF-βRⅠ) and type Ⅱ (TGF-βRⅡ) receptors[30]. Upon TGF-β1 binding there is phosphorylation and activation of TGF-βRⅠ by the constitutively active and auto-phosphorylating TGF-βRⅡ. TGF-βRⅠ in turn phosphorylates two proteins, termed Smad 2 and Smad3. Once phosphorylated, Smad2 and Smad3 associate with Smad4 and translocate to the nucleus where Smad protein complexes participate in transcriptional control of target genes[30]. This pathway has been reported to be antagonized by inflammatory cytokines, which can impair the regulatory functions of TGF-β1 and alter immune homeostasis[31,32]. Therefore, it is conceivable that, during chronic inflammatory processes, there may be deficient TGF-β1 activity despite this cytokine being highly produced. In line with this, Benahmed *et al*[33] have recently shown that TGF-β1 inhibited the proliferative response of normal intestinal IELs and lamina propria

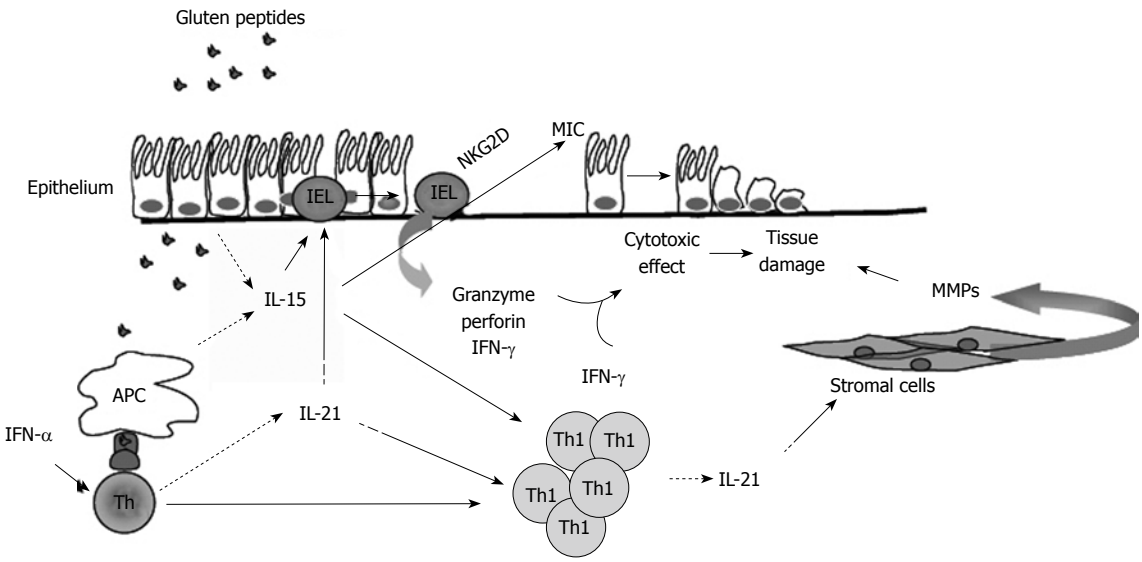

Figure 1 Hypothetical view of the pathogenic effects of IL-15 and IL-21 in celiac disease. In active celiac disease mucosa, IL-15 is produced by epithelial cells and antigen presenting cells (APC) in response to gluten stimulation. APC also produce interferon (IFN)-α, which stimulates CD4$^+$ T cells to polarize along the Th1 pathway. IL-21 is produced by activated CD4$^+$ T cells, including Th1 cells. IL-15 activates various pathways, which lead to the lysis and death of epithelial cells. IL-15 augments the production of granzyme B by IELs and IFN-γ by IELs and Th1 cells; enhances the expression of the activating NGK2D receptor on IELs; induces the NKG2D ligand, MICA, on epithelial cells. IL-21 seems to contribute to the tissue-damaging immune response in CD, given that this cytokine is able to enhance the production of perforin by IELs and IFN-γ by T cells, and to stimulate stromal cells to make extracellular matrix-degrading metalloproteinases.

lymphocytes induced by IL-2 but not that triggered by IL-15. The inhibitory effect of IL-15 on TGF-β1 activity was substantiated further by the demonstration that IL-15 impaired the formation of Smad3-DNA complexes in response to TGF-β1 stimulation[33]. In contrast, IL-15 enhanced the activation of the Janus kinase (JNK) pathway thus promoting the inhibitory effect of phospho-c-jun on the formation of Smad-DNA complexes. Further analysis revealed that the transcription levels of tristetraprolin, a TGF-β target gene under the control of the Smad3 pathway, were decreased in duodenal biopsies from patients with active CD compared with controls. This defect was associated with no significant change in the intracellular levels of phosphorylated Smad3 and Smad7, clearly indicating that initial steps of TGF-β signaling are preserved in patients with active CD[33]. This is in contrast to the Smad7-dependent inhibition of TGF-β1/Smad3 signaling described in patients with inflammatory bowel diseases[34,35].

IELs, lamina propria lymphocytes, and epithelial cells of active CD contain high levels of phospho-c-jun, and inhibition of this protein in organ cultures of CD biopsies enhances tristetraprolin[33]. Moreover, treatment of *ex vivo* organ cultures of CD biopsies with anti-IL-15 reduces phospho-c-jun and increases tristetraprolin, thus confirming the prominent role of IL-15 in the phospho-c-jun-mediated inhibition of TGF-β1 signaling[33].

While NKG2C and NKG2D are activating NK receptors, NKG2A is considered an inhibiting NK receptor. NKG2A can associate with CD94 and bind the non-classical MHC Ib molecule, HLA-E, which is expressed on epithelial cells of CD patients but not on epithelial cells of healthy individuals[36,37]. Although NKG2A competes with NKG2C for its interaction with HLA-E, the former has a higher binding affinity for HLA-E compared with NKG2C. Upon binding to this ligand, CD94/NKG2A delivers negative signals to cytotoxic cells[38]. A higher percentage of small intestinal TcRγδ IELs express CD94/NKG2A compared with TcRαβ IELs, and TcRγδ IELs exert negative effects on the IL-15-mediated induction of IFN-γ, granzyme B, and NKG2D in CD8$^+$ TcRαβ IELs[39]. These observations suggest that TcRγδ IELs have the ability to suppress the cytotoxic programming of TcRαβ IELs. This inhibitory effect requires interaction of NKG2A with its ligand HLA-E, a phenomenon which is followed by enhanced secretion of TGF-β1. Notably, blockade of TGF-β1 activity with a neutralizing human TGF-β1 antibody partially abrogates the suppressive capability of TcRγδ IELs, and exogenous TGF-β1 dose-dependently inhibits the IL-15-mediated induction of IFN-γ, granzyme B, and NKG2D in CD8$^+$ TcRαβ IELs[39]. Therefore, in this cell context, IL-15 is not sufficient to abrogate the immunosuppressive action of TGF-β1. These findings conflict with the aforementioned data published by Benahmed *et al*[33]. It is likely that this discrepancy may simply be due to differences in cell culture conditions used in these two studies. Another possibility is that the IL-15-mediated negative regulation of TGF-β1 signaling occurs only in specific cell types. Since TGF-β1 can trigger both Smad-dependent and -independent intracellular pathways[39,40], it is also plausible that IL-15 can interfere with some and not all TGF-β1-activated signals.

Expression of NKG2A is decreased on TcRαβ IELs and TcRγδ IELs from CD patients as compared to normal subjects[39-41]. The mechanism that removes this negative regulator from CD IELs remains unknown. NKG2A possesses in its promoter a binding site for

Smad3, and there is evidence that TGF-β1 cooperates with the TcR in enhancing the expression of NKG2A on CD8$^+$ T cells[18,42]. Therefore, molecules that disrupt TGF-β1 signaling could, at least in theory, contribute to the down-regulation of NKG2A in CD. IL-15 is not able to directly regulate NKG2A expression[43], even though it remains possible that it could inhibit the TGF-β1-induced NKG2A expression.

IL-21 as a positive regulator of Th1 cell response in CD

The demonstration that CD lesions are associated with a marked infiltration of IFN-γ-secreting cells has boosted intensive research aimed at identifying the factors that promote the ongoing mucosal Th1 cell response. Paradoxically, IL-12, the major Th1-inducing factor in man, is not over-produced in CD mucosa[12]. However, analysis of transcription factors that drive Th cell differentiation revealed that in active CD mucosa there is enhanced expression of T-bet, a member of the T-box family of transcription factors that directs Th1 lineage commitment and is essential for IFN-γ production in CD4$^+$ T cells[43]. In contrast, no increase in GATA3 and active STAT6, two transcription factors that specifically regulate Th2 differentiation, is seen in active CD samples as compared to controls[43]. Interestingly, active CD biopsies do not exhibit enhanced activation of STAT4[43], an IL-12-dependent Th1-inducing transcription factor, thus confirming the lack of IL-12 activity. These data strongly support the existence of a regulatory pathway for Th1 differentiation that starts from activation of T-bet and is independent of IL-12-driven STAT-4 signaling. So the critical question is: what induces and sustains T-bet in CD? We have recently shown that in the duodenal mucosa of patients with active but not inactive CD there is enhanced production of IL-21[44]. Interestingly, neutralization of IL-21 activity in *ex vivo* organ cultures of CD biopsies reduces both T-bet expression and IFN-γ production[44]. Since IFN-γ enhances T-bet *via* a STAT1-dependent mechanism[43], it is tempting to speculate that IL-21 is part of a positive feedback loop that helps amplify and stabilize the committed Th1 cell phenotype in CD (Figure 1).

Recent genome-wide association studies have provided convincing evidence that the chromosomal 4q27 region harboring the *IL-2* and *IL-21* genes is associated with CD[7]. A similar genetic association has been described in other immune-mediated diseases, such as psoriasis, type 1 diabetes, and inflammatory bowel diseases[45,46]. However, it is not yet known if such polymorphisms can influence the tissue levels of IL-21.

Biopsies taken from CD patients on a gluten-free diet over-express IL-21 when challenged *in vitro* with gluten peptides[44]; however, the basic mechanisms that control the expression of IL-21 in CD mucosa remain to be elucidated. Previously, we showed that IFN-α is up-regulated in the mucosa of active CD patients, where it most probably contributes to intensifying IFN-γ production[47]. Indeed, neutralization of IFN-α activity drastically reduces the gliadin peptide-driven IFN-γ expression in biopsies of inactive CD patients[48]. Interestingly, IFN-α enhances the mRNA expression of IL-21 in activated human T cells[49], thus suggesting a role for this cytokine in the positive control of IL-21 in CD (Figure 1).

Potential involvement of IL-21 in the activity of other mucosal cells types

The biological functions of IL-21 are mediated by a cell-surface class I cytokine receptor, formed by the common γ-chain subunit (shared with IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 receptors) and its own unique receptor (designated IL-21 receptor)[50]. Since this receptor is expressed by both immune and non-immune cells, it is plausible that IL-21 maintains chronic inflammation and/or favors tissue damage in CD by targeting additional cell types other than Th1 lymphocytes. In fact, IL-21 has been shown to stimulate epithelial cells to secrete chemokines and facilitate recruitment of immune cells within the inflamed tissue, induce fibroblasts to make tissue-damaging proteases, and make effector CD4$^+$ T cells resistant to regulatory T cell-mediated immunosuppression[51-53]. In light of the role of IL-21 in the control of B cell and plasma cell function[54], IL-21 may also contribute to the production of CD-associated autoantibodies. Since its discovery, it has become clear that IL-21 is also able to modulate the proliferation and/or effector function of CD8$^+$ T cells and NK cells[55]. Using IELs isolated from human jejunal mucosa, Ebert has recently shown that IL-21 increases perforin-mediated cytotoxicity and serine esterase release without affecting the growth and survival of these cells[56]. The relevance of this finding for CD pathogenesis remains to be ascertained, although IL-21-mediated cytotoxic T cell activation could contribute to the intestinal epithelial cell death and villous atrophy in CD.

CONCLUSION

A growing body of evidence suggests that IL-15 and IL-21 may play important roles in the immune response associated with inflammation and tissue damage in CD, even though some intriguing questions remain to be resolved. For instance, it remains to be determined whether these two cytokines contribute qualitatively and quantitatively differently to the initiation and progress of the inflammatory cascade in CD. The fact that IL-15 and IL-21 share a receptor subunit and target the same cell types raises the possibility that these cytokines may cooperate in regulating specific cell immune responses. Indeed, both IL-15 and IL-21 are known to stimulate the growth of T cells, enhance the production of IFN-γ by Th1 cells, and promote the activation of cytotoxic cells[21,44,50,57] (Figure 1). As outlined in this article, these two cytokines might also act in concert to disrupt local mechanisms of immune tolerance. This hypothesis is supported by the demonstration that IL-15 inhibits TGF-β1 activity, and IL-21 renders effector CD4$^+$ T cells resistant to the suppressive effects of CD4$^+$CD25$^+$ regulatory T cells[33,53]. Nonetheless it is noteworthy that

IL-15 and IL-21 seem to differentially modulate some aspects of both innate and adaptive immunity, such as dendritic cell maturation and T cell apoptosis[50,57]. Therefore, we cannot exclude the possibility that, at least in some stages of the inflammatory process, IL-15 and IL-21 can exert opposing effects on the gluten-driven immune response.

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EDITORIAL

Anemia and digestive diseases: An update for the clinician

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Abstract

Anemia and iron deficiency are so common in digestive diseases that often are underestimated and undertreated. Our goal is to review from classification to treatment of the diverse types of anemias in different digestive diseases to update our knowledge on diagnosis and treatment. With the goal of improving the prognosis and quality of life of digestive diseases patients, we will review current transfusion, intravenous iron, and erythropoietin roles in the treatment of anemia.

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Key words: Anemia; Iron deficiency; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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The human mind is a marvellous albeit complex tool. It is not easy to understand how it works[1] with no exception for the clinician[2]. Inflammatory bowel diseases (IBD) are complex, often difficult to manage, and anemia is so common around the world[3] that perhaps even the most astute clinician simply cannot always see that specific tree in the forest[4]. However, anemia is really important for patients, and we should not forget this. Since publication of the landmark article by Gasche[5], a study[6] has shown the importance of anemia that should be actively looked for and treated. Anemia in IBD is a good example[6], and we will dedicate our specific attention to it, but anemia can also be a key point in many different clinical scenarios of gastroenterology and hepatology. Therefore, in this short article, we will attempt to review several aspects we consider interesting for the practicing clinician. There are many possible approaches and we will take a look at different perspectives. Hematologists, gastroenterologists, hepatologists and even nephrologists will contribute to our task.

Anemia is both a simple word and a complex one. There are many different diseases that could be included in the World Health Organization definition of anemia (a patient has anemia if his or her blood hemoglobin level is below standard for age and sex). However, the simple final result can be due to the consequence of many different causes and mechanisms that can even overlap in the same patient and at the same time. Our first goal is to furnish our readers with an up-to-date classification of anemia. To do this work, we have chosen the hematologists' point of view.

Iron is not only the most abundant chemical element on Earth, but also a key element for life from very ancient ancestors. Life beings often compete for iron, a key to the control of redox reactions in many living organisms[7]. Perhaps, due to evolutional reasons, iron regulation is important in inflammation. In many digestive diseases, iron is a principal player because absorption, loss and regulation of iron metabolism, can all be affected in different diseases. Most likely, the paradigm is Crohn's disease[8], in which malabsorption, inflammation and blood loss, all contribute to iron deficiency and iron deficiency anemia in some cases. The regulation of iron metabolism has been a very interesting topic in the last few years, and some new molecules have been developed. Hepcidin is a raising star, but it is not alone[9]. The clinician should know the basics of these mechanisms to understand the treatment modalities, and so we have asked the experts to summarize this topic.

It is a bit counterintuitive, but luminal contents are out of our body, in a bacterial world (in fact all the earth is a bacterial world). To get iron in, it has to be absorbed as many other elements or substances. The role of malabsorption in anemia is a very interesting topic for gastroenterologists.

Anemia is a rather common problem in liver units as well. Patients with severe liver disease often have anemia and need specific treatment. Blood transfusion is common

(perhaps too common) in these patients. Anemia in patients with liver disease has interesting particularities, such as the role of portal hypertension, renal failure or antiviral drugs. Thus, a specific article has been devoted to anemia in liver disease.

The story of intravenous iron is a complex one[10]. Some old preparations are difficult to manage and cause significant risks in patients. So, when new safer preparations[11] that are easier to use appear, clinicians are simply fearful of using intravenous iron. However, with increasing experience in nephrology, oncology, gastroenterology, and gynecology, IV iron is becoming a standard treatment for patients at all ages and conditions[12]. If we want this to be a real life standard, clinicians need clear rules, namely "who", "when", and "how" are the classic questions that are addressed in a specific article on IV iron.

Intravenous iron is not always enough to treat anemia since erythropoietin can be very useful if used judiciously. For this topic, we prefer the nephrologists' point of view because they have the most important experience with both sides: Dr. Jeckyll (the improvement in quality of life) and Mr. Hyde (mortality associated with a high hemoglobin level), who will tell us about their experience, which is very interesting with rather complex economic implications[13].

Blood transfusion can be lifesaving in many clinical situations, and has been one of the greatest advances in medicine. However, blood transfusion is not a risk and/or cost-free, and may have been overused in many situations. Very important evidence from traumatology, surgical and intensive care units has been published in recent years[14]. We should apply this update knowledge in our patients, following the most recent guidelines. The hematologists' point of view can be important for the gastroenterologist and the hepatologist.

Anemia, intravenous iron, blood transfusion, iron metabolism, *etc*, have been a few of the changing topics over the last few years. To help our patients, we still need to know many things about this very old tree with many roots and branches. We have selected to update our knowledge on some of them, with the aim of helping our patients. In Darwin's year, it was always tempted to use the tree image, and so in a Freudian way to recognize him as the father of modern biology.

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, *Series Editors*

An update on iron physiology

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Abstract

Iron is an essential micronutrient, as it is required for adequate erythropoietic function, oxidative metabolism and cellular immune responses. Although the absorption of dietary iron (1-2 mg/d) is regulated tightly, it is just balanced with losses. Therefore, internal turnover of iron is essential to meet the requirements for erythropoiesis (20-30 mg/d). Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency (ID) and iron-deficiency anemia. Hepcidin, which is made primarily in hepatocytes in response to liver iron levels, inflammation, hypoxia and anemia, is the main iron regulatory hormone. Once secreted into the circulation, hepcidin binds ferroportin on enterocytes and macrophages, which triggers its internalization and lysosomal degradation. Thus, in chronic inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling, which results in hypoferremia and iron-restricted erythropoiesis, despite normal iron stores (functional ID), and anemia of chronic disease (ACD), which can evolve to ACD plus true ID (ACD + ID). In contrast, low hepcidin expression may lead to iron overload, and *vice versa*. Laboratory tests provide evidence of iron depletion in the body, or reflect iron-deficient red cell production. The appropriate combination of these laboratory tests help to establish a correct diagnosis of ID status and anemia.

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Key words: Iron metabolism; Iron deficiency; Functional iron deficiency; Hepcidin; Anemia of chronic disease

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INTRODUCTION

Erythropoiesis is a part of the larger process of hematopoiesis. In the normal adult human, the daily turnover of red blood cells (RBCs) exceeds 10^{11} cells. In periods of increased RBC loss caused by hemolysis or hemorrhage, the production of RBCs increases rapidly and markedly. However, an overproduction of RBCs (i.e. rebound polycythemia) does not occur even after the most severe loss of RBCs. Thus, erythropoiesis is a finely regulated yet rapidly responsive process that maintains the normal number of circulating RBCs within a narrow range[1].

The major stages of differentiation in human erythropoiesis are depicted in Figure 1. The commitment of multipotent hemopoietic stem cells to erythroid progenitors is driven by several growth factors, such as stem cell factor, thrombopoietin, and interleukin (IL)-3[2]. The most immature stage of committed erythroid progenitors is the burst-forming unit-erythroid, which differentiates into colony-forming unit-erythroid (CFU-E) in approximately 7 d, with declining proliferative potential as the progenitors approach CFU-Es. Each CFU-E develops a single cluster of 8-64 mature erythroblasts within 7 d, after several differentiation stages (proerythroblast, basophilic erythroblast, polychromatic erythroblast, and orthochromatic erythroblast). Orthochromatic erythroblasts do not divide but they enucleate, and form nascent RBCs, called reticulocytes, which are release into the bloodstream. After 1 d of circulation in the peripheral blood, reticulocytes mature into RBCs[3]. The normal proliferation and differentiation of erythroid progenitor cells require several essential

nutrients, such as iron, folate, and vitamin B12, the interaction with the stromal cells in the bone marrow, and stimulation by erythropoietin (EPO)[3].

IRON HOMEOSTASIS

For a 70-kg male individual, total body iron is about 3.5 g (50 mg/kg). Most of the iron in the body is distributed within RBC hemoglobin (65%; 2300). Approximately 10% is present in muscle fibers (in myoglobin) and other tissues (in enzymes and cytochromes) (350 mg). The remaining body iron is stored in the liver (200 mg), macrophages of the reticuloendothelial system (RES; 500 mg), and bone marrow (150 mg). In premenopausal women, total body iron (especially the stored fraction, 250-300 mg) is lower than in men. The normal diet contains 15-20 mg of iron, and the body absorbs 1-2 mg/d of dietary iron. This is balanced with losses *via* sloughed intestinal mucosal cells, menstruation and other blood losses. Therefore, internal turnover of iron is essential to meet the bone marrow requirements for erythropoiesis (20-30 mg/d)[3-5].

On the other hand, the body has no effective means of excreting iron and thus the regulation of absorption of dietary iron from the duodenum plays a critical role in iron homeostasis in the body[5]. This is extremely important as iron is essential for cellular metabolism and aerobic respiration, whilst cellular iron overload leads to toxicity and cell death *via* free radical formation and lipid peroxidation, thus, iron homeostasis requires tight regulation[3,4,6]. A summary of proteins involved in iron homeostasis, as well as their most frequently used acronyms, is given in Table 1.

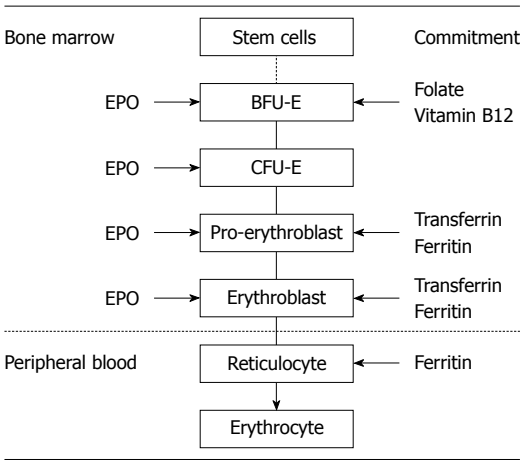

Figure 1 Major stages of human erythropoiesis showing the point of commitment, the period of EPO dependence and the requirements for essential nutrients. BFU-E: Burst-forming unit-erythroid; CFU-E: Colony-forming unit-erythroid; EPO: Erythropoietin.

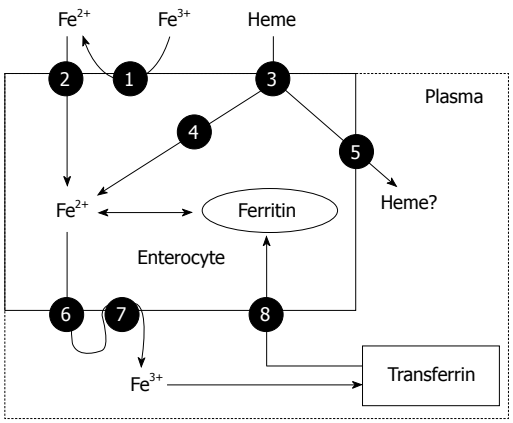

Figure 2 Main pathways of iron absorption by enterocytes in mammals. 1: Ferrireductase; 2: Divalent metal transporter 1 (DMT-1); 3: Heme protein carrier 1 (HPC1); 4: Heme oxygenase; 5: Heme exporter; 6: Ferroportin (Ireg-1); 7: Hephaestin; 8: Transferrin receptor-1 (TfR1) (for details see Table 1).

Iron absorption

Nearly all absorption of dietary iron occurs in the duodenum. Several steps are involved, including the reduction of iron to a ferrous state, apical uptake, intracellular storage or transcellular trafficking, and basolateral release (Figure 2). Dietary iron is found in heme (10%) and non-heme (ionic, 90%) forms and their absorption occurs at the apical surface of duodenal enterocytes *via* different mechanisms. Dietary non-heme iron primarily exists in an oxidized (Fe^{3+}) form that is not bioavailable, and must first be reduced to the Fe^{2+} form by a ferrireductase enzyme, before it is transported across the intestinal epithelium by a transporter called divalent metal transporter 1 (DMT-1), which also transports other metal ions such as zinc, copper and cobalt by a proton-coupled mechanism[3,4]. There is also a siderophore-like iron uptake pathway mediated by lipocalin-2 (that seems to exert an innate immune response to bacterial infection by sequestrating iron) but its physiological role is not fully worked out.

The absorption of non-heme can be diminished by co-administration of tetracyclines, proton pump inhibitors and antacid medication, phytates (high-fiber diets), calcium, and phenolic compounds (coffee and tea). In addition, infection with *Helicobacter pylori* (*H pylori*) produces gastric atrophy that, even in the absence of significant bleeding, can lead to profound iron-deficiency anemia (IDA). As expected, this anemia is poorly responsive to oral iron therapy, but can be corrected by eradication of *H pylori* infection[7].

Heme iron is absorbed into enterocytes by a putative, not totally identified heme carrier protein 1, which is a membrane protein found in the proximal intestine, where heme absorption is greatest[8]. Once internalized in the enterocytes, it is likely that most dietary heme iron is released as ferrous iron by heme oxygenase to enter a common pathway with dietary non-heme iron before it leaves the enterocytes[3,4] (Figure 2). However, it remains uncertain whether some heme might traverse the cells intact, leaving the enterocytes through the action of the recently characterized heme exporters, Bcrp/Abcg2 and feline leukemia virus C receptor (FLVCR)[8]. If this does occur, the subsequent disposition of plasma heme

Table 1 Main proteins involved in iron homeostasis in mammals

Protein name (alternative name)	Acronyms	Function	Localization
Divalent metal transporter 1 (divalent cation transporter 1, NRAMP 1 and 2)	DMT1 DCT1 NRAMP1* NRAMP2	Traffics divalent metal ions such as iron, zinc, copper and cobalt across the membrane by a proton coupled mechanism	Enterocyte (apical membrane) Erythroblast (siderosome) Macrophage (plasma membrane, phagocytic vesicles*) Hepatocyte Kidney cells
Ferrireductase	Dcytb STEAP3*	Reduction of Fe^{3+} to Fe^{2+}	Enterocyte (apical membrane) Erythroblast (siderosome*)
Heme carrier protein 1	HCP1	Putative transporter that traffics heme across the membrane by an unknown mechanism	Enterocyte (apical membrane) Hepatocyte Kidney cells
Heme oxygenase		An enzyme that disassembles heme to liberate iron	Enterocyte (microsomal fraction) Macrophages
Ferroportin 1 (Iron regulatory protein 1)	FPN 1 Ireg1 MTP1	Transmembrane Fe^{2+} transporter (exporter)	Enterocyte (basolateral membrane) Macrophages Hepatocytes
Hephaestin	Hp	Membrane-bound multicopper ferroxidase, similar to plasma ceruloplasmin, which oxidizes Fe^{2+} to Fe^{3+} to load it onto transferrin Maintenance of cell-surface localization of ferroportin	Enterocyte (basolateral) Macrophage Hepatocyte?
Transferrin	Tf	Plasma Fe^{3+} binding protein Ligand for transferrin receptors 1 and 2	Plasma
Transferrin receptor 1	TfR1	Cellular uptake of transferrin bound iron	Ubiquitously expressed
Transferrin receptor 2	TfR2	Sensor for diferric transferrin; regulates hepcidin expression; may participate in a signaling complex with HFE	Enterocyte Hepatocyte Erythroblast
Mitoferrin	SLC25A37	Mitochondrial iron importer that plays a critical role in supplying iron to ferrochelatase for insertion in protoporphyrin IX to form heme	Erythroblast (mitochondria)
Ferritin	Ft	Iron storage protein (H and L chains) Ferroxidase activity (H chain)	Enterocyte Erythroblast Macrophage Hepatocyte Myocytes and cardiomyocytes
Hemosiderin		Iron storage protein; breakdown product of ferritin that occurs when iron levels are high	Macrophage (lysosomes) Hepatocytes (lysosomes)
Heme exporters	LFLVCR* Bcrp/Abcg2** Abcb6***	ATP-independent heme export at the cell membrane* ATP-dependent heme export at the cell membrane** and mitochondrial membrane***	Erythroblast* Ubiquitously expressed**,***
HFE	HFE	Regulates hepcidin expression, mechanism uncertain; may participate in a signaling complex with TfR2; interacts with TfR1 & β-2-microglobulin	Enterocyte Macrophage Hepatocyte
Hemojuvelin	HFE2	Acts as a BMP co-receptor to stimulate hepcidin transcription	Hepatocytes
Lipocalin 2		Mediates a siderophore-like iron uptake pathway. Its an innate immune response to bacterial infection by sequestrating iron, but its physiologic role in iron absorption is not fully worked out	Enterocytes (apical membrane) Macrophages Adipocyte
Hepcidin	HEP HAMP LEAP1	Iron regulatory hormones, binds ferroportin to cause its internalization and degradation	Hepatocytes Adipocytes (low secretion) Enterocytes?
Erythropoietin	EPO	Upregulates the expression ferroportin in macrophages, TfR1 in erythroblasts, and DMT1 and hephaestin in enterocytes Downregulates hepcidin expression in hepatocytes and DMT1 expression in macrophages	Kidney (interstitial peritubular cells) Hepatocytes (low secretion)

BMP: Bone morphogenetic protein.

is unknown. In addition, it is not yet known whether heme carrier protein 1 has physiological roles in tissues other than the intestine. The protein is also expressed in the kidneys and liver, which suggests that it may act at those sites. It might, for example, scavenge free heme or mediate cellular uptake of heme from its circulating carrier protein, hemopexin[9].

Once inside the intestinal epithelial cell, iron may either remains in the cell for use or storage (this iron is never absorbed into the body; rather, it is lost when enterocytes senesce and are sloughed into the gut lumen) or exported across the basolateral membrane of the enterocyte into the circulation (absorbed iron). Ferroportin 1 is the only putative iron exporter identified to date. Ferrous iron once exported across the basal membrane by ferroportin 1, is then oxidized

by a multi-copper oxidase protein called hephaestin (an enzymatic protein similar to plasma ceruloplasmin) before being bound by plasma transferrin. Ferroportin 1 is also the putative iron exporter in macrophages and hepatocytes (Figure 2)[3,4].

The absorption of iron is dependent on the body's iron stores, hypoxia and rate of erythropoiesis. Two models have been proposed to explain how the absorption of iron is regulated: the crypt programming model and the hepcidin model.

The crypt programming model: This model proposes that enterocytes in the crypts of the duodenum take up iron from the plasma. The intracellular iron level of the crypt cells corresponds to the body's iron stores, which in turn determines the amount of iron absorbed from the gut lumen, as these crypt cells migrate upwards to become absorptive cells at the brush border. The crypt cells express both transferrin receptor 1 (TfR1) and TfR2, which mediate the cellular uptake of transferrin-bound iron from plasma[3,5].

TfR1 is expressed ubiquitously and transferrin mediated iron uptake is thought to occur in most cell types. HFE, an MHC-class 1-like molecule that interacts with β2-microglobulin and forms a complex with TfR1, is highly expressed in crypt cells. Its role in the regulation of TfR1-mediated transferrin-bound iron uptake remains unclear, but it seems to enhance transferrin-bound iron uptake from the plasma into crypt cells *via* TfR1, and may also inhibit the release of iron from the cell *via* ferroportin 1. In contrast, TfR2 is restricted to hepatocytes, duodenal crypt cells and erythroid cells, which suggests a more specialized role in iron metabolism. The intracellular iron concentration controls the interaction of cytosolic iron regulatory proteins (IRPs) 1 and 2 with iron regulatory elements (IREs; which act as iron sensors in mammalian cells and regulate translation or stability of mRNA-encoding proteins) in the 3' and 5' regions of different mRNA molecules. In the absence of iron, IRP1 binds to IREs of TfR1, DMT-1, and ferroportin 1 mRNA, the transcript is stabilized, translation proceeds, and the proteins are synthesized. Thus, a high IRP binding activity reflects low body iron stores and results in upregulation of these proteins in the duodenum and increased dietary iron absorption. When IRPs bind to IRE of ferritin mRNA, translation of the transcript is blocked and synthesis is halted. Thus, ferritin levels are regulated reciprocally - being increased in iron-replete states and decreased in iron-deplete states[3].

The hepcidin model: Liver hepcidin is a 25-amino-acid cysteine-rich peptide with antimicrobial properties, which is regulated by a number of factors such as liver iron levels, inflammation, hypoxia and anemia. The hepcidin model proposes that hepcidin is secreted into the blood and interacts with villous enterocytes to regulate the rate of iron absorption, by controlling the expression of ferroportin 1 at their basolateral membranes. The binding of hepcidin to ferroportin 1 results in internalization of ferroportin 1 and loss of its function. Ferroportin 1 molecules present in macrophages and liver are also targets for hepcidin. Thus, it is hypothesized that when hepcidin levels are increased in iron overload (by the uptake of transferrin bound iron *via* TfR1/HFE and TfR2) or inflammation (*via* IL-6), iron release from intestinal crypt cells, liver and macrophages is reduced. In contrast, when hepcidin levels are reduced, as in iron deficiency (ID), anemia or hypoxia, it is likely that ferroportin 1 expression and iron release from intestinal cells, liver and cells of reticuloendothelial system is increased[10]. In contrast, a mutation in the *ferroportin 1* gene is responsible for type Ⅳ hemochromatosis.

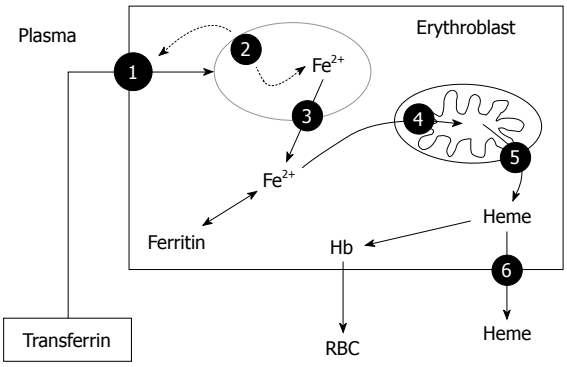

Figure 3 Main pathways of iron utilization by erythroblasts in mammals. 1: TfR1; 2: Diferric-transferrin-TfR1 complex; 3: Natural resistance macrophage protein (NRAMP-1); 4: Mitoferrin; 5: Mitochondrial heme exporter (Abcb6); 6: Heme exporter (FLVCR, Bcrp/Abcg2) (for details see Table 1).

There is evidence to support both models and it is possible that both control mechanisms may contribute to the regulation of iron absorption. In this regard, there is emerging evidence that hepcidin may act directly on mature villous enterocytes rather than crypt enterocytes. There are several situations (e.g. acute phase response) when iron absorption can be modulated more rapidly (within hours) than can be accounted for *via* the mechanism that involves the programming and maturation of crypt enterocytes (lag time of days)[5].

Iron distribution

Iron released into the circulation binds to transferrin and is transported to sites of use and storage. Transferrin has two binding sites, binding one iron atom each (thus three forms can be found in plasma: apo-transferrin which contains no iron, monoferric-transferrin and diferric-transferrin). About 30%-40% of these sites are occupied under normal physiological conditions. Thus, transferrin-bound iron is about 4 mg, but this is the most import dynamic iron pool[11]. Transferrin-bound iron enters target cells - mainly erythroid cells, but also immune and hepatic cells- through a process of receptor-mediated endocytosis (Figure 3). As diferric-

transferrin has a much higher affinity for TfR than does monoferric-transferrin, it binds to the TfR at the plasma membrane, and patches of cell-surface membrane that carry receptor-ligand complexes invaginate to form clathrin-coated endosomes (siderosomes)[11]. After clathrin is removed, the siderosomes become acidified through an ATP-dependent proton influx, which leads to conformational changes in transferrin and TFR1, and promotes iron release of Fe^{3+} from transferrin. Fe^{3+} is then reduced to Fe^{2+} by a ferrireductase and transported to the cytoplasm through the DMT-1, whereas the TfR is recycled to the cell membrane and transferrin shed back to the circulation[3,12] (Figure 3). Production of hemoglobin by the erythron accounts for most iron use. High-level expression of TfR1 in erythroid precursors ensures the uptake of iron into this compartment. To make heme, iron must again cross an ion-impermeable membrane to enter the mitochondria. The mitochondrial iron importer was recently identified as mitoferrin (also known as SLC25A37), a transmembrane protein that plays a crucial role in supplying iron to ferrochelatase for insertion into protoporphyrin IX to form heme[12] (Figure 3). Recently, different human heme exporters have been identified in erythroblasts, and their activity seems to be essential for erythropoiesis, by transferring heme from the mitochondria to citosol (Abcb6) and removing the excess of heme from the erythroid cells (FLVCR, Bcrp/Abcg2) (Figure 3).

In the erythroid precursors, the expression of TfR1, DMT-1 and ferritin are regulated reciprocally through IRP1 and IRP2, which act on the IRE present in their RNA. Thus, when increased iron uptake is needed, the expression of TfR1 and DMT-1 is increased, whereas the synthesis of ferritin is halted[3]. In addition, there is evidence that EPO activates IRP-1, leading to upregulation of TfR1 expression in the erythroid precursors, which is maintained along with the differentiation process, and DMT-1 and hephaestin gene expression in the duodenum[13]. To date, three patients have been reported with DMT-1 mutations that cause microcytic hypochromic anemia, as a result of decreased erythroid iron utilization, but lead to increased liver iron storage[14].

A truncated form of the TfR can be detected in human serum. The serum concentration of this soluble form of TfR (sTfR; normal median concentration: 1.2-3.0 mg/L, depending on the assessment kit used) is proportional to the total amount of surface TfR. Increased sTfR concentrations indicate ID even during the anemia of chronic disease (ACD), as well as increased erythropoietic activity without ID, whereas lower sTfR concentrations may reflect decreased numbers of erythroid progenitors[3,15].

Iron storage

Hemoglobin iron has substantial turnover, as senescent erythrocytes undergo phagocytosis by RES macrophages. Within the phagocytic vesicles, heme is metabolized by

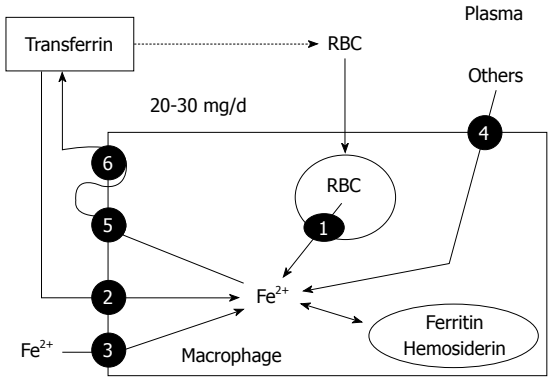

Figure 4 Main pathways of iron storage and exportation by macrophages in mammals. 1: NRAMP-1; 2: TfR1; 3: DMT-1; 4: Others: others: bacteria, lactoferrin, hemoglobin-haptoglobin, heme-hemopexin; 5: Ferroportin (Ireg-1); 6: Hephaestin (for details see Table 1).

heme oxygenase and the released iron is exported to the cytoplasm through the action of natural resistance-associated macrophage protein-1, a transport protein similar to DMT-1 (Figure 4). Macrophages can also obtain iron from bacteria and apoptotic cells, from plasma through the action of DMT-1 and TfR1, and from other sources (Figure 4). Within the cell, iron can be stored in two forms: in the cytosol as ferritin and, after breakdown of ferritin within the lysosomes, as hemosiderin. Hemosiderin represents a very small fraction of normal body iron stores, mostly in macrophages, but increases dramatically in iron overload[11]. Iron export from macrophages to transferrin is accomplished primarily by ferroportin 1, the same iron-export protein expressed in duodenal enterocytes, and hephaestin[3] (Figure 4). The amount of iron required for daily production of 300 billion RBCs (20-30 mg) is provided mostly by recycling iron by macrophages[4]. Importantly, iron storage at the macrophages is safe, as it does not lead to oxidative damage. EPO reduces iron retention in macrophages by decreasing DMT-1 and increasing ferroportin 1 expression[16].

The liver is the other main storage organ for iron. In iron overload, free radical formation and generation of lipid peroxidation products may result in progressive tissue injury and eventually cirrhosis or hepatocellular carcinoma[17]. Iron is sequestrated in hepatocytes predominantly in the form of ferritin or hemosiderin. The uptake of transferrin-bound iron by the liver from plasma is mediated by TfR1 and TfR2 (Figure 5). In iron overload, TfR1 is downregulated in hepatocytes[5]. TfR2 is expressed highly in human liver and is likely to play an important role in liver iron loading in iron overload states. Unlike TfR1, TfR2 lacks an IRE and thus is not regulated reciprocally in response to the level of plasma iron. Instead, TfR2 protein expression is regulated by transferrin saturation (TSAT), and is upregulated in iron overload. In normal and iron-loaded conditions, expression of TfR2 exceeds that of TfR1, which suggests that TfR2 plays an important role in hepatic iron loading in hemochromatosis[5].

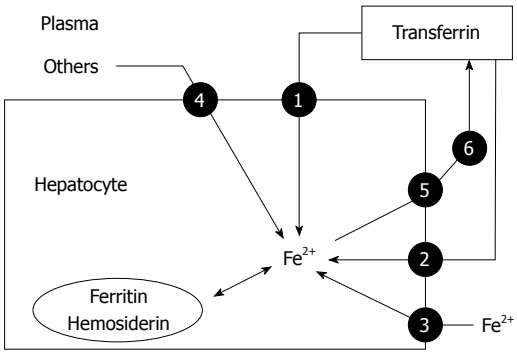

Figure 5 Main pathways of iron storage and exportation by hepatocytes in mammals. 1: TfR1; 2: TfR2; 3: DMT-1; 4: Others: hemoglobin, heme, ferritin; 5: Ferroportin (Ireg-1); 6: Ceruloplasmin (for details see Table 1).

In fact, a mutation in TfR2 is responsible for type 3 hemochromatosis[14].

As transferrin becomes saturated in iron overload states, excess iron is also found as non-transferrin-bound iron is transported across the hepatocyte membrane *via* a carrier-mediated process consistent with DMT-1. The hepatocytes may also store iron from ferritin, hemoglobin-haptoglobin complexes, and heme-hemopexin complexes. In contrast, once again, ferroportin 1 is likely to be the only protein that mediates the transport of iron out of hepatocytes, which is then oxidized by ceruloplasmin and bound to transferrin[3,4] (Figure 5).

Iron storage within cardiomyocytes is also of outstanding interest, as cardiac failure is the leading cause of death among patients with untreated hereditary hemochromatosis or transfusion-associated hemosiderosis[3]. In cardiac cells, excess iron may result in oxidative stress and alteration of myocardial function because of DNA damage by hydrogen peroxide through the Fenton reaction[3].

EFFECTS OF INFLAMMATION ON IRON HOMEOSTASIS AND ERYTHROPOIESIS

Anemia is a frequent complication of chronic inflammatory diseases (e.g. cancer, rheumatoid arthritis, inflammatory bowel diseases, and congestive heart failure), as well as sepsis and chronic renal failure. In addition to blood loss, hemolysis, hepatic or endocrine disorders, nutritional deficiencies, bone marrow infiltration (cancer cells), or vitamin consumption (bacteria), this anemia may be the result of activation of the immune system by the underlying process, and certain immune and inflammatory cytokines including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-1, 6, 8 and 10[18,19].

As for chronic inflammatory diseases (and sepsis), these inflammatory mediators lead to anemia through several of the following pathophysiological mechanisms[19] (Figure 6): (1) decreased RBC half-life because of dyserythropoiesis, RBC damage and increased erythrophagocytosis (TNF-α); (2) EPO responses are inadequate for the degree of anemia in most, but not

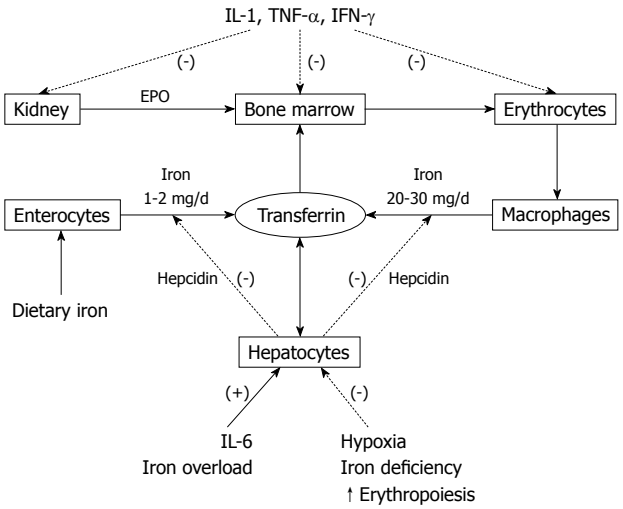

Figure 6 Effects of inflammation on erythropoiesis and iron homeostasis in mammals. (-): Negative effect; (+): Positive effect.

all (e.g. systemic-onset of juvenile chronic arthritis) (IL-1 and TNF-α)[20]; (3) impaired responsiveness of erythroid cells to EPO (IFN-γ, IL-1, and TNF-α); (4) inhibited proliferation and differentiation of erythroid cells (IFN-γ, IL-1, TNF-α, and α-1-antitrypsin); and (5) pathological iron homeostasis caused by increased DMT-1 (IFN-γ) and TfR (IL-10) expression in macrophages, reduced ferroportin 1 expression (IFN-γ and IL-6-induced high hepcidin levels) in enterocytes (inhibition of iron absorption) and macrophages (inhibition of iron recirculation), and increased ferritin synthesis (TNF-α, IL-1, IL-6, IL-10) (increased iron storage). All these lead to hypoferremia through iron diversion to the RES [functional iron deficiency (FID) that is characterized by low serum iron and decreased TSAT], iron-restricted erythropoiesis, and mild-to-moderate anemia.

Thus, the immunological pathophysiology of ACD includes disturbances of iron homeostasis, impaired proliferation of erythroid progenitor cells, and a blunted EPO response to anemia[19]. However, the pathophysiology of acute inflammation-related anemia (e.g. trauma or surgery) is somewhat different. In this setting, inflammatory responses are mediated mainly by IL-6 and IL-8 (with transient contribution of TNF-α and IL-1 in some visceral surgery, such as gastrointestinal or cardiac procedures), whereas IFN-γ plasma levels are undetectable or within the normal range[21-23]. Therefore, in most of these conditions, the two major mechanisms that lead to anemia are perioperative or traumatic blood loss and blunted erythropoiesis caused by decreased iron availability (caused by IL-6-induced high hepcidin levels), whereas EPO levels are normal or near-to-normal[24]. Finally, with persisting decreased iron absorption and/or chronic blood loss, ACD may evolve to ACD with true ID (ACD + ID).

On the other hand, it must be borne in mind that iron is not only required for erythropoiesis and oxidative metabolism. Cellular immune responses are also

dependent on the presence of iron, and specific defects in cell-mediated immunity have been described in detail, even in mild ID, including the impaired proliferation and function of lymphocytes and natural killer cells, and a depressed neutrophil respiratory burst[25,26]. Thus, ID or FID may lead not only to a blunted erythropoiesis and chronic fatigue, but also to an inappropriate immune response. For this reason, systemic inflammatory response episodes last longer in critically ill patients with FID, and result in prolonged stay in the intensive care unit and increased morbidity[27]. On the other hand, the effectiveness of the administration of iron sucrose, alone or in combination with EPO, has been assessed in a population of anemic, critically ill patients[28]. Compared to those in the control group who only received folic acid, patients treated with iron sucrose experienced an amelioration of systemic inflammatory response [decreased C-reactive protein (CRP) levels]. These beneficial effects were not as evident in patients who received iron sucrose plus recombinant human EPO (rHuEPO), probably because of the persistence of FID caused by rHuEPO-enhanced erythropoietic activity.

LABORATORY ASSESMENT OF IRON STATUS

Under physiological conditions, there is a balance between iron absorption, iron transport and iron storage in the human body. However, ID and IDA are common conditions among medical, surgical and critically ill patients, and result from the interplay of three distinct risk factors: increased iron requirements [e.g. growth or use of erythropoiesis-stimulating agents (ESAs), pregnancy and post-bleeding recovery], limited external supply (e.g. malnutrition, malabsorption caused by inflammatory bowel disease, use of gastric antiacid agents, infection with *H pylori*, even in the absence of significant bleeding), and increased blood loss (e.g. chronic gastrointestinal bleeding)[28]. ID can be either absolute or functional. In absolute ID, iron stores are depleted; in FID, iron stores, although replete, cannot be mobilized as fast as necessary from the macrophages of the RES to the bone marrow. As stated above, FID occurs in anemia of inflammatory diseases because iron is trapped in the RES[3,29].

Thus, laboratory tests for investigating ID fall into two categories: measurements providing evidence of iron depletion in the body, and measurements reflecting iron-deficient RBC production[30] (Table 2). The appropriate combination of these laboratory tests will help to establish a correct diagnosis of anemia and ID status[19].

Table 2 Main laboratory tests for the diagnosis of anemia and iron deficiency

Laboratory test	Normal values, units	Conversion to SI units
Iron depletion in the body		
Serum iron	50-180 μg/dL	× 0.179 μmol/L
Transferrin	200-360 mg/dL	× 0.01 g/L
TSAT	20%-50%	
Ferritin	30-300 ng/mL	× 2.247 pmol/L
sTfR	0.76-1.76 mg/L	6.4-25.7 nmol/L
Ratio of sTfR to serum ferritin (sTfR/log ferritin)	< 1	
Iron deficient red cell production		
Hemoglobin	12-16 g/dL (female) 13-17 g/dL (male)	× 0.6206 mmol/L
MCV	80-100 fL	
RDW	11-15	
MCH	28-35 pg	
HYPO	< 5%	
CHr	28-35 pg	

TSAT: Transferrin saturation; sTfR: Soluble transferrin receptors; MCV: Mean corpuscular volume; RDW: Red cell distribution width; MCH: Mean corpuscular Hb; HYPO: Hypochromic red cells; CHr: Reticulocyte Hb content.

ID without anemia

Normal hemoglobin level does not exclude ID, because individuals with normal body iron stores must lose a large amount of body iron before the hemoglobin falls below the laboratory definition of anemia. According to WHO criteria, laboratory definition of anemia is Hb < 12 g/dL for female and < 13 g/dL for male. However, although we will refer to these values in the following paragraph, it is worth noting that WHO criteria have been challenged recently. Analysis of the large NHANES-III (the third US National Health and Nutrition Examination Survey) and Scripps-Kaiser databases has indicated that a hemoglobin concentration < 13.7 g/dL in a white man aged between 20 and 60 years would have only an approximately 5% chance of being a normal value. The corresponding value for female of all ages would be 12.2 g/dL[31]. Should these new definitions of anemia be applied to our inflammatory bowel disease patient population, the prevalence of anemia would be higher than that reported in several previous studies.

In non-anemic patients, the most important clinical clue of ID is the symptom of chronic fatigue (iron is required for the enzymes involved in oxidative metabolism). However, it is of little screening value because clinicians rarely consider the presence of ID in patients who are not anemic, and therefore ID is invariably diagnosed in the laboratory[30]. A normal hemoglobin level with a mean corpuscular hemoglobin (MCH) in the lower limit of normality (normal range: 28-35 pg), or an increased RBC distribution width (RDW, normal range: 11-15) point to mild ID without anemia, but the main laboratory finding is low ferritin level. Although ferritin is an intracellular iron storage protein, small amounts of ferritin are secreted into the circulation and can be measured in the laboratory, and 1 ng/mL serum ferritin corresponds to approximately 8 mg of stored iron. Thus, measurement of ferritin

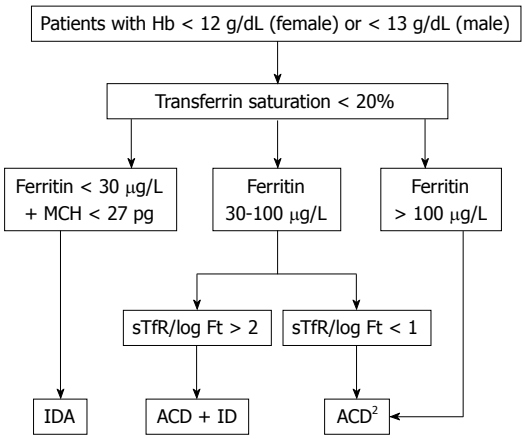

Figure 7 A simplified algorithm for the diagnosis of IDA (modified from Weiss *et al*[19]).

provides the aID can be defined by a ferritin level < 30 ng/mL in the absence of inflammation (normal serum concentrations of CRP: < 0.5 mg/dL) (true ID). In the presence of inflammation, a normal ferritin level (acute phase reactant) does not exclude ID, and TSAT also should be measured. As transferrin is the only iron-binding protein involved in iron transport, TSAT reflects iron availability for the bone marrow. Thus, in the presence of inflammation, ID should be better defined by normal ferritin concentrations and low TSAT (FID). FID may also occur in response to the therapeutic use of ESAs, such as epoetin or darbopoetin, which place a significant demand on iron stores that may surpass the iron-release capacity of the RES[32].

IDA

Patients should be considered to suffer from IDA when they presented with low Hb (male < 13 g/dL and female < 12 g/dL), TSAT (< 20%) and ferritin concentrations (< 30 ng/mL) but no signs of inflammation[30]. The MCH rather than mean corpuscular volume (MCV) has become the most important RBC marker for detecting ID in circulating RBCs (Figure 7). MCV is a reliable and widely available measurement but is a relatively late indicator in patients who are not actively bleeding. In addition, patients may present with IDA and without microcytosis, with coexisting vitamin B12 or folate deficiency. sTfR levels are usually high or very high, but they are not usually required for the diagnosis of uncomplicated IDA.

ACD

Patients should be considered to suffer from ACD when they have: (1) evidence of chronic inflammation (high CRP level); (2) hemoglobin concentration < 13 g/dL for male and < 12 g/dL for female; and (3) low TSAT < 20%, but normal or increased serum ferritin concentration (> 100 ng/mL) or low serum ferritin concentration (30-100 ng/mL), and an sTfR/log ferritin ratio < 1[19,33-35] (Figure 7). ACD, as well as FID, are frequent among patients with inflammatory disease without apparent blood loss (e.g. rheumatoid arthritis, renal failure or chronic hepatitis).

On the other hand, although ACD is typically mild to moderate, and RBCs may not show any stigmata of ID (normochromic, normocytic anemia), the underlying iron etiology is evident: macrophages that normally recycle iron are found to sequester it, intestinal iron absorption is interrupted, and erythroid precursors respond very rapidly when iron-transferrin is made available, especially by the administration of iv iron preparations. Thus, it can be speculated that the normocytic RBCs result from the combination of iron insufficiency and an as-yet-unexplained tendency to macrocytosis (e.g. alterations in folate or B12 metabolism in response to inflammation)[12].

ACD + ID

Patients should be considered to have ACD + ID when they have: (1) chronic inflammation (high CRP level); (2) hemoglobin concentration < 13 g/dL for male and < 12 g/dL for female; and (3) low TSAT < 20%, serum ferritin concentration > 30 and < 100 ng/mL, and an sTfR/log ferritin ratio > 2[19,33-35] (Figure 7). This type of anemia is more frequent in patients with inflammatory diseases and chronic blood losses (e.g. inflammatory bowel disease). There are two important hematological indices that may also help in the diagnosis of ACD + ID: reticulocyte hemoglobin content (CHr) and hypochromic red blood cells (HYPO). In non-ferropenic patients, the 2.5 percentile values were 28 pg for CHr and 5% for HYPO[15]. However, these hematological indices are only available in specific hematological analyzers. The Advia 120 hematology analyzer determines CHr and RBC hemoglobin content (⩾ 27 pg), whereas the Sysmex XE-2100 hematology analyzer determines RET-Y, which can be considered as the reticulocyte hemoglobin equivalent, as well as RBC-Y, which can be considered as the RBC hemoglobin equivalent[36,37]. These hematological indices (CHr and HYPO) are direct indicators of FID, in contrast to the majority of biochemical markers, which measure FID indirectly *via* iron-deficient erythropoiesis and demonstrate weaknesses in the diagnosis of FID as defined by hematological indices[15]. New hematological indices are being developed for other hematological analyzers (e.g. Beckman-Coulter LH 750) and their clinical utility in the diagnosis of ID will be evaluated in the near future.

Although hepcidin affects iron traffic in ACD and ACD + ID, individuals suffering from ACD + ID have significantly lower hepcidin levels than ACD subjects, and ACD + ID individuals, in contrast to ACD subjects, are able to absorb some dietary iron from the gut and to mobilize some iron from macrophages. Thus, hepcidin determination may also aid differentiation between ACD and ACD + ID and in selecting appropriate therapy for these patients[33].

CONCLUSION

Iron is an essential micronutrient, as it is required for an adequate erythropoietic function, oxidative metabolism and cellular immune response. Although the absorption of dietary iron (1-2 mg/d) is regulated tightly, it is just balanced with losses. Therefore, internal turnover of iron is essential to meet the requirements for erythropoiesis (20-30 mg/d). Hepcidin, which is primarily made in hepatocytes in response to liver iron levels, inflammation, hypoxia and anemia, is the main iron regulatory hormone for iron absorption and recirculation. Increased iron requirements, limited external supply, and increased blood loss may lead to ID and IDA. During inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling, which results in hypoferremia and iron-restricted erythropoiesis, despite normal iron stores, and finally, in ACD, which can later evolve to ACD + ID. An appropriate combination of laboratory tests that provides evidence of iron depletion or reflects iron-deficient RBC production will help to establish a correct diagnosis of ID status and anemia[38].

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, *Series Editors*

Classification of anemia for gastroenterologists

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Abstract

Most anemia is related to the digestive system by dietary deficiency, malabsorption, or chronic bleeding. We review the World Health Organization definition of anemia, its morphological classification (microcytic, macrocytic and normocytic) and pathogenic classification (regenerative and hypo regenerative), and integration of these classifications. Interpretation of laboratory tests is included, from the simplest (blood count, routine biochemistry) to the more specific (iron metabolism, vitamin B12, folic acid, reticulocytes, erythropoietin, bone marrow examination and Schilling test). In the text and various algorithms, we propose a hierarchical and logical way to reach a diagnosis as quickly as possible, by properly managing the medical interview, physical examination, appropriate laboratory tests, bone marrow examination, and other complementary tests. The prevalence is emphasized in all sections so that the gastroenterologist can direct the diagnosis to the most common diseases, although the tables also include rare diseases. Digestive diseases potentially causing anemia have been studied in preference, but other causes of anemia have been included in the text and tables. Primitive hematological diseases that cause anemia are only listed, but are not discussed in depth. The last section is dedicated to simplifying all items discussed above, using practical rules to guide diagnosis and medical care with the greatest economy of resources and time.

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Key words: Anemia; Microcytic anemia; Iron; Deficiency diseases; Macrocytic anemia; Normocytic anemia

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INTRODUCTION

Erythrocytes and the digestive system are linked closely from the very beginning of life. The yolk sack is the origin of the first generation of erythrocyte precursors. Yolk-sac-derived progenitor cells may seed the developing liver *via* the circulation and produce mature red blood cells that are required to meet the metabolic needs of the fetus. By week 8, liver-derived red cells are evident and the liver is the only source of erythrocytes until the 18th week of gestation. Afterwards, the spleen and bone marrow take over, but a small percentage of hepatic erythropoiesis remains until the postnatal week 6.

In adult life, the esophagus, stomach, bowel and liver are involved in the pathogenesis of different types of anemia caused by nutritional deficiency, bleeding or chronic inflammation.

Several diseases of the esophagus may produce chronic or acute bleeding: varicose veins, diverticula, diaphragmatic hernia, tumors, ulcers, esophagitis and Mallory-Weiss syndrome. The stomach plays a decisive role in the protection of vitamin B12 until it is absorbed in the final portion of the ileum, and contributes to an optimum absorption of iron reducing Fe^{3+} to Fe^{2+}. The stomach also may be a source of bleeding in peptic ulcer, gastritis and tumors, and treatment with acetylsalicylic acid and nonsteroidal anti-inflammatory drugs. The absorption of iron is a sophisticated and specialized process located mostly in the duodenum. The integrity of the small-intestinal mucosa is essential for folate absorption. Vitamin B12 absorption takes place chiefly in the ileum. In consequence, several gastrointestinal diseases, hereditary or acquired, including surgical resection, may produce nutritional deficiency anemia. Small-bowel inflammatory disease is a widespread cause of chronic disease anemia. Chronic bleeding is a crucial

sign in the diagnosis of malignant and benign lesions (such as diverticula) in the colon and rectum.

Splenomegaly is a relatively common cause of chronic hemolysis, and Zieve's syndrome is an infrequent cause of acute hemolytic crisis. Last, but not least, the liver plays an essential role in the control of iron metabolism through the hepcidin pathway, and accounts for most vitamin B12, folate and iron stores.

The links between anemia and digestive system are strong and multiple. Because of this, hematologist and the specialist in digestive system should work together to acquire a profound know of common pathogenic processes.

CONCEPT OF ANEMIA

Anemia is defined as a lower than normal hemoglobin concentration. Low hematocrit is a subrogate value for anemia, but it is not measured directly by the hematological analyzer. Instead, it is calculated from hemoglobin and other parameters. The erythrocyte count may be misleading in the evaluation of anemia. In fact, in some cases of microcytic anemia, such as thalassemia, there is usually an elevated erythrocyte count (spurious polycythemia).

In order to make a generalized approach to the diagnosis of anemia, the World Health Organization (WHO) has established a reference range for normal blood hemoglobin concentration, depending on age and sex[1]. According to this criterion, anemia is present if the blood concentration of hemoglobin falls below 130 g/L in men or 120 g/L in women. This rule does not apply to infants, children and pregnant women, who have their own tables of lower limits of hemoglobin concentration. The WHO criterion has been accepted widely for diagnosis and publication, but its universal application has been questioned mainly because of racial differences. Beutler has proposed a lower limit of hemoglobin (1-2 g less) in African Americans than in Caucasians. The reference range of hemoglobin concentration in blood may vary depending on the population analyzed, age, sex, environmental conditions and food habits[2,3].

Anemia causes tissue hypoxia and triggers compensating mechanisms. Both processes together produce the signs and symptoms characteristics of anemic syndrome. Patients with anemia may present with fatigue, dizziness and dyspnea; however, mild anemia shows few clinical signs or symptoms. The sings of anemia include pallor of the conjunctivae, face, nail beds and palmar creases, although the absence of pallor does not rule out anemia[4].

Anemia is one of the most frequent causes of medical visits because of the high incidence in children, young women and elderly people, especially if malnutrition is present. Moreover, anemia is one of the leading sings in many diseases or is the first evidence of disease observed in routine blood cell enumeration. Anemia is unusually prevalent in developing countries because of malnutrition, and genetic, parasitic or infectious diseases[5,6]. The prevalence of anemia varies greatly, from 2.9% to 61%, depending on population, age, sex, and normal limits of hemoglobin used by the author[7].

The most frequent cause of chronic anemia is gastrointestinal pathology, and this explains why patients with anemia are often sent for consultation to a gastroenterological specialist[8].

Anemia is a syndrome, not a disease, and therefore, the etiology must always be investigated and therapy must be directed mainly to the causal disease, and not only to restoring a normal hemoglobin concentration. In a patient with anemia, whose clinical and laboratory data are insufficient to find the cause, examination of the digestive system is a priority for two reasons. The first one is the high frequency of anemia in bowel disease, and the second is the opportunity to diagnose a malignant disease before it is too advanced[9-11].

CLASSIFICATION

Anemia can be classified from three points of view: pathogenesis, red cell morphology, and clinical presentation. All are important to guide the diagnosis. Pathogenic mechanisms involved in the production of anemia are very simple: inadequate production and loss of erythrocytes a a result of bleeding or hemolysis. Based on these pathogenic mechanisms, anemia can be divided into two types. (1) Hypo-regenerative: when bone marrow production is decrease as a result of impaired function, decreased number of precursor cells, reduced bone marrow infiltration, or lack of nutrients; (2) Regenerative: when bone marrow responds appropriately to a low erythrocyte mass by increasing production of erythrocytes.

In practice, classification based on basic parameters of red cell morphology such as mean corpuscular volume (MCV), allows for a quicker diagnostic approach.

Anemia also can be classified according to the form of clinical presentation as acute (usually bleeding or hemolysis) or chronic.

Anemia can be classified as microcytic, normocytic or macrocytic, depending on MCV. As stated above, it can be hypo-regenerative or regenerative, which depends on the number of reticulocytes. Using both, the list of possible diagnoses in the individual patient is reduced considerably. Both parameters can be supplied routinely by most of the automatic hematological cell counters.

PATHOGENIC CLASSIFICATION

The reticulocyte count is useful to distinguish anemia in which there is an appropriate bone marrow response from that in which there is a decrease in the production of erythrocytes. The concentration of reticulocytes reports on the bone marrow response to anemia. This approach is especially useful when MCV is normal.

A decrease in hemoglobin stimulates erythropoiesis through an increase in circulating erythropoietin. Therefore, when the bone marrow shows a normal

regenerative capacity, there should be an inverse relationship between the decrease in hemoglobin and the increased number of reticulocytes (regenerative anemia). The expected reticulocyte count is much higher than normal to compensate for the anemia. However, when the hemoglobin decreases and the bone marrow does not have regenerative capacity, the expected increase in reticulocytes fails, despite the increase in erythropoietin plasma level (hypo-regenerative anemia)[12].

The reticulocyte count is expressed as a proportion of the number of erythrocytes, which must be corrected for anemia, and for increased lifespan of reticulocytes in peripheral blood. Most hematological analyzers provide directly the number of reticulocytes per mm^3, which is a better estimation of erythropoietic activity than a percentage. However, the absolute reticulocyte enumeration per mm^3 overestimates the actual activity of erythropoiesis, since reticulocytes are released earlier and remain longer in the circulating blood. To avoid these confounding events, it is recommended to calculate the reticulocyte index.

The next step is the calculation of reticulocyte production index (RPI):

RPI = correcteds reticulocyte count (%) × (haematocrit observed/normal hematocrit) : F (reticulocyte maturation times *in vivo*)

RPI in a healthy person is 1. An RPI > 3 in a patient with anemia suggests an appropriate bone marrow response, and therefore, regenerative anemia[13,14]. Nevertheless, the best estimate of the actual erythropoietic activity[15], and easiest to calculate, is to divide by two the number of reticulocytes per mm^3.

Regenerative anemia

This is characterized by increased generation of erythropoietin in response to decreased hemoglobin concentration, and generally reflects a loss of erythrocytes, due to bleeding or hemolysis (Table 1). In both cases, there is a typical increase in reticulocytes. The bleeding can be intense, with a sharp drop of hematocrit and obvious clinical signs; or of small intensity and chronic, with progressive decrease in hematocrit and MCV, which may go unnoticed. Over time, chronic hemorrhagic anemia becomes hypo-regenerative and microcytic because of depletion of iron stores.

Hemolysis, can be acute (usually intravascular) or chronic (usually extravascular). Acute hemolysis is characterized by sudden episodes with very obvious clinical signs (fever, chills, back pain, dark urine) and typical laboratory data (hemoglobinuria and reduced plasma haptoglobin)[16].

Hypo-regenerative anemia

This is caused by alteration of bone marrow progenitor cells, which can be located at different stages of differentiation and maturation. The impairment of pluripotent stem cells usually produces pancytopenia (anemia, leukopenia and thrombocytopenia). Pancytopenia may be caused by intrinsic [bone marrow aplasia, leukemia, myelodysplastic syndrome (MDS) or myelofibrosis] or extrinsic (metastasis, Gaucher disease and other thesaurismosis, tuberculosis, histoplasmosis, viral and parasitic infections). All of them are capable of displacing normal hematopoiesis or changing the microenvironment necessary for regeneration, differentiation and proliferation of stem cells[16,17].

Less frequently, progenitor cells committed to the erythroid line (burst-forming unit-erythroid and colony-forming unit-erythroid) are affected selectively, and the result is pure red cell aplasia. When progenitor red cells are impaired selectively, bone marrow erythroblasts are much reduced or absent, while other hematological cellular lines remain normal. Pure red cell aplasia, hereditary or acquired, is very rare. However, a qualitative alteration of the red cell line (dyserythropoiesis) is often seen in clinical practice. In contrast to pure red cell aplasia, the bone marrow is rich in erythroblasts in patients with dyserythropoiesis. In these cases, the erythropoiesis is abnormal morphologically and functionally. Erythroblastopenia as dyserythropoiesis may have a hereditary or acquired origin. The former is extremely rare. Thymoma is the most common cause of acquired pure red cell aplasia. MDS is the most common cause of acquired dyserythropoiesis. In MDS patients, the lesion is located at the level of very primitive multipotent progenitors, and thus, the morphological and functional alterations can affect all blood cell lines (erythrocyte, granulocyte-monocyte and megakaryocyte)[18-20].

Table 1 Etiopathogenic classification of anemia

Regenerative anemia
Acute or chronic bleeding
Hemolytic anemia
Hereditary (hemoglobinopathy, enzymopathy, membrane-cytoskeletal defects)
Acquired (autoimmune, mechanical destruction, toxic-metabolic, drugs, infectious, PNH, hypersplenism)
Hypo-regenerative anemia
Bone marrow failure caused by stem cell pathology
Quantitative disorder
Selective: erythroblastopenia (pure red cell aplasia)
Global: aplastic anemia
Qualitative disorder (dysmyelopoiesis)
Inherited: hereditary dyserythropoiesis
Acquired: myelodysplasia
Bone marrow infiltration
Leukemia, lymphoma, multiple myeloma
Solid tumors
Myelofibrosis
Thesaurismosis (Gaucher disease)
Inflammatory chronic diseases, microorganisms (Histoplasma, HIV)
Drugs, hypothyroidism, uremia
Erythropoietic factors deficiency
Iron
IDA
ACD
Cobalamin and folate
Megaloblastic anemia
Hormones: erythropoietin, thyroid hormones, androgens, steroids

PNH: Paroxysmal nocturnal hemoglobinuria; IDA: Iron-deficiency anemia; ACD: Anemia chronic disease.

Table 2 Morphological classification

Microcytic anemia (MCV < 82 fL)
IDA
Thalassemia
Non thalassemic conditions associated with microcytosis
ACD (e.g. rheumatoid arthritis, Hodgkin's lymphoma, chronic infection, neoplasia)
Sideroblastic anemia (e.g. hereditary, lead poisoning)
Normocytic anemia (MCV = 82-98 fL)
Nutritional anemia (iron deficiency, cobalamin y/o folate)
Anemia of renal insufficiency
Hemolytic anemia
Red cell intrinsic causes: membranopathy, enzymopathy, hemoglobinopathy
Red cell extrinsic causes: immune-mediated, microangiopathic, associated with infection, chemical agent (spider venoms), metabolic
ACD
Primary bone marrow disorder
Causes that are intrinsic to hematopoietic stem cells: bone marrow aplasia (idiopathic, PNH, Fanconi syndrome), pure red cell aplasia (acquired, congenital, Diamond-Blackfan syndrome), myelodysplastic syndrome
Extrinsic causes: drugs, toxins, radiation, viruses, immune-mediated, bone marrow infiltration (metastatic and lymphoma)
Macrocytic anemia (MCV > 98 fL)
Drugs (hydroxyurea, zidovudine, methotrexate)
Nutritional (vitamin B12 and folate deficiency)
Drug-induced hemolytic anemia
Dyserythropoiesis, myelodysplastic syndrome, clonal hematologic disorder
Hereditary hematologic disorders
Mild macrocytosis (MCV = 100-110 fL)
Reticulocytes
Excess alcohol intake, liver disease, smoking
Hypothyroidism, Waldenström's macroglobulinemia
Copper deficiency, bone marrow aplasia, erythroblastopenic anemia
Down syndrome
Chronic obstructive pulmonary disease

MCV: Mean corpuscular volume.

Anemia caused by nutritional deficiencies or decreased production of erythropoietin is much more frequent than that caused by a primitive defect in bone marrow.

MORPHOLOGICAL CLASSIFICATION

Pathogenic classification is very important to understand the mechanisms involved in the genesis of anemia (Table 2). However, in daily clinical practice, it is more useful to start with the analytical parameters of the hemogram. MCV allows us to classify anemia as microcytic (MCV < 82 fL), normocytic (MCV = 82-98 fL) and macrocytic (MCV > 98 fL)[21,22].

MCV has a relationship with mean corpuscular hemoglobin (MCH), which reports on the mean hemoglobin per erythrocyte expressed in picograms (normal range: 27-32 pg). Therefore, MCV and MCH decrease (microcytic, hypochromic anemia) or increase (macrocytic, hyperchromic anemia) jointly. The MCH concentration (MCHC) reports on the average concentration of hemoglobin in each erythrocyte expressed as a percentage (normal range: 32%-36%), and its variations are very small, even in the presence of hypochromia. MCHC increases only in a few rare diseases such as hereditary spherocytosis, and therefore, its practical utility is scarce.

Table 3 Classification of anemia as RDW and MCV

	↓ MCV	Normal MCV	↑ MCV
Normal RDW	β-thalassemia α-thalassemia	Normocytic	Bone marrow aplasia
Increased RDW	Iron deficiency	Inflammatory anemia Hypothyroidism	Megaloblastic anemia

RDW: Red blood cell distribution width.

It should always be borne in mind that MCV is an average value and therefore does not provide information about the homogeneity of the erythrocyte population. To resolve this problem, hematological analyzers provide the erythrocyte distribution curve, with an index of dispersion: red blood cell distribution width (RDW) (normal range: 10%-14%). RDW is a rough indicator of anisocytosis and is an essential complement to MCV (Table 3).

Microcytic anemia

Faced with microcytic anemia, the three main diagnostic possibilities include iron deficiency anemia (IDA), thalassemia, and anemia of chronic disorders (ACD). A fourth possibility, sideroblastic anemia, is so rare that is not considered in the initial diagnosis, unless there is a history of contact with lead (Figure 1).

Iron deficiency is the most common cause of anemia, so the first step in diagnosis should be directed toward confirmation or exclusion of IDA. Serum ferritin allows us to confirm the diagnosis. Despite ferritin being an acute phase reactant, the diagnosis of IDA is unlikely with normal or elevated ferritin levels. Other parameters of iron metabolism (serum iron, total transport capacity of iron, and transferrin) are unable to distinguish with certainty IDA from ACD[23].

IDA occurs in 2%-5% of adult men and postmenopausal women in the developed world[24,25], and is a common cause of referral to a gastroenterology clinic 4%-13%[26].

It is important to note that microcytosis without anemia is characteristic of thalassemia trait, but also of polycythemia associated with iron deficiency[27].

The simple analysis of different parameters provided by the hematological analyzer gives a diagnosis of microcytic anemia. RDW helps to distinguish thalassemia from IDA. RDW is normal in thalassemia; on the contrary, microcytic anemia with RDW > 15 is probably IDA.

In recent years, the importance of serum soluble transferrin receptor has been recognized in differential diagnosis of IDA and ACD. Serum soluble transferrin receptor is increased in IDA, without interference in case ACD is present[28,29]. Intra-erythrocytic ferritin and erythrocyte zinc-protoporphyrin also help to differentiate IDA from ACD. These parameters allow the diagnosis

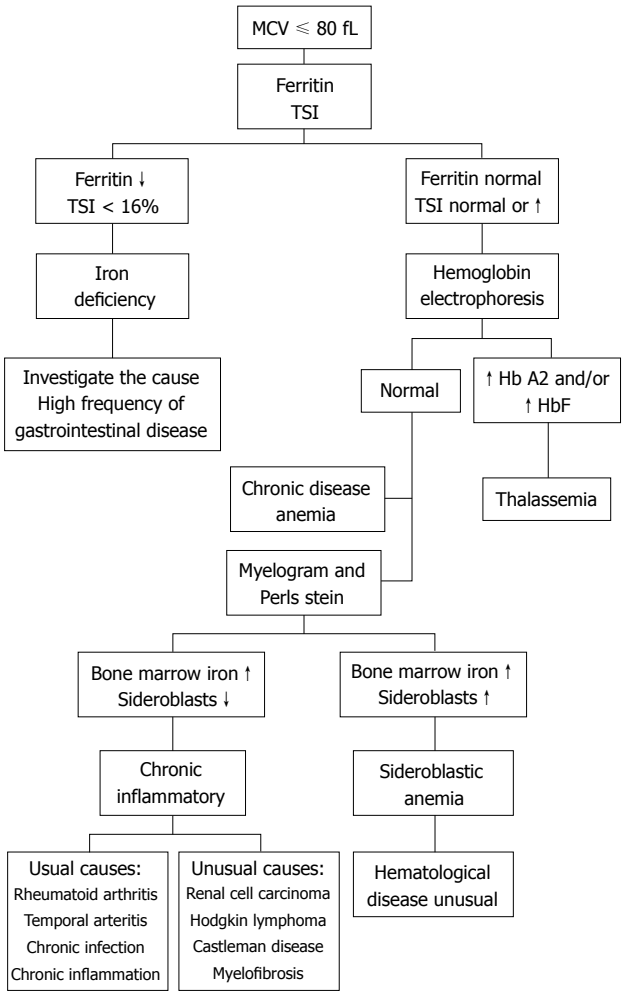

Figure 1 Evaluation of microcytic anemia. TSI: Transferrin saturation index; MCV: Mean corpuscular volume; Hb: Hemoglobin.

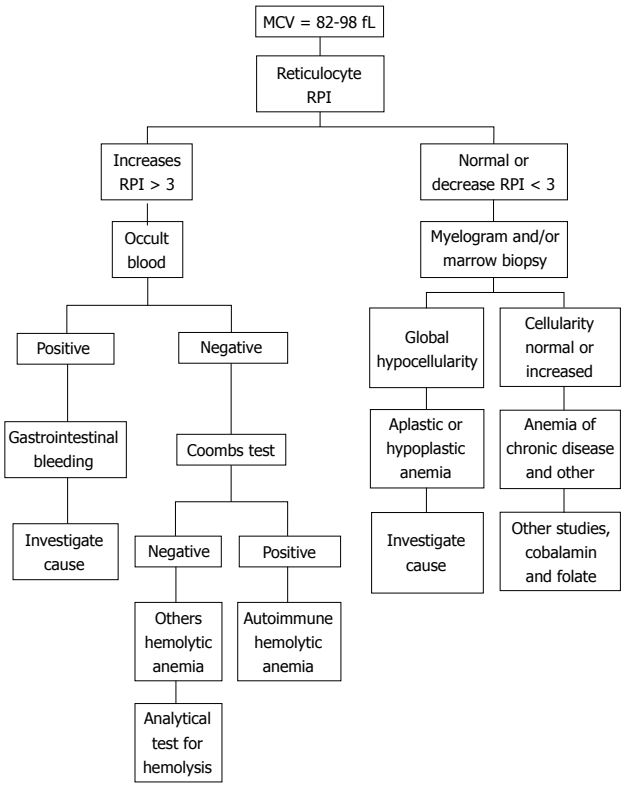

Figure 2 Evaluation of normocytic anemia. RPI: Reticulocytes production index.

of borderline patients who, at another time, would have undergone unnecessary investigation of iron in the bone marrow[30-32].

In men and postmenopausal women, the bigger concern should be to rule out the presence of occult bleeding. If positive, the first exploration should be a colonoscopy, mainly in men, because of the frequent association of occult blood with adenocarcinoma[33,34]. If the colonoscopy does not reveal the cause of the anemia, gastroscopy should be performed. The study of the small bowel is more controversial, but it is convenient to keep in mind that celiac disease is a cause of IDA[35].

In premenopausal women, genital bleeding is the most frequent cause of anemia. Therefore, gastrointestinal exploration is controversial. An anamnesis directed towards the characteristics of menstrual bleeding, although a subjective criterion, may be useful for distinguishing a subgroup of women without excessive genital bleeding, who require direct assessment of occult bleeding, followed by gastrointestinal exploration if positive. In cases in which occult bleeding is negative, the exploration should be directed to the genital system[36-38].

Microcytosis associated with normal ferritin guides the diagnosis toward hereditary diseases, mainly thalassemia. If there are no family antecedents of microcytosis, it is necessary to investigate acquired causes of non-iron deficiency microcytosis, mainly ACD and sideroblastic anemia. Normal RDW guides the diagnosis toward the former and a high RDW toward the latter.

Normocytic anemia

The fundamental question in normocytic anemia is to recognize the causes and susceptibility to treatment as soon as possible. Among the causes are nutritional deficiency, renal failure and hemolytic anemia[21]. Nutritional mixed anemia that combines deficiency of vitamin B12, folic acid and iron is frequent. In consequence, these three parameters should be requested in the initial phase of the diagnosis of normochromic anemia.

In order to differentiate among regenerative (hemolysis and bleeding) or hypo-regenerative anemia (bone marrow aplasia, chronic disease, nutrition deficiency and hemopathy), it is necessary to determine the corrected RPI (Figure 2). In anemia of renal failure, morphological alterations in the blood are scarce and serum erythropoietin may be normal, but inappropriately low for the degree of anemia. The severity of anemia is not evident until the disease is very advanced.

It will always be necessary to rule out hemolysis, by performing easily accessible laboratory tests [lactate dehydrogenase (LDH), indirect bilirubin, haptoglobin and reticulocytes]. These parameters do not inform us

Table 4 Serum levels that differentiate ACD, IDA and mixed anemia

	ACD	IDA	Mixed anemia
Iron	↓	↓	↓
Transferrin	↓ or N	↑	↓
Transferrin saturation	↓	↓	↓
Ferritin	N or ↑	↓	↓ or N
Serum transferrin receptor	N	↑	N or ↑
Ratio: soluble receptor of transferrin/log ferritin	< 1	> 2	> 2
Cytokine levels	↑	N	↑

N: Normal; ↑: Increase; ↓: Decrease.

about the origin of hemolysis, and it is necessary to evaluate others, such as schistocytes in peripheral blood (intravascular hemolysis), the Coombs test (autoimmune hemolysis), tests of osmotic fragility (extravascular hemolysis), and tests to rule out hemolysis induced by drugs[39]. A detailed anamnesis and Coombs test, with and without the suspected drugs, are very useful in the investigation of drug-induced hemolytic anemia.

When the cause of normocytic anemia is not any of the previously mentioned causes, it is necessary to guide the diagnosis toward a chronic disease or to primitive hematological disease[21,22]. It is difficult to arrive at the definitive diagnosis. In this situation, it is fundamental that a careful clinical evaluation is carried out to rule out other causes of normocytic anemia: alcoholism (more frequently macrocytic), effects of drugs (chemotherapy, immunosuppression), radiotherapy, neoplasia (bone marrow infiltration), infections (mainly in hospitalized patients), surgery, or recent trauma (first phase of bleeding). The association with pathological concomitant processes, elevation of erythrocyte sedimentation rate (ESR) and the absence of morphological alterations in peripheral blood smears supports the suspicion of ACD[40]. ACD is the most frequent cause of anemia after ferropenia[41,42]. It is observed in patients that have immune hyperactivity. Activation of cytokines and the reticuloendothelial system induces changes in iron homeostasis, erythroid precursor proliferation, erythropoietin secretion, and erythrocyte life span[43]. All of these contribute to the pathogenesis of anemia.

ACD can be complicated with chronic bleeding, and in this case, the diagnosis is more difficult because of the presence of microcytosis and ferropenia. The biochemical parameter that better differentiates ACD from IDA is serum ferritin[44] (Table 4).

The hematologist must revise the blood smear if primitive disease of bone marrow is suspected. Depending on the result, a bone marrow study may be necessary to detect hematological diseases or metastasis. The biopsy, bone marrow smear, or both inform us of: the morphology of the hematopoietic cells, on their quantitative distribution, especially the myelo-erythroid ratio (normal 3:1); fibrosis; the presence of non-hematopoietic cell; or possible bone marrow aplasia. Perls' specific iron stain informs us of the state of iron stores, and it allows quantification of siderocytes and sideroblasts. In a patient with anemia and fever of unknown origin, we must carry out careful microscopic examination of bone marrow, some selective staining, and bone marrow cultures to diagnose any underlying infectious diseases. Some infectious or parasitic diseases that are diagnosed with relative frequency are: tuberculosis, histoplasmosis, kala-azar and malaria[45-47].

Macrocytic anemia

Macrocytosis is observed frequently using blood cell analyzers (Figure 3). Its prevalence is 1.7%-3.9%, but 60% of the patients with macrocytosis do not have anemia[48]. On the other hand, macrocytosis can be physiological in some circumstances (infants, pregnancy, some families). Even keeping in mind the precedent data is convenient to make a careful evaluation of macrocytosis in every patient, to rule out any underlying pathology. In vitamin B12 and folic acid deficiency, as well as in other diseases, macrocytosis (blood) is accompanied by megaloblastosis (bone marrow). In such cases, both terms can be used interchangeably[49].

The starting point in the diagnostic process for macrocytic anemia will be to rule out therapy with drugs that interfere with nucleic acid metabolism, such as.hydroxyurea, methotrexate, trimethoprim, zidovudine or 5-fluorouracil, as well as habitual intake of alcohol[49,50]. Hydroxyurea is the drug that increases most the MCV (> 110 fL); the other drugs and alcohol induce a moderate macrocytosis (100-110 fL).

In the absence of intake of any of the drugs mentioned above or alcohol, the most frequent cause of microcytic anemia is nutritional deficiency[50]. Therefore, serum vitamin B12 and folic acid levels should be evaluated. The last can be modified by the previous day's folic acid intake. A better alternative is intra-erythrocyte folate, which remains stable during the lifetime of red blood cells and gives a better indication of possible chronic folic acid deficiency. The measurement of erythrocyte folate is a difficult technique that is not available in every laboratory. The determination of homocysteine is an accessible alternative to intra-erythrocyte folate. In the absence of folic acid, homocysteine levels increase rapidly because it cannot be converted into methionine. Normal serum levels of homocysteine are highly unlikely in folate deficiency[21].

In the same way, vitamin B12 deficiency is correlated with low serum levels, although there are clinical situations in which low serum levels are not correlated with vitamin B12 deficiency (pregnancy, elderly patients, and those with low white blood cell counts). In these circumstances, normal serum methyl-malonic acid levels, in the absence of congenital errors of metabolism or renal failure, preclude a deficiency of vitamin B12[51].

To complete the study of vitamin B12 deficiency, it is necessary to look for antibodies to intrinsic factor, which if positive, confirms the diagnosis of pernicious anemia. If not present, it is necessary to investigate malabsorption by performing the Schilling test[52] (Table 5).

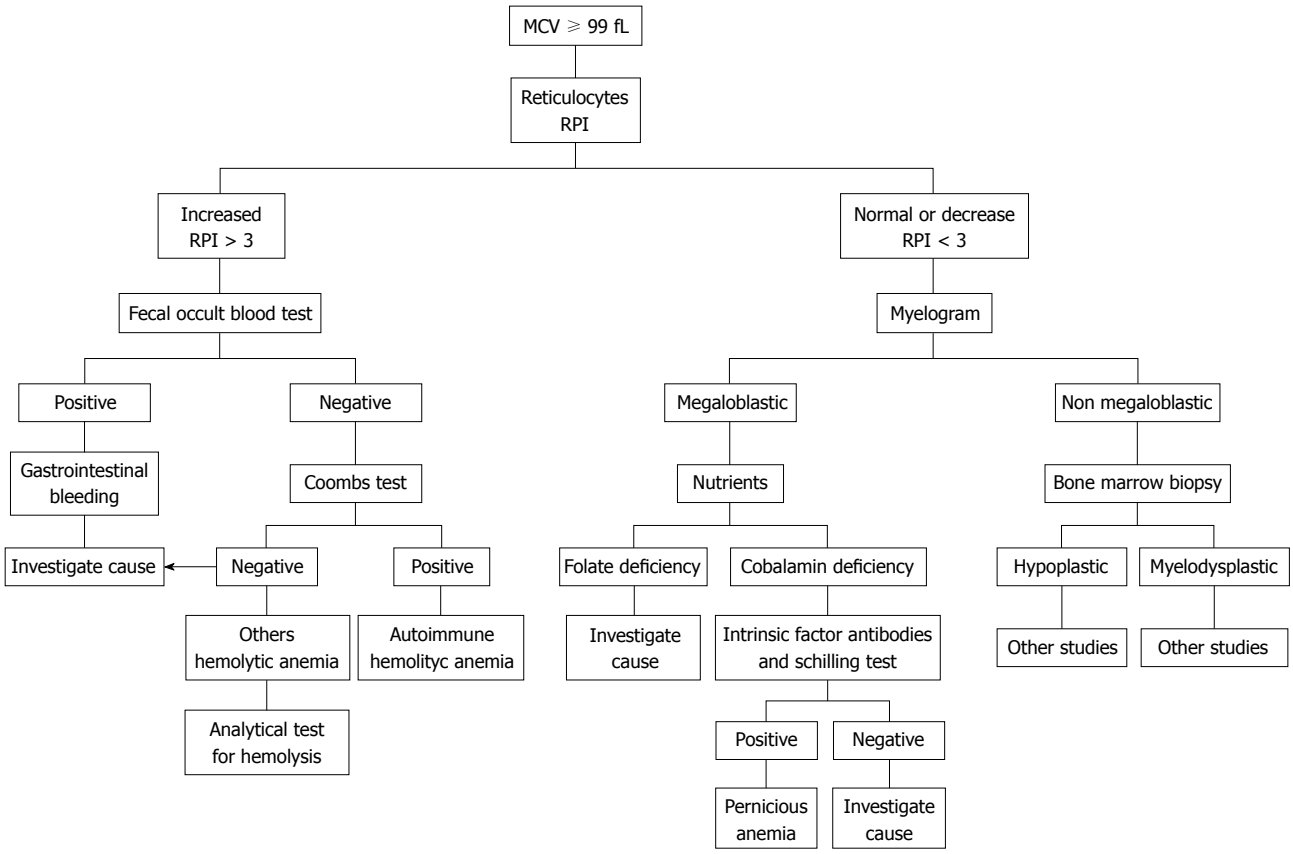

Figure 3 Evaluation of macrocytic anemia.

ANEMIA, A PRACTICAL APPROACH FOR THE GASTROENTEROLOGIST: STRATEGY TO DIAGNOSE PATIENTS WITH ANEMIA, AND PREVALENCE OF DIFFERENT TYPES OF ANEMIA

The strategy for establishing a diagnosis of anemia by gastroenterologists must follow a classic pattern that includes a clinical interview, a basic physical examination, and careful selection of complementary tests.

The clinical history should emphasize the causes of blood loss, including non-gastrointestinal diseases, recent history of gastrointestinal symptoms, gastrointestinal surgery, use of nonsteroidal anti-inflammatory drugs, acetylsalicylic acid and anticoagulant therapy, family history of hematologic diseases and colorectal neoplasia[53-55]; all symptoms should be recorded, and not only the gastrointestinal ones.

A single blood sample can give information on the concentration of hemoglobin (essential for the diagnosis of anemia according to WHO criteria), the erythrocyte indices MCV and MCH (useful for the morphological classification of anemia), RDW (estimate of anisocytosis) and ESR, which reports on possible ACD.

The hematologist should examine a blood smear if any abnormal erythrocyte indices have been detected. A clotted blood sample (to obtain serum) allows the determination of the rest of the analytical parameters that are useful in diagnosis, which are iron metabolism (mainly ferritin), urea, creatinine, bilirubin and total protein. In cases of suspected IDA, the concentration of soluble transferrin receptor should be included as a key parameter in differentiation between IDA and ACD[56]. The most probable causes of microcytic anemia are IDA, thalassemia or thalassemia trait. RDW and iron metabolism are clues to differentiate between these processes in the initial step, as has been mentioned above. The most likely causes of normocytic anemia are ACD, renal failure and primitive hematological diseases (least frequently). Iron metabolism and routine biochemical tests may help to guide the diagnosis, but in some cases, erythropoietin serum levels and bone marrow examination are necessary.

In cases of macrocytic anemia, serum levels of vitamin B12 and serum and/or erythrocyte levels of folate will guide the diagnosis to deficiency of one of these. Most causes of folic acid deficiency are nutritional, although malabsorption should always be kept in mind. In contrast, vitamin B12 deficiency is almost always the result of malabsorption[49,57]. The few exceptions are in strict vegetarians and hereditary transcobalamin Ⅱ deficiency (a very infrequent disease). Among malabsorption causes of B12 vitamin deficiency, the most frequent is pernicious anemia. Therefore, the first step is to establish whether the serum levels of gastrin are highly elevated, because

Table 5 Causes of megaloblastic anemia

- Cobalamin deficiency
  - Poor diet
  - Deficiency of intrinsic factor
    - Pernicious anemia
    - Total or partial Gastrectomy
    - Ingestion of caustic (lye)
  - Functional defect of intrinsic factor
  - Alteration of ileal (microenvironment)
    - Insufficient pancreatic protease activity
      - Inactivation enzyme (Sd. Zollinger-Ellison)
    - Competition for cobalamin
  - Alteration of ileal mucosa
    - Acquired
      - Surgical resection or by-pass
      - Regional enteritis (Crohn's disease)
      - Tropical esprue
      - Celiac disease, Tuberculosis
      - Lymphomatous infiltration
    - Induced by drugs
    - Congenital
      - Sd Immerslund-Gräsbeck
  - Congenital transcobalamin Ⅱ deficiency or abnormality
  - Congenital methylmalonic acidemia and aciduria
  - Hemodialysis
  - Urinary losses (congestive heart failure)
- Folate deficiency
  - Dietary
    - Old age, infancy, poverty, alcoholism, chronic invalids, psychiatrically disturbed, scurvy and kwashiorkor
  - Excess utilization or loss
    - Physiologic: pregnancy and lactation, prematurity
    - Pathologic: Hematologic diseases
    - Malignant diseases
    - Inflammatory disease
    - Metabolic disease
    - Excess urinary loss, congestive heart failure, active liver disease
    - Hemodialysis, peritoneal dialysis
  - Malabsorption
    - Congenital
    - Anti-folate drugs
    - Alcoholism
    - Tropical sprue, gluten-induced enteropathy
    - Extensive jejune resection, Crohn's disease, partial gastrectomy, systemic bacterial infection, Whipple's disease
  - Congenital abnormalities of folate metabolism
    - Cyclohydrolase, methionine synthetase
- Combined deficit of folate and cobalamin
  - Celiac disease
  - Regional enteritis (Crohn's disease)
- Congenital disorder of DNA synthesis
- Disorders of DNA synthesis induced by drugs
  - Anti-folate
  - Purine antagonists
  - Pyrimidine antagonists
  - Alkylating
- Eritroleucemia

they are > 1000 pg/mL only in atrophic gastritis type A (pernicious anemia) and Zollinger-Ellison syndrome. In pernicious anemia, pepsinogen I levels are low, gastric pH is high and gastric acid secretion does not respond to stimulation with histamine or pentagastrin. Serum antibodies against intrinsic factor and parietal cells are not sensitive or specific enough to confirm the diagnosis. The exploration that leads directly to diagnosis is gastroscopy, with mucosal biopsy and study of antiparietal cell antibodies. Gastric biopsy has the added advantage of giving information about possible metaplasia or precancerous lesions[58]. In the absence of gastric disease, the most likely cause of malabsorption of vitamin B12 is Crohn's disease, with involvement of the terminal ileum[59]. Hereditary causes of malabsorption (Immerslund syndrome) or transport (transcobalamin Ⅱ deficiency) are extremely infrequent.

Table 6 Differential diagnosis of anemia from a gastrointestinal point of view

- Gastrointestinal causes of anemia
  - Microcytic anemia
    - Iron deficiency
      - Decreased iron absorption
        - Frequent: Celiac disease, gastrectomy, *H pylori* colonization
        - Infrequent: Bowel resection, bacterial overgrowth
      - Occult gastrointestinal blood loss
        - Frequent: aspirin and nonsteroidal anti-inflammatory drug use, colonic carcinoma, gastric ulceration, angiodysplasia, inflammatory bowel diseases
        - Infrequent: esophagitis, esophageal carcinoma, gastric antral vascular carcinoma, small bowel tumors, ampullary carcinoma, *Ancylomasta duodenale*
      - Non-gastrointestinal blood loss
        - Frequent: menstruation, blood donation, ACD
        - Infrequent: Hematuria, epistaxis
    - Sideroblastic anemia (alcohol, lead, drugs), vitamin B6
    - ACD
  - Normocytic anemia
    - Frequent: ACD (liver disease, renal insufficiency, malignancy, nutritional deficiency, drug effects, alcoholism, recent trauma or surgery, iron deficiency
    - Infrequent: primary bone marrow disorder
  - Macrocytic anemia
    - Non-megaloblastic
      - Systemic disease:
        - Frequent: liver disease, alcoholism
        - Infrequent: primary bone marrow disease (myelodysplastic syndrome, aplastic anemia), metastatic infiltration, hemolytic anemia, hypothyroidism
    - Megaloblastic anemia
      - Vitamin B12 deficiency: pernicious anemia, gastrectomy, hereditary deficiency of intrinsic factor, inflammatory bowel disease, primary intestinal malabsorptive disorders, parasitic colonization, nutritional deficiencies
      - Folate deficiency: diet poor in folates, regional enteritis, Whipple's disease, scleroderma, amyloidosis, increase requirements (liver disease, hemolytic anemia)
      - Antifolate drugs: methotrexate

The list of causes of anemia is long and cumbersome, but most can fit into a very small number of diseases on which we must concentrate as a first diagnostic option (Table 6)[60-66].

IDA is the most prevalent form of anemia worldwide, especially in women and children. Thirty-percent of the world's population, some 1300 million people, suffer from anemia. However, the prevalence of anemia worldwide is unequal (36% in underdeveloped and 8% in developed countries) About half of the patients with anemia have IDA, which is most prevalent in the general population and in outpatients. The most likely cause of IDA is malnutrition in children, bleeding in adult males (especially gastrointestinal), menstruation or lactation in fertile

women, and bleeding in the elderly. The distribution of nutrient-deficiency anemia in the elderly is: 48% iron alone, 19% folate alone, 17% vitamin B12 alone, and the rest have combined deficiencies. Therefore, in young male adults and in both sexes older than 65 years, the most likely cause of IDA is chronic bleeding, especially from gastrointestinal lesions[67].

The hemogram, the concentration of iron, ferritin and transferrin in blood and investigation of fecal occult blood are sufficient to obtain a diagnosis in most cases.

ACD is the most prevalent form of anemia after IDA in the general population[40,68], but is even more common in hospitalized patients, regardless of sex and age[42,67,69,70].

The underlying causes of ACD vary greatly: acute and chronic infections, 18%-95%; cancer, 30%-77%; autoimmune diseases (including chronic inflammatory bowel disease), 8%-71%; rejection of solid organ transplantation, 8%-70%; and chronic renal disease, 23%-50%. ACD is found in 20% of elderly people with anemia (30% excluding nutritional deficiency), and a further 4.3% suffer from ACD and kidney failure. Therefore, we should keep in mind ACD as the most likely diagnosis in elderly and inpatients, and as the second most likely diagnosis in outpatients under 65 years of age[68]. The laboratory tests needed to confirm the diagnosis include serum iron, transferrin, transferrin saturation, ferritin, urea and creatinine.

The gastroenterologist must rule out chronic inflammatory bowel disease, tumors of the digestive tract and liver disease. In some patients, the cause of anemia is complex (chronic disease and chronic hemorrhage). In these cases, it is difficult to confirm the diagnosis and additional testing is required: soluble transferrin receptor and microscopic examination of bone marrow with special stains for iron.

The most common type of anemia after IDA and ACD is megaloblastic anemia (vitamin B12 deficiency, folic acid or both). It is important in gastroenterology because intestinal malabsorption and pernicious anemia are common causes of this form of anemia. The diagnosis is suspected when MCV is elevated, and is confirmed by measuring serum vitamin B12 and serum and erythrocyte folate.

Hemolytic anemia follows next in order of frequency. Acute hemolysis presents a distinctive clinical picture, but chronic hemolysis may go unnoticed. If hemolytic anemia is suspected, the appropriate laboratory tests are antiglobulin test (direct and indirect), LDH, haptoglobin, total and conjugated bilirubin in serum, and salts and bile pigments in urine. These tests can give us the generic diagnosis of hemolytic anemia, immune or not immune, but the etiological diagnosis should be resolved in collaboration with the hematologist.

Anemia is common in hematological malignancies, but with limited gastrointestinal symptoms, except for gastric or intestinal bleeding caused by thrombocytopenia or lymphoma. It is rare, but it should be noted that gastric or intestinal lymphoma sometimes presents as mild anemia with nonspecific gastrointestinal symptoms. Endoscopy usually provides the diagnosis.

Hereditary anemia is extremely rare, except for thalassemia and sickle cell disease in certain geographical areas. Sickle cell disease is restricted to blacks. When one suspects hereditary anemia, the cooperation of a hematologist should be sought, because the diagnosis is difficult and the laboratory tests should be carefully selected.

CONCLUSION

More than 100 diseases may cause anemia, but 90% belong to three groups: nutritional deficiencies (iron, vitamin B12 and folic acid), ACD (chronic inflammation, tumors), and bleeding (excluding chronic bleeding, which produces iron deficiency). Hemolytic anemia, although less frequent than the other, is the last option before considering the diagnosis of rare diseases.

A few laboratory tests, such as blood count, ESR, serum ferritin, and serum iron and transferrin, are sufficient to focus the diagnosis. According to initial results, additional laboratory tests should be ordered: serum vitamin B12 and serum and erythrocyte folate (in case of macrocytosis), tumor markers and acute phase reactants (if ACD is suspected), LDH, haptoglobin and antiglobulin test (if hemolytic anemia is suspected).

With these key data, the doctor can put in place more specific tests: imaging, endoscopy and additional laboratory tests. The next step should be to correct the cause of anemia when possible or correct anemia itself when the cause cannot be treated. The advantages are clear if the cause is a cancer that can be diagnosed at an early stage. On the other hand, we can improve the quality of life of patients by correcting the anemia with medical treatment or regular transfusions.

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TOPIC HIGHLIGHT

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A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases

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Abstract

Iron deficiency (ID), with or without anemia, is often caused by digestive diseases and should always be investigated, except in very specific situations, as its causes could be serious diseases, such as cancer. Diagnosis of ID is not always easy. Low serum levels of ferritin or transferrin saturation, imply a situation of absolute or functional ID. It is sometimes difficult to differentiate ID anemia from anemia of chronic diseases, which can coexist. In this case, other parameters, such as soluble transferrin receptor activity can be very useful. After an initial evaluation by clinical history, urine analysis, and serological tests for celiac disease, gastroscopy and colonoscopy are the key diagnostic tools for investigating the origin of ID, and will detect the most important and prevalent diseases. If both tests are normal and anemia is not severe, treatment with oral iron can be indicated, along with stopping any treatment with non-steroidal anti-inflammatory drugs. In the absence of response to oral iron, or if the anemia is severe or clinical suspicion of important disease persists, we must insist on diagnostic evaluation. Repeat endoscopic studies should be considered in many cases and if both still show normal results, investigating the small bowel must be considered. The main techniques in this case are capsule endoscopy, followed by enteroscopy.

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Key words: Anemia; Iron-deficiency; Iron deficiency anemia

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INTRODUCTION

Iron deficiency (ID) is defined as the decrease of the total content of iron in the body. Iron deficiency anemia (IDA) occurs when ID is sufficiently severe to reduce erythropoiesis. This type of anemia is the most frequent chronic anemia. ID may be the result of either excessive loss or, less frequently, decreased absorption. In general, the iron absorbed daily equals the amount needed to compensate its loss, so that the overall iron pool remains stable. This fine balance is easily broken, because the capability to absorb iron orally is limited. When the inputs are less than necessary or, more frequently, when the outputs increase and cannot be compensated for, ID and finally IDA, develops. In many cases, these alterations will be secondary to gastrointestinal disease. IDA occurs in 2%-5% of adult males and postmenopausal women in the developed world[1,2]. ID, with or without anemia, is even more frequent. It is a common cause of consulting a gastroenterologist (4%-13% of all referred patients)[3]; however, there is only one clinical guide published that deals with this clinical entity and the evidence level for most conclusions is medium-low, due to the paucity of reliable clinical data[1]. In addition, for similar reasons, many of the patients treated by gastroenterologists will develop ID and IDA. This data underscores the importance of anemia in our clinical practice. Using a therapeutic iron course has been suggested as diagnostic test of ID. This is a reasonable approach only for people at high risk of physiological ID, such as adolescents and pregnant women[4]. However, in daily clinical practice it is not unusual to find patients who have received several cycles of iron treatment before assessing the potential cause of anemia. This strategy leads to belated evaluation, especially in young females and in patients with a

prior history of anemia, resulting in significant delay in etiological diagnosis of anemia[5]. In this sense, except in very specific situations, ID with or without anemia should always be investigated because it can be caused by potentially serious diseases[1]. We will review the etiology and laboratory diagnosis of chronic ID and IDA to establish recommendations on the practical management of this clinical entity.

ETIOLOGY OF ID OR IDA

There are many potential causes of ID and IDA and some of them are very relevant. These causes are clearly different in developing and in developed countries. Deficient intake is the most frequent etiology in the former, whereas other important diseases are potentially implicated in the case of the latter.

In developed countries, the likeliest cause of anemia in each patient depends on age and sex. In women of childbearing age, excessive menstrual loss is the most frequent etiology, while in postmenopausal women and in males, digestive diseases are the main causes[3,6]. Taking both these data and a detailed clinical history into account, we will plan diagnostic strategy in each case. However, these assumptions should not lead to errors in dealing with IDA or ID, such as not to investigate anemia in women assuming non-diagnosed gynaecological problems, because that attitude leads to a significant delay in detecting important diseases[5].

In addition to digestive disorders, gynaecological diseases, urological diseases and other specific situations, such as intravascular hemolysis, the etiology of IDA (Table 1) includes aspects that exceed the purposes of this article. Focusing on digestive diseases, the etiology of ID and IDA of gastrointestinal origin can be divided into two groups: situations with increased loss of iron (the most common in developed countries), and those with decreased iron absorption. In the former, the blood loss can occur in the form of visible bleeding (melena, hematemesis, rectal bleeding) or hidden bleeding, which might be more difficult to diagnose. Among the diseases causing the blood loss we should emphasize, by frequency and importance, benign or malignant gastrointestinal tumors of the colon, stomach, esophagus and small intestine, peptic ulcer and reflux disease, use of non-steroidal anti-inflammatory drugs (NSAIDs), and inflammatory bowel disease. The possible existence of a malignancy as the source of anemia, which leads to early completion of endoscopic examinations in this clinical scenario, is of great concern. In the National Health and Nutrition Examination Survey and Epidemiologic Follow-up Study carried out in the USA on a cohort of 9024 individuals (aged between 25 to 74 years old without prior diagnosis of gastrointestinal malignancy), hemoglobin levels and iron saturation were determined. No case of ID in premenopausal women was determined to be due to malignancies. Among men and postmenopausal women with IDA [Relative Risk (RR) = 31, 95% confidence interval (CI): 9-107] or ID without anemia (RR = 5, 95% CI: 1-21) an increased risk of being diagnosed with cancer within the subsequent two years was observed[7]. Therefore, gastrointestinal malignancies are uncommon in premenopausal women with ID or IDA, but in men and postmenopausal women with ID or IDA gastrointestinal malignancies are more common than in individuals with normal hemoglobin and iron levels.

Table 1 Causes of iron deficiency anemia

Digestive disorders
Increased losses of iron
Cancer/polyp: colon, stomach, esophagus, small bowel
Peptic ulcer, esophagitis
NSAID use
Inflammatory bowel disease: ulcerative colitis, Crohn's disease
Intestinal parasites
Vascular lesions: angiodysplasia, watermelon stomach
Meckel's diverticulum
Reduced iron absorption
Celiac disease
Bacterial overgrowth
Whipple's disease
Lymphangiectasia
Gastrectomy (partial and total) and gastric atrophy
Gut resection or bypass
Urological and gynecological disorders
Intravascular hemolysis
Prosthetic valves and cardiac myxomas, paroxysmal nocturnal hemoglobinuria, marathon runners, multiple blood donations
Deficient iron intake

Reduced iron absorption is the second category of ID causes of digestive origin, and can be caused by celiac disease, atrophic gastritis, and postsurgical status (gastrectomy, intestinal resection) among others. Celiac disease is very relevant and specific evaluation to exclude it must be performed. In a study on patients referred to a specialized gastroenterological consultation because of ID or IDA, celiac disease was finally the diagnosis in 10% of cases[8]; other authors described that at least 2%-3% of patients with IDA are finally diagnosed as celiac disease[3,6]. The prevalence of this disease worldwide is approximately 1%, and it is probably under diagnosed[9]. Microscopic alterations in the duodenal mucosa in non-treated celiac disease patients will lead to them becoming refractory to oral iron treatment. This has also been described in patients with autoimmune atrophic gastritis and gastritis due to *Helicobacter pylori (H pylori)*[10-12]. Gastroscopy with biopsies, allowing us to detect the presence of atrophy with or without *H pylori*, is essential. The positivity of autoantibodies (anti-intrinsic factor or anti-parietal cell) supports the diagnosis of autoimmune atrophic gastritis[13]. Regarding the possible role of *H pylori* in IDA, a recent meta-analysis indicated that the infection is associated with depleted iron deposits. The mechanism by which *H pylori* induces this alteration is not clear, but it appears to involve gastrointestinal blood loss, diminished iron absorption from the diet, and increased consumption of iron by the bacteria. The authors suggest that the impact of eradication of *H pylori* in the improvement of the iron deposits must be evaluated in large controlled trials[14]. Finally, it must be pointed out that in our environment,

a deficit of dietary iron not associated with any other pathology will rarely be the cause of ID or IDA.

CLINICAL MANIFESTATIONS

The clinical picture varies greatly from one case to another, and it is produced both by the anemia itself and by the lack of iron, which is essential for cellular energy metabolism. Symptoms depend greatly on the speed of onset of anemia, its severity, and the characteristics of the patient. Thus, IDA or ID can be detected in an asymptomatic individual on a screening-analysis, or in a person with symptoms that include general weakness, fatigue, irritability, poor concentration, headache, and intolerance to exercise. These symptoms appear even in the figures for ID with normal hemoglobin levels. Patients often show relatively few symptoms spontaneously. Although the impact of ID on the quality of life of the subject is high, they often get used to their symptoms and these are assumed as normal. The patient becomes aware of an improvement only when the symptoms disappear. Some iron-deficient patients, with or without anemia, might have alopecia, atrophy of lingual papillae, or dry mouth due to loss of salivation. Other symptoms, such as weakness or digging fingernails (koilonychia), chlorosis, or the syndromes of Plummer-Vinson or Paterson-Kelly (dysphagia with esophageal membrane and atrophic glossitis) have virtually disappeared. These changes were caused by reduction of iron-containing enzymes in the epithelia and the gastrointestinal tract. Pica, the eating disorder in which there is an irresistible desire to lick or eat non-nutritive and unusual substances, such as soil, chalk, gypsum, ice (pagophagia) or paper, might appear in some cases. Pagophagia is considered quite specific to ID and it responds quickly to treatment. In a study on a group of patients referred to a gastroenterology consultation, more than half had pagophagia. It was especially frequent in women, and was not related to the cause of bleeding[15].

Physical examination might be normal or show pallor of varying intensity, there may be a systolic murmur in cardiac auscultation, and abdominal and rectal exploration will allow us to rule out the existence of masses at those locations.

LABORATORY DIAGNOSIS: ID WITH OR WITHOUT ANEMIA

The diagnosis of anemia is simple and objective: the World Health Organization defines it as the decline in blood hemoglobin to a concentration below 13 g/dL in men and 12 g/dL in women. However, to confirm that ID is the origin of the anemia is not always easy. Sometimes the simple blood cell count strongly suggests this origin, the typical pattern being microcytosis, hypochromia (perhaps the most important, even more than the microcytosis), and elevation of red cells distribution width (RDW). However, up to 40% of "pure" IDA cases are normocytic. Therefore, a normal mean corpuscular volume (MCV) does not exclude ID from being the cause of the anemia. Moreover, the presence of microcytosis does not necessarily imply ID and can be produced by other anemias (chronic process, sideroblastic anemia) and diseases (e.g. thalassemia). RDW measures the degree of anisocitosis (size difference) of the population of red cells and its elevation is neither sensitive nor specific for ID. The next step is to determine the so-called iron metabolism (in addition to all other necessary determinations, including levels of vitamin B12 and folic acid) and in many cases the level of C-reactive protein. A typical pattern is a decrease in sideremia, plasma ferritin, and transferrin saturation. However, this is not the usual case. The least reliable parameter for diagnosis of ID is probably the determination of sideremia, because it could be detected as an artefact of contamination of laboratory equipment, it has a nocturnal rhythm and it can normalize hours after ingestion. Serum ferritin, in the absence of inflammation (usually defined as a normal C-reactive protein level), reflects total body iron deposits. Thus, a low serum ferritin (< 30 ng/L) unequivocally means ID, whether accompanied by anemia or not. However, as serum ferritin is an acute phase reactant, a normal or even elevated ferritinemia does not exclude the presence of ID. Thus, in the presence of an inflammatory process (usually defined by an elevated C-reactive protein level), ID could exist even with levels of ferritin up to 100 ng/mL. Another parameter of the normal "iron metabolism", especially useful when the determination of ferritin is equivocal, is the transferrin saturation index. This shows the percentage of transferrin that transports iron and thus a decrease (< 20%) implies ID, either absolute or functional.

In some cases, even taking into account all these determinations, ID can be difficult to diagnose. It generally occurs in situations where the anemia has a multifactorial origin. This is typical in cases of anemia of mixed origin, a chronic process that coexists with ID, which is a frequent scenario in gastrointestinal inflammatory disease or cancer. Even with all the determinations previously described, it can be difficult to estimate the role of each factor in the genesis of anemia in this setting. In these cases, other values can help us to assess the pathogenesis of anemia. These other factors include the determination of soluble transferrin receptor, reticulocyte hemoglobin concentration, the percentage of hypochromic red cells, the concentration of erythropoietin (and its relation to the expected values), and even the determination of hepcidin. The soluble transferrin receptor is one of the most useful as it is the least influenced by the presence of inflammation and it correlates well with concentration of transferrin receptor in the cell plasma membrane[16]. If the levels are high, ID is likely to be a major component of anemia, while in those cases with normal or low levels; anemia is probably not associated with ID. In Table 2, the values of different determinations in different clinical scenarios are shown.

Table 2 Differences between the serum values of iron-deficiency anemia, anemia of chronic diseases, and anemia of mixed origin

	Iron-deficiency anemia	Anemia of chronic diseases	Anemia of mixed origin
Serum iron	Decreased	Decreased	Decreased
Transferrin	Increased	Decreased or normal	Decreased
Transferrin saturation	Decreased	Decreased	Decreased
Ferritin[1]	Decreased (< 30 ng/mL)	Increased (> 100 ng/mL)	Normal
Soluble transferrin receptor	Increased	Normal	Increased or normal
C-reactive protein	Normal	Increased	Increased
Erythropoietin	Increased	Normal or slightly increased for the degree of anemia	Increased or normal

[1]Ferritin values between 30 and 100 ng/mL: Make other determinations to differentiate the two entities.

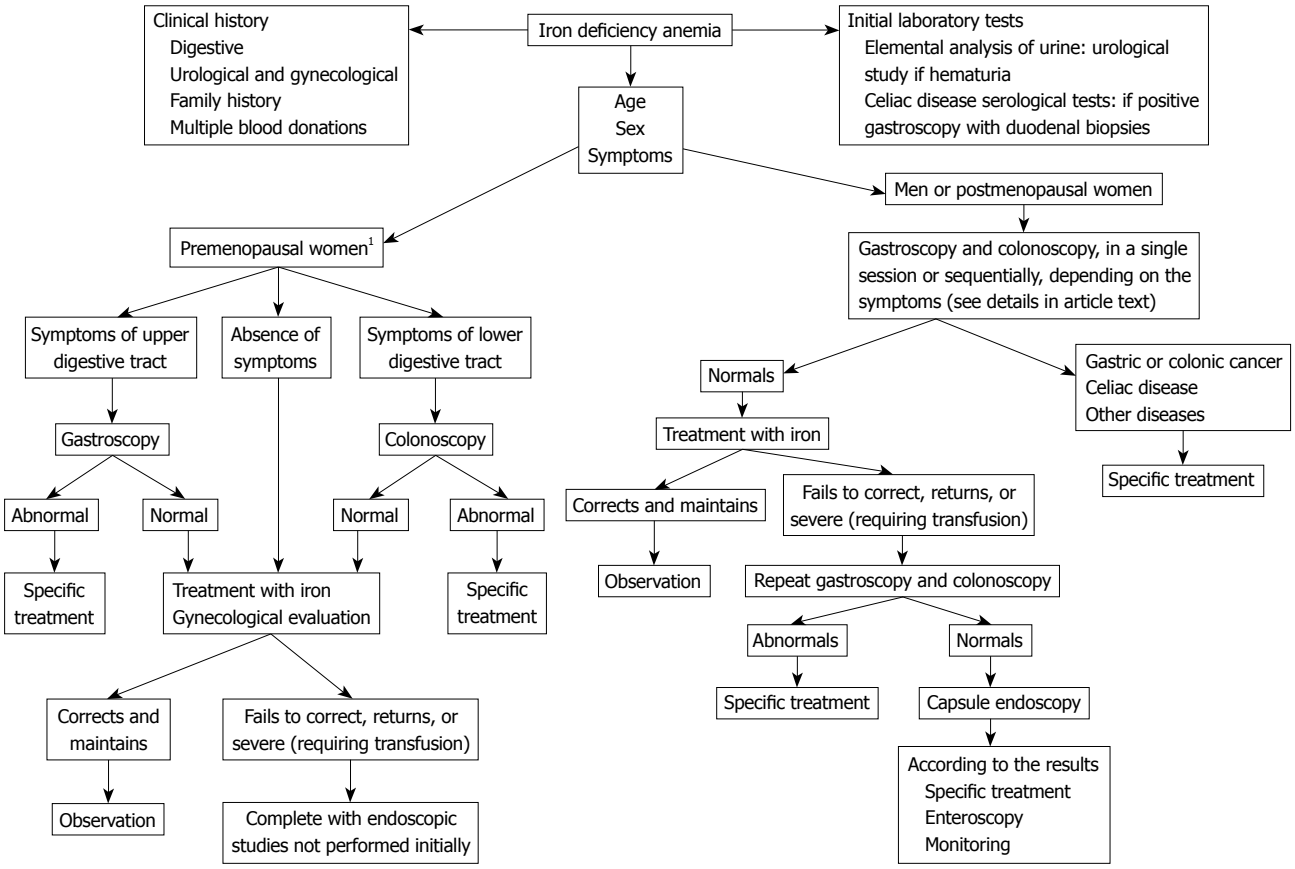

Figure 1 Etiologic diagnosis of iron deficiency anemia. [1]We must always consider gynecological cause.

ETIOLOGIC DIAGNOSIS OF ID ANEMIA

Once the diagnosis of IDA or ID without anemia has been established, it is necessary to investigate its origin (Figure 1), because it can be caused by very serious diseases. Taking into account the age and sex of the patient, and of course an adequate clinical history, we will plan a diagnostic strategy in every individual case.

In the clinical history, we should investigate any sign of digestive or urologic bleeding, and in the case of women, gynaecological history and symptoms should be included. Personal history of peptic ulcers and a family history of colon cancer or celiac disease should be investigated. The existence of suggestive symptoms in a location is a predictor of disease in that location. Therefore, the initial evaluation can be accompanied by the location of symptoms[17].

The initial studies should include laboratory tests, with an elementary analysis of urine (up to one third of the renal carcinomas have anemia). In the case of positive serological tests for celiac disease, it is also necessary to perform a gastroscopy with distal duodenum biopsies. In most patients, a dilemma will arise; is it necessary or mandatory to perform a gastroscopy, a colonoscopy or both? In patients over 50 years old, a colonoscopy is preferred to gastroscopy, and on the other hand, many of the patients under study for ID might have an indication of preventive colonoscopy screening, either because of family history or age (over 50 years)[18]. It has been described that in the presence of a low ferritin value, the probability of finding any pathology at colonoscopy will be quadrupled[19]. This supports the need for colonoscopy in the study of IDA and ID. It is important to underline that up to 15% of cases may have synchronous lesions in the upper and lower digestive tract, which means that it is necessary to perform both techniques, except in those cases in which celiac disease or a neoplasm is diagnosed

in the initial examination[1]. Gastrointestinal endoscopies (gastroscopy and colonoscopy) might be performed in the same session or sequentially, according to clinical history (or serologic data). The combination of gastroscopy and colonoscopy is highly sensitive and specific for locating gastrointestinal lesions that produce anemia[3,20,21]. However, the combination of both endoscopic techniques only determines the final cause of anemia in a little more than half of the patients. In a prospective study carried out on 100 consecutive patients with IDA, gastrointestinal endoscopies revealed at least one lesion potentially responsible for the loss of blood in 62 patients, 36% by gastroscopy, 25% by colonoscopy, and 1% by both[17]. If examinations are normal, the anemia is not severe and symptoms do not suggest significant disease. The next step might be clinical follow-up, oral iron treatment (but iron supplementation may be harmful in some patients, e.g. in those with renal diseases) and cessation of any NSAIDs or aspirin consumption. Patients not responding to treatment with oral iron, those with severe anemia, or suspected serious illness, will require re-evaluation[1,22]. Some authors describe that in the elderly, low MCV (≤ 60 fL) and a positive test for fecal occult blood are predictors of the presence of potentially bleeding lesions in endoscopy in patients with anemia without gastrointestinal symptoms[23]. In premenopausal women, the most frequent endoscopic findings are gastritis by *H pylori* and celiac disease, and it has been suggested that in these patients initial diagnostic approach to IDA may include, in addition to the serologic celiac tests, a ^{13}C-urea breath test, reserving endoscopic studies for cases where these tests are negative or anemia persists despite the eradication of *H pylori*[24]. Finally, in the study of ID or IDA it must be pointed out that a barium enema is not a useful tool, being clearly inferior to colonoscopy[25].

In those patients whose non-favourable clinical course after negative endoscopic studies advises further evaluation, repeating the endoscopic studies is justified because a proportion of lesions within the reach of conventional endoscopes might not be detected for several reasons. Repeating a gastroscopy might show erosions in a large hiatus hernia (Cameron lesions), peptic ulcer and vascular ecstasy not detected in a previous exploration[26]. Repeating a colonoscopy has a slightly lower yield, but it is a reasonable and necessary option if the previous colonoscopy was suboptimal because of incomplete or poor preparation. It has been shown that colonoscopy can fail in the diagnosis in 5% of colorectal tumors due to several reasons: an incomplete exploration, poor bowel preparation, misinterpretation of findings, inadequate biopsies of lesions[27], or just not seeing the lesion.

In those cases in which repeated endoscopic examinations are all negative, we should investigate the small bowel as the source of anemia. In this scenario, the best initial approach is probably a capsule endoscopy[28], reserving enteroscopy (single and double-balloon) for cases in which it is necessary to apply a treatment or to obtain biopsies of lesions localized by the capsule[29,30]. This strategy significantly reduces the number of patients requiring an alternative study after an initial investigation of the small intestine. Capsule endoscopy is a technique that explores the entire small intestine, something which is not always possible with enteroscopy. Capsule endoscopy has the disadvantage that it does not allow biopsies of detected lesions. The most common findings in patients with bleeding of obscure origin and/or IDA are angiodysplasia and Crohn's disease[31]. According to the results of the meta-analysis of Triester *et al*[32], diagnostic yield of capsule endoscopy (63%) in a study of patients with gastrointestinal bleeding of obscure origin was higher than that of push enteroscopy (26%), and of contrast studies with barium (8%). Enteroscopy should be considered as a second line technique in patients with a positive capsule endoscopy requiring sampling for histology or performing therapeutic endoscopy, and in patients in whom the suspicion of a small intestine lesion is high, despite the negativity of the capsule[33].

Classical imaging studies of the small intestine (bowel through and enteroclysis) are much less sensitive for detecting lesions potentially causing anemia[17] and have been reserved for those centres where the previous techniques are not available or are contraindicated. Radiological studies of the small bowel with computed tomography or magnetic resonance imaging techniques, which are very useful in the characterization of tumors from these locations and in the diagnosis of inflammatory bowel disease could, in experienced hands, increase the diagnostic yield[34].

Finally, we should bear in mind that enteropathy by NSAIDs affects a significant number of people using these drugs, and that the amount of blood lost with the regular use of NSAIDs, such as ibuprofen, can be quite large[35]. Injuries by the use of classic NSAIDs and selective inhibitors of COX-2 are very common (50%-70%) in the evaluation of the small intestine, i.e. by capsule endoscopy[36,37]. We must remember that despite treatment with gastro-protective agents (proton pump inhibitors), aspirin and NSAIDs can cause gastrointestinal lesions of the lower bowel and thus cause bleeding and chronic ID.

CONCLUSION

IDA and ID are quite frequent in digestive pathology and they must always be taken into consideration for two reasons: (1) they have a clear impact on the patient's quality of life, and therefore they require adequate treatment and (2) they can be the consequence of significant or severe diseases, so it is essential to investigate their origin. Initial etiological evaluation must include celiac disease serological tests. In many patients, the usual endoscopic studies (gastroscopy and colonoscopy) will be prescribed, as they are necessary to rule out more severe diseases and they allow identification of the origin of anemia in more than half of the cases. In the rest of the patients, if anemia is severe or it does not respond to oral iron treatment, the first step will be to repeat those studies. If the results are still normal, it is necessary to investigate the existence of lesions in the small bowel by using capsule endoscopy. With all the diagnostic means

available nowadays, very few IDAs should be left without a diagnosis.

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, *Series Editors*

A short review of malabsorption and anemia

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Abstract

Anemia is a frequent finding in most diseases which cause malabsorption. The most frequent etiology is the combination of iron and vitamin B12 deficiency. Celiac disease is frequently diagnosed in patients referred for evaluation of iron deficiency anemia (IDA), being reported in 1.8%-14.6% of patients. Therefore, duodenal biopsies should be taken during endoscopy if no obvious cause of iron deficiency (ID) can be found. Cobalamin deficiency occurs frequently among elderly patients, but it is often unrecognized because the clinical manifestations are subtle; it is caused primarily by food-cobalamin malabsorption and pernicious anemia. The classic treatment of cobalamin deficiency has been parenteral administration of the vitamin. Recent data suggest that alternative routes of cobalamin administration (oral and nasal) may be useful in some cases. Anemia is a frequent complication of gastrectomy, and has been often described after bariatric surgery. It has been shown that banding procedures which maintain digestive continuity with the antrum and duodenum are associated with low rates of ID. *Helicobacter pylori* (*H pylori*) infection may be considered as a risk factor for IDA, mainly in groups with high demands for iron, such as some children and adolescents. Further controlled trials are needed before making solid recommendations about *H pylori* eradication in these cases.

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Key words: Anemia; Celiac disease; *Helicobacter pylori*; Cobalamin deficiency; Gastrectomy

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INTRODUCTION

Anemia is a frequent finding in most digestive diseases which cause malabsorption. In this review we will focus on some frequent entities producing anemia and malabsorption, which are of current interest because of recent advances regarding each of them. Celiac disease (CD) may be considered as an archetypal malabsorption syndrome, and it is a frequent cause of anemia without associated intestinal symptoms. Anemia due to isolated cobalamin deficiency is a frequent finding in the elderly, and its etiology goes beyond the classical pernicious anemia concept. On the other hand, physicians should be aware that anemia often follows gastric surgery procedures, mainly after bariatric surgery, in order to implement prevention strategies. *Helicobacter pylori* (*H pylori*) infection is being recognized as a frequent cause of iron deficiency (ID), both in developed and developing countries. In these cases eradication of the infection could be a successful therapy of the anemia.

ANEMIA IN CD

CD is an immunologically-mediated enteropathy, triggered in genetically susceptible subjects, by the intake of certain proteins of wheat, barley and rye, and resulting in small-bowel mucosal villous atrophy with crypt hyperplasia[1].

Anemia has frequently been reported as the only manifestation or the most frequent extra-intestinal symptom of CD[2-4]. Although folate and cobalamin deficiency are known complications of CD, the most common nutritional anemia associated with CD is ID. ID anemia (IDA) was reported in up to 46% of patients with subclinical CD in one study, and its prevalence was higher in adults than in children[4]. In a recent case-control study, only anemia (OR: 26.3; 95% CI: 6-120) and diarrhea

Table 1 Prevalence of CD in patients presenting with IDA

Study, yr	*n*	Positive serology (%)	Positive biopsy (%)	Comments
Corazza *et al*[16], 1995	200	8	5	IDA. 8.5% if only obscure IDA considered. All patients with positive serology were biopsied
Carroccio *et al*[17], 1998	85	5.8	5.8	IDA. 20% if only refractory obscure IDA considered. All patients with positive serology were biopsied. 80% of CD cases were women
Unsworth *et al*[18], 2000	483	6.6	4.6	Blood donors with anemia, most with IDA. Not all seropositive patients were biopsied. 95% of CD cases were women
Haslam *et al*[19], 2001	216	2.3	n/a	Pregnant women with anemia. Four of five anemic women with positive serology have IDA. Only serology was performed
Annibale *et al*[20], 2001	190	n/a	13.7	Obscure IDA. All patients were biopsied. 85% of CD cases were women
Howard *et al*[21], 2002	258	10.9	4.7	Majority of patients with previously not studied IDA (4% folate deficiency). Not all seropositive patients were biopsied. 83% of CD cases were women
Ransford *et al*[22], 2002	484	3.5	2.3	Previously not studied IDA. Not all seropositive patients were biopsied. 73% of CD cases were women
Grisolano *et al*[23], 2004	103	n/a	8.7	Previously not studied IDA. All patients were biopsied. 100% of CD cases were women
Mandal *et al*[24], 2004	504	n/a	1.8	IDA with normal upper gastroscopy. Not all patients were biopsied
Hershko *et al*[25], 2005	150	5.3	5.3	Previously not studied IDA. All seropositive patients were biopsied
Kalayci *et al*[26], 2005	135	4.4	4.4	Previously not studied children with IDA. All seropositive patients were biopsied
Zamani *et al*[27], 2008	206	15	14.6	Obscure IDA. All patients were biopsied
Carter *et al*[28], 2008	116	n/a	6.5	Previously not studied premenopausal women with IDA. All patients were biopsied

n/a: Not available data; IDA: Iron deficiency anemia; CD: Celiac disease.

(OR: 4.5; 95% CI: 2-10) were identified as independent predictors of an eventual diagnosis with CD among the different clinical presentations in general practice during the 5 years prior to the diagnosis of CD[5]. Similarly, among patients identified by population screening, 50% were anemic as the primary presentation[6].

Iron is absorbed in the proximal small intestine and the absorption is dependent upon several factors, including an intact mucosal surface and intestinal acidity[7,8]. ID in CD primarily results from its impaired absorption as a result of the villous atrophy of the intestinal mucosa. Consequently, IDA develops. The concept of abnormal iron absorption is supported by the failure to increase serum iron following oral iron supplementation.

Occult gastrointestinal bleeding has been described in CD in correlation with the severity of villous atrophy[9]. More recent studies have found, however, that the rate of positive occult blood tests in CD is low and does not exceed that of the general population[10,11].

Anemia of chronic disease has also been described in CD[12,13]. It is well known that pro-inflammatory cytokines play an essential role in the pathogenesis of CD[14]. In this regard, both interferon-γ and interleukin-6 are powerful mediators of hypoferremia in inflammation, leading to the abnormalities in iron homeostasis associated with the anemia of chronic disease[15]. Accordingly, Harper *et al*[12] described that in the majority of CD patients with anemia, low serum ferritin levels were an indicator of ID. However, in 13% of patients with anemia, serum ferritin levels were increased, such abnormalities reverting to normal after a gluten-free diet. In a recent study, refined precision laboratory methods to identify anemia of inflammation were used[13]. Among 65 anemic CD patients, 45 had IDA, two had cobalamin or folate deficiency, and 11 had anemia of chronic disease alone or in combination with ID, which implies a prevalence of 17%. After 12-mo on a gluten-free diet, the response was equally favorable in the patients with either IDA or anemia of chronic disease, indicating that the suppression of inflammatory intestinal changes by the diet improves anemia both by correcting iron absorption and by blunting the inflammatory response.

CD is frequently diagnosed in patients referred for evaluation of anemia, and subclinical CD appears to be a relatively common cause of IDA. Studies using serologic tests and small-bowel biopsies in patients referred for evaluation of IDA have reported CD in 1.8%-14.6% of patients (Table 1)[16-28]. These studies are heterogeneous in design, as some included only patients with IDA whereas others included both folate and iron deficient patients, and used different methods for diagnosis of CD, often in selected referral populations. Highest frequencies of CD were observed in patients with obscure IDA, and frequency may be as high as 20% in patients with refractory obscure IDA[17]. The frequency of CD in women with IDA is higher than in other risk groups of CD, with a female:male ratio of 2:1[4,29], and several studies have reported that 73%-100% of IDA patients diagnosed with CD were adult pre-menopausal women[17,18,20-23]. The higher iron demand in adult pre-menopausal women as a result of menstrual loss in a condition of chronic iron malabsorption attributable to CD probably explains this higher prevalence of CD in this group of patients.

Clinicians should consider CD as a possible cause of anemia in all subjects with IDA of unknown origin, even in menstruating women. Recent guidelines from the British Society of Gastroenterology recommend that duodenal biopsies should be taken during endoscopy if no obvious case of ID can be found[30]. The treatment of IDA associated with CD is primarily a gluten-free diet with iron supplementation until the iron stores have been restored.

Table 2 Stages of cobalamin metabolism and corresponding causes of cobalamin deficiency

Stage of cobalamin metabolism	Causes of cobalamin deficiency
Intake solely through food	Strict vegetarianism without vitamin supplementation
Digestion brings into play haptocorrin, gastric secretions (hydrochloric acid and pepsin), pancreatic and biliary secretions, enterohepatic cycle	Gastrectomy; pernicious anemia (Biermer's disease); food-cobalamin malabsorption
Absorption brings into play intrinsic factor and cubilin	Ileal resection; malabsorption; pernicious anemia; Imerslund syndrome
Transport by transcobalamins	Congenital deficiency of transcobalamin Ⅱ
Intracellular metabolism based on various intracellular enzymes	Congenital deficiency of various intracellular enzymes

Several studies have shown that many untreated patients with CD are folate deficient[7]. Deficiency of vitamin B12 is also common in CD and frequently results in anemia[7]. The main site of vitamin B12 absorption is the distal ileum; a small proportion is also absorbed passively along the entire small bowel. The causes of vitamin B12 deficiency in CD may include associated autoimmune gastritis, bacterial overgrowth, decreased gastric acid or decreased efficiency of mixing with transfer factors in the intestine.

ANEMIA AND COBALAMIN DEFICIENCY

Literature of the last 10 years has provided several definitions of cobalamin (vitamin B12) deficiency, depending mainly on the population studied and on the particular assay kits used. Cobalamin deficiency is defined in terms of the serum values of cobalamin and of homocysteine and methylmalonic acid, two components of the cobalamin metabolic pathway. High homocysteine levels (hyperhomocystinemia) may also be caused by folate or vitamin B6 deficiencies, and these should be excluded before a diagnosis of cobalamin deficiency is made[31]. Accordingly, definitions of cobalamin deficiency are[32]: (1) Serum cobalamin levels < 150 pmol/L (< 200 pg/mL) with clinical features and/or hematological anomalies related to cobalamin deficiency; (2) Serum cobalamin levels < 150 pmol/L on two separate occasions; (3) Serum cobalamin levels < 150 pmol/L and total serum homocysteine levels > 13 μmol/L or methylmalonic acid levels > 0.4 μmol/L (in the absence of renal failure and folate and vitamin B6 deficiencies); (4) Low serum holotranscobalamin levels < 35 pmol/L.

Epidemiological studies show that in the general population of industrialized countries, cobalamin deficiency has a prevalence of around 20%, ranging from 5% to 60% depending on the definition of cobalamin deficiency used[31,32].

Cobalamin metabolism and corresponding causes of deficiency

The metabolic pathway starts when dietary cobalamin, obtained through animal foods, enters the stomach bound to animal proteins. A typical Western diet contributes 3-30 μg of cobalamin per day towards the recommended dietary allowance of 2.4 μg/d for adults[33]. Pepsin and hydrochloric acid in the stomach split the animal protein, releasing free cobalamin. Most of the free cobalamin is then bound to R-protein which is released from the parietal and salivary cells. Intrinsic factor is also secreted in the stomach, but its binding to cobalamin is weak in the presence of gastric and salivary R-protein. In the duodenum, dietary cobalamin bound to R-protein is joined by cobalamin-R-protein complexes that have been secreted in the bile. Pancreatic enzymes degrade both biliary and dietary cobalamin-R-protein complexes, releasing free cobalamin. The cobalamin then binds with intrinsic factor. The cobalamin-intrinsic factor complex remains undisturbed until the distal 80 cm of the ileum, where it attaches to mucosal cell receptors (cubilin) and the cobalamin is bound to transport proteins designated transcobalamin Ⅰ, Ⅱ and Ⅲ. Transcobalamin Ⅱ, although it represents only a small fraction (about 10%) of the transcobalamins, is the most important because it is able to deliver cobalamin to all cells in the body. The cobalamin is subsequently transported systemically *via* the portal system. Within each cell, the transcobalamin Ⅱ-cobalamin complex is taken up by means of endocytosis and the cobalamin is liberated and then converted enzymatically into its two coenzyme forms, methylcobalamin and adenosylcobalamin. The causes of cobalamin deficiency according to the stage of cobalamin metabolism are described in Table 2[31,32].

Cobalamin deficiency in the elderly: clinical entities

Vitamin B12 or cobalamin deficiency occurs frequently among elderly patients, but it is often unrecognized or not investigated because the clinical manifestations are subtle[33]. However, the potential seriousness of the complications (particularly neuropsychiatric and hematological) requires investigation of all patients who present with vitamin or nutritional deficiency. In elderly patients, cobalamin deficiency is caused primarily by food-cobalamin malabsorption and pernicious anemia[31]. Food-cobalamin malabsorption accounts for about 60%-70% of the cases among elderly patients, and pernicious anemia accounts for 15%-20% of the cases. Other causes included dietary deficiencies (< 5%), malabsorption (< 5%) and hereditary cobalamin metabolism diseases (< 1%).

Intrinsic factor, which is released by parietal cells in the stomach, binds to vitamin B12 in the duodenum. This vitamin B12-intrinsic factor complex subsequently plays a role in the absorption of vitamin B12 in the terminal ileum. This mechanism is responsible for 60% of the absorption of cobalamin. In addition, an alternate system exists that is independent of intrinsic factor or

even an intact terminal ileum: cobalamin is absorbed by simple diffusion or mass action independent of intrinsic factor if 300-1000 μg/d cobalamin is administered orally or intramuscularly to patients with pernicious anemia. Approximately 1%-5% of free cobalamin (or crystalline cobalamin) is absorbed along the entire intestine by passive diffusion[33,34].

Food-cobalamin malabsorption: Food-cobalamin malabsorption syndrome is characterized by the inability to release cobalamin from food or from intestinal transport proteins, particularly in the presence of hypochlorhydria, where the absorption of "unbound" cobalamin remains normal. This syndrome is defined by cobalamin deficiency in the presence of sufficient food-cobalamin intake and a negative Schilling test, where the latter rules out malabsorption or pernicious anemia. Thus in this syndrome, patients can absorb "unbound" cobalamin through intrinsic factor or passive diffusion mechanisms. The recognition of the syndrome permits new developments of oral cobalamin therapy[31-33,35]. Researchers have supported the existence of this syndrome by using a modified Schilling test, which employs radioactive cobalamin bound to animal proteins and reveals malabsorption when the results of a standard Schilling test are normal.

Food-cobalamin malabsorption is caused primarily by gastric atrophy. Over 40% of patients older than 80 years have gastric atrophy that may or may not be related to *H pylori* infection[31]. Other factors that contribute to food-cobalamin malabsorption in elderly people include: intestinal microbial proliferation; long term ingestion of biguanides (metformin) and antacids, including H2-receptor antagonists and proton pump inhibitors; chronic alcoholism; surgery or gastric reconstruction; partial pancreatic exocrine failure; and Sjögren's syndrome[31,32].

The partial nature of this form of malabsorption may produce a more slowly progressive depletion of cobalamin than does the more complete malabsorption engendered by disruption of the intrinsic factor-mediated absorption. The slower progression of cobalamin depletion probably explains why mild, preclinical deficiency is more frequently associated with food-cobalamin malabsorption than with pernicious anemia[32,33,35]. When associated with *H pylori* infection, eradication of the infection alone may correct cobalamin levels[36].

Pernicious anemia: This is the classic cause of cobalamin deficiency and one of the most frequent among elderly patients. Pernicious anemia is an autoimmune disease characterized by the destruction of the gastric mucosa, especially fundal mucosa, by a primarily cell-mediated process[31,37]. Gastric secretions are neutral to slightly acidic even in the presence of gastrin and contain little or no intrinsic factor. The disease is also characterized by the presence of two antibodies, particularly in plasma and gastric secretions: few people who do not have the disease have antibodies (specificity 98%), but only about 50% of patients will have anti-intrinsic factor antibodies (IFA) (sensitivity 50%). Anti-gastric parietal cell (GPC) antibodies can also be measured in the serum (sensitivity > 90% but specificity 50%). Moderate hypergastrinemia, and sometimes major hypergastrinemia, have also been associated with pernicious anemia, but this is not a pathognomonic finding. A positive Schilling test with the addition of a test for IFA virtually confirms the diagnosis (specificity > 99%)[31].

The optimal testing strategy remains unclear and considerable controversy still exists as to whether both indirect immunofluorescence testing for GPC and enzyme linked immunosorbent assay for IFA need to be performed as screening tests for pernicious anemia. It has been suggested that both autoantibodies do not need to be tested simultaneously, as the finding of IFA alone is very rare. GPC testing is therefore the most appropriate means of screening for pernicious anemia, with IFA testing as a more specific, but less sensitive test, being reserved for confirmatory testing[38].

Treatment

The classic treatment of cobalamin deficiency has been parenteral administration, usually by intramuscular injection, of the vitamin (in the form of cyanocobalamin and, more rarely, hydroxocobalamin or methylcobalamin). The recommended practice involves administration of 1000 μg of cyanocobalamin per day for 1 wk, followed by 1000 μg/wk for 1 mo, and then the dose is reduced to 1000 μg/mo, normally for the rest of the patient's life[31,33].

Alternative routes of cobalamin administration have been used: oral[32-35] and nasal[32,33]. These other routes of administration have been proposed as a way of avoiding the discomfort, inconvenience and cost of monthly injections. An evidence-based analysis supports the efficacy of oral cobalamin therapy[32,33,35,39]. Sublingual therapy (2000 μg/d for 7-12 d) is another treatment modality for cobalamin deficiency, applicable in patients who refuse parenteral treatment and present either diarrhea or vomiting, and/or are unable to take oral medication[34].

The procedure for oral cobalamin therapy has, however, not been completely validated yet in clinical practice, most notably the long-term efficacy. The current literature does not suggest a strategy in terms of the optimal form (hydroxy- or cyanocobalamin), frequency or duration of treatment. The therapeutic schema for use of oral cyanocobalamin would be[33]: (1) Intensification treatment: cyanocobalamin 1000 μg/d for 1 mo; (2) Maintenance treatment: cyanocobalamin 125-500 μg/d for intake deficiency and food-cobalamin malabsorption, and cyanocobalamin 1000 μg/d for pernicious anemia.

ANEMIA AND GASTRIC SURGERY

Gastrectomy, previously used for peptic ulcer and its complications, is the preferred operation for palliation of gastric cancer either as total or partial gastrectomy.

Anemia is a frequent complication of gastrectomy. There are many reports addressing iron, vitamin B12 or folate deficiencies either alone or in combination after gastric surgery. The most frequent is the combination of iron and vitamin B12 deficiency[40]. Impaired absorption of iron following gastrectomy is probably due to operative bypass of the duodenum and to rapid intestinal transit. Reduction in gastric acid (necessary for the absorption of food iron), a common consequence of subtotal gastrectomy, has also been incriminated. Vitamin B12 deficiency develops as a consequence of the decreased production of intrinsic factor which is essential for vitamin B12 absorption in the lower small bowel, and also because of a defect in the separation of vitamin B12 from its transporter protein. It is a frequent deficiency which will appear 2-4 years or even longer after gastrectomy, when the vitamin stores are exhausted. Thus, gastrectomized patients should be followed carefully to avoid iron and vitamin B12 deficiencies and anemia.

Over the last few decades, bariatric surgery has been suggested as an effective treatment for obesity. There are several different procedures, including gastric bypass, laparoscopic adjustable gastric banding, vertical banded gastroplasty, biliopancreatic diversion, and biliopancreatic diversion with duodenal switch[41]. All of these procedures may be associated with long-term sequelae including iron, vitamin B12 and folate deficiencies[41,42]. ID and anemia can have a strong impact on quality of life, especially in menstruating women who make up the majority of bariatric surgery patients. Most studies report ID, ranging from 6% to 50% within months to years of follow-up[43-45]. Vitamin B12 deficiency may appear 1-9 years after gastric bypass, and its prevalence has been estimated to be 12%-33%[42].

The main causes of ID after bariatric surgery are similar to those described after gastrectomy; diminished gastric acid secretion and exclusion of the duodenum. In gastric bypass, patients experience decreased gastric acid production in their proximal pouch and, in addition, the duodenum is excluded from digestive continuity. Thus, banding procedures which maintain digestive continuity with the antrum and duodenum are associated with low rates of ID and other nutritional deficiencies[41]. Conversely, the biliopancreatic diversion with duodenal switch, a gastric bypass procedure that may preserve some function of the proximal duodenum, may offer protection from ID, as compared with biliopancreatic diversion (which excludes the duodenum)[46,47].

Physicians should be aware that folate, vitamin B12, and iron deficiencies occur after gastric bypass, though the time to development is variable. In an attempt to prevent nutritional deficiencies, multivitamin preparations are in general prescribed to all patients. Systematic prescription of such supplements may prevent most nutritional deficits. However, vitamin B12 and iron deficits require specific supplementation. In spite of a multivitamin, ID still develops postoperatively in some patients. Adherence to oral iron supplements is often low because of digestive intolerance, and unresponsive IDA can be an important problem in these patients. Parenteral iron treatment is recommended in those patients refractory to oral iron supplementation. Intramuscular vitamin B12 supplementation is recommended only when a deficiency becomes clinically apparent.

ANEMIA AND *H PYLORI* INFECTION

The current evidence regarding the association between *H pylori* infection and either ID or IDA is mainly based on case reports[48-53], observational epidemiologic studies[54], and a very limited number of intervention trials[55-64]. The mechanisms of *H pylori*-related anemia have not been well defined and it is not known why only a small population develops IDA despite a significant worldwide *H pylori* infection rate[65]. Individuals with increased demands of iron needed for growth and tissue building such as children, pregnant, postpartum or premenopausal women, or those with chronic inflammatory disorders such as CD, are thought to be more likely to develop IDA associated with *H pylori* infection.

H pylori infection has been shown to be associated with ID in asymptomatic *H pylori*-infected subjects in several cross-sectional studies. However, a great variability was found across the studies and most of these have been conducted in countries with high prevalence of *H pylori* infection. Recently, Muhsen *et al*[54] performed two different meta-analyses of observational epidemiologic studies aimed at examining the association between *H pylori* infection and either ID or IDA. These analyses yielded a 2.8-fold increased risk for IDA (95% CI: 1.9-4.2), and an 1.38-fold increased risk for ID (95% CI: 1.16-1.65) in *H pylori*-infected subjects as compared with non-infected subjects.

In clinical and interventional trials, the participants were mostly children and adolescents, and only three trials were conducted among ill people (those presenting with symptoms for investigation in clinical settings)[58,59,62]. Small sample sizes[56-59,62], lack of control groups, and other methodological issues, including the use of validated tests to confirm active *H pylori* infection, are among factors that limit the interpretation and ability to generalize the relevance of the results of these studies[54]. Several studies reported resolution of IDA with eradication of *H pylori* infection, regardless of the absence of iron supplementation. The study of Kurekci *et al*[59], in which all participants received *H pylori* eradication therapy without a control group, emphasized that resolution of both ID and IDA associated with *H pylori* infection may be achieved by *H pylori* eradication treatment alone. The antagonistic effect of asymptomatic *H pylori* infection on the response to iron supplementation was investigated in India among participants of a randomized, controlled trial of iron supplementation (n = 169, age 1-10 years). It was found that asymptomatic *H pylori* infection was not associated with higher rates of anemia or ID, but had a significant adverse effect on response to iron supplementation among children[53].

Sarker *et al*[61] completed a population-based,

randomized, double-blind, and placebo-controlled trial to evaluate the response of iron *plus* anti-*H pylori* therapy in children with IDA (n = 200). This trial was performed in Bangladesh, an area highly endemic for ID and *H pylori* infection. Results failed to observe any additional impact of combined anti-*H pylori plus* iron therapy over the iron therapy alone. These findings support those obtained by an Alaskan trial[60], in another highly prevalent *H pylori* infection area, where a large therapeutic, randomized, controlled, unblinded trial in children (n = 219) was performed. There were no significant differences between the intervention and the control groups in the rates of ID and anemia, during 14 mo follow-up period. The authors hypothesized that 14 mo was too early to resolve *H pylori*-induced gastric damage. Consequently, in a follow-up study performed at 40 mo, 176 children were reevaluated. Re-infection occurred among 52% of children who had initially cleared their infection. However, *H pylori*-negative children had lower prevalence of ID (RR: 0.62; 95% CI: 0.38-1.01) and IDA (RR: 0.22; 95% CI: 0.03-1.50), compared with *H pylori*-positive children. It was concluded that the resolution of *H pylori* infection for > 14 mo modestly reduced the prevalence of ID and substantially reduced the prevalence of ID and IDA[64].

In the light of the above mentioned studies, *H pylori* infection may be considered as a risk factor for IDA, mainly in groups with high demands for iron, such as some children and adolescents. However, the relationship between *H pylori* and ID may be stronger than that described, since most of the above mentioned studies have been performed in geographical areas where both ID and *H pylori* infection are highly prevalent, and where the etiology of ID is possibly multifactorial (malnutrition, vitamin deficits, chronic parasitic infections, malaria)[66]. In this setting, poor response to *H pylori* eradication should be viewed with caution. Thus, further large and well-controlled trials will be of value. Both the impact of anti-*H pylori* therapy on the improvement of iron stores and the role the infection plays in interfering with iron supplementation in patients with IDA require further evaluation before making solid recommendations.

The biologic mechanism

The relationship between refractory IDA and *H pylori* infection may be explained by several hypotheses. One of the possible explanations is the gastrointestinal blood loss that may range from chronic occult bleeding from discrete gastric erosions[67] to massive bleeding from peptic ulcers[68] and gastric carcinomas. However, most patients with *H pylori*-associated IDA have no evidence of gastrointestinal bleeding. Data obtained through case series and case reports mostly described patients with IDA after a comprehensive investigation including laboratory tests, imaging, and endoscopic studies, and *H pylori* gastritis was the only pathologic finding[55,61-63].

Intragastric acidic pH plays an important role in the reduction of the ferric to the ferrous form. This reaction is enhanced by ascorbic acid. Gastric juice and mucosal ascorbic acid concentrations were significantly lowered in *H pylori*-infected subjects as compared to those non-infected; the lower concentrations were associated with more severe gastritis[69]. This negative influence of *H pylori* on gastric ascorbic acid was reversed after eradication of the infection[70].

The results of some studies support the hypothesis that the infection by *H pylori* influences iron absorption directly, and that iron absorption improves significantly after clearance of the infection[71,72]. It has been suggested that there is a competition for iron between the bacteria and the host. Iron is an essential nutrient for bacterial growth. Therefore, an efficient iron uptake system is an important factor for the maintenance of virulence[73]. Many pathogens use the siderophore-mediated iron acquisition system that removes iron from lactoferrin or transferrin. The regulation of iron uptake systems in *H pylori* are different from other bacteria since they are constitutively expressed, most probably as a part of the germ adaptation to the conditions in the human stomach, where iron starvation and iron overload can be encountered frequently[74]. The siderophore of *H pylori* has not yet been identified. Several studies support this hypothesis[75-78].

Bacterial genetic factors related to *H pylori*-associated IDA pathogenesis have been studied. Non-IDA and IDA strains can be distinguished by their protein expression profiles, suggesting that polymorphisms in *H pylori* strains may be one of the factors determining the occurrence of *H pylori*-associated IDA[79]. A mutation in the *H pylori pfr* gene causing overproduction of *H pylori* ferritin protein (Pfr) has been proposed as playing a role in the imbalance of body iron[80]. However, an analysis of the complete coding region of the *pfr* gene revealed three sites of mutation with no differences in the mutation among *H pylori*-positive patients with or without IDA[80]. On the other hand, three heme-binding iron-repressible outer membrane proteins that may be involved in the uptake of heme from the host by *H pylori* were described in the presence of poor iron environment[81]. Recently, *feoB* gene product, which was regarded as a high-affinity ferrous iron transporter, was proposed as a possible pathway related to the bacterium itself; however, its relation to IDA remains unclear[82]. Although *H pylori* CagA strains were proposed to be involved in alteration of the host's iron stores in some studies, more recent work does not support this hypothesis[72,83,84]. However, seropositivity to *H pylori* CagA antibodies was inversely associated with gastric ascorbic acid concentrations[84].

CONCLUSION

CD should be considered as a possible cause of anemia in all subjects with IDA of unknown origin, even in menstruating women. Duodenal biopsies should be taken during endoscopy if no obvious case of IDA can be found. Vitamin B12 or cobalamin deficiency occurs frequently among elderly patients, but it is often unrecognized or not investigated because the clinical manifestations are subtle. In this age group, cobalamin

deficiency is caused primarily by food-cobalamin malabsorption (60%-70% of cases) and pernicious anemia (15%-20% of cases). The classic treatment of cobalamin deficiency has been parenteral administration of the vitamin. However, recent data support the efficacy of oral cobalamin therapy. Folate, vitamin B12, and iron deficiencies occur after gastric bypass, with an incidence ranging from 6% to 50% within months to years of follow-up. *H pylori* infection may be considered as a risk factor for IDA, mainly in groups with high demands for iron, such as some children and adolescents. However, further large and well-controlled trials will be required before making solid recommendations about *H pylori* eradication in these cases.

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, *Series Editors*

Spectrum of anemia associated with chronic liver disease

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Abstract

Anemia of diverse etiology is a common complication of chronic liver diseases. The causes of anemia include acute or chronic gastrointestinal hemorrhage, and hypersplenism secondary to portal hypertension. Severe hepatocellular disease predisposes to hemorrhage because of impaired blood coagulation caused by deficiency of blood coagulation factors synthesized by hepatocytes, and/or thrombocytopenia. Aplastic anemia, which is characterized by pancytopenia and hypocellular bone marrow, may follow the development of hepatitis. Its presentation includes progressive anemia and hemorrhagic manifestations. Hematological complications of combination therapy for chronic viral hepatitis include clinically significant anemia, secondary to treatment with ribavirin and/or interferon. Ribavirin-induced hemolysis can be reversed by reducing the dose of the drug or discontinuing it altogether. Interferons may contribute to anemia by inducing bone marrow suppression. Alcohol ingestion is implicated in the pathogenesis of chronic liver disease and may contribute to associated anemia. In patients with chronic liver disease, anemia may be exacerbated by deficiency of folic acid and/or vitamin B12 that can occur secondary to inadequate dietary intake or malabsorption.

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Key words: Anemia; Liver disease; Liver failure; Aplastic anemia; Pegylated interferon; Ribavirin; Alcohol

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INTRODUCTION

Chronic liver diseases frequently are associated with hematological abnormalities. Anemia of diverse etiology occurs in about 75% of patients with chronic liver disease[1].

A major cause of anemia associated with chronic liver disease is hemorrhage, especially into the gastrointestinal tract. Patients with severe hepatocellular disease develop defects of blood coagulation as a consequence of endothelial dysfunction, thrombocytopenia, deficiencies of coagulation factors and various associated disorders[2].

In severe hepatocellular disease, decreased synthesis of liver-produced plasma proteins leads to reduced serum levels of several blood clotting factors. Hemorrhage may occur as a complication of chronic liver disease because of a lack of one or more liver-produced blood clotting factors, thrombocytopenia, and/or defective platelet function. Hemorrhage in such patients may also occur from esophageal or gastric varices secondary to portal hypertension. The biosynthetic pathways of blood coagulation factors Ⅱ, Ⅶ, Ⅸ and Ⅹ are within the hepatocyte and are dependent on vitamin K[3]. Low serum levels of these factors are associated with prolongation of the prothrombin time (PT). When attributable to hepatocellular disease, they are not improved by administration of vitamin K; correction of the associated impaired blood coagulation necessitates infusion of preparations of the deficient factors.

Splenomegaly, which is usually caused by portal hypertension in patients with chronic liver disease, may lead to secondary hemolysis, an increase in plasma volume, macrocytosis and megaloblastic anemia. Alcohol, a common etiologic factor of chronic liver disease, is toxic to the bone marrow. Alcoholics often develop secondary malnutrition, a manifestation of

which may be anemia caused by folic acid deficiency. In some patients, bone marrow failure and aplastic anemia develop after an episode of hepatitis. Finally, anemia is a recognized complication of treatment of chronic hepatitis C with a combination of interferon and ribavirin: anemia in this context is predominantly caused by ribavirin-induced hemolysis[4].

The frequent association of anemia with chronic liver disease and/or hepatocellular failure provides a rationale for examining the role of the liver in the formation and destruction of erythrocytes. Indeed, the liver itself may be implicated in a variety of different mechanisms that contribute to the development of anemia in patients with chronic liver disease. This paper provides an overview of anemia that may complicate chronic liver diseases and the mechanisms responsible.

The frequent association of anemia with chronic liver disease and/or hepatocellular failure provides a rationale for examining the role of the liver in the formation and destruction of red blood cells. Indeed, a variety of different mechanisms may be implicated in the development of anemia in patients with liver disease.

PORTAL HYPERTENSION AND ANEMIA

Acute gastrointestinal hemorrhage is a common and potentially serious complication of portal hypertension[5-8]. It is usually caused by rupture of an esophageal varix. Hemorrhage caused by this mechanism is the second most common cause of mortality in patients with cirrhosis. In such patients, a ruptured esophageal varix is the cause of approximately 70% of episodes of upper gastrointestinal hemorrhage[6]. Acute hemorrhage may induce severe hypovolemia and subsequently secondary iron deficiency anemia. The initial aim of treatment is correction of hypovolemia and restoration of stable hemodynamic function; minimal values for mean arterial pressure and for hemoglobin of 80 mmHg and 8 g/100 mL, respectively, should be maintained. Initially, gelatin-based colloids or solutions of human albumin may be infused to correct hypovolemia. However, infusions of packed erythrocytes in plasma are ideal in this context since such infusions have the potential of correcting, not only hypovolemia, but also secondary anemia. First-line management involves institution of both medical and endoscopic treatments (Table 1)[6]. Medical therapy includes administration of vasoactive drugs, such as somatostatin, octreotide or terlipressin. Optimal endoscopic treatment involves ligation of esophageal varices and obturation of gastric varices with tissue adhesives.

In some patients with cirrhosis, chronic hemorrhage into the gastrointestinal tract occurs. Esophageal and gastric varices and/or portal hypertensive gastropathy may be associated with slow chronic loss of blood into the gut and development of chronic iron deficiency anemia. The most important approach to management is prevention of variceal hemorrhage[5,7,8]. The annual incidence of initial variceal hemorrhage in patients with cirrhosis is estimated to be about 4%, but for the group with medium-sized or large varices, the incidence is about 15%[6]. β-blockers or isosorbide 5-mononitrate may reduce the rate of transformation of small varices into large varices and decrease the incidence of variceal hemorrhage in patients with small varices[5]. In patients who survive a first episode of variceal hemorrhage, the risk of recurrent hemorrhage is > 60%. Accordingly, all patients surviving variceal hemorrhage should receive active treatment aimed at preventing recurrence. Non-selective β-blockers or isosorbide 5-mononitrate and endoscopic therapy, including ligation of and/or sclerotherapy of varices, are the first-line treatments for preventing recurrence of variceal hemorrhage[5,8]; a combination of both these approaches constitutes optimal management. Additional treatment with oral iron supplementation is indicated for iron deficiency anemia caused by chronic blood loss. In some cases of advanced chronic liver disease, intravenous iron formulations may be administrated to increase plasma levels and tissue deposits of iron.

Table 1 Management of acute gastrointestinal bleeding

Treatment of variceal bleeding
Pharmacological therapy
Somatostatin: initial bolus (24-48 h) and a perfusion (5 d)
Octreotide: initial bolus (24-48 h) and a perfusion (5 d)
Terlipressin: important side effects
Primary prophylaxis
β-blockers non-selective: propranolol, nadolol
Isosorbide 5-mononitrate
Secondary prophylaxis
β-blockers non-selective: propranolol, nadolol
Isosorbide 5-mononitrate
Endoscopy therapy: band ligation, sclerotherapy

Hypersplenism secondary to portal hypertension is another mechanism of anemia in patients with chronic liver disease. Hypersplenism is associated with splenomegaly. In addition to chronic liver disease, thrombosis of the splenic vein may also be a cause of an increase in pressure within the portal venous system, which can lead to secondary hypersplenism. The main characteristics of hypersplenism are those attributable to pancytopenia. Hemolytic anemia occurs because of intrasplenic destruction of erythrocytes. Destruction of megakaryocytes and leukocyte precursors results in thrombocytopenia and leukopenia[9]. Symptoms and signs of hypersplenism are influenced by the primary underlying disease; they include abdominal pain and/or discomfort, and, in advanced cases, gastrointestinal hemorrhage secondary to portal hypertension. There may be hyperplasia of the progenitor cells in the bone marrow. It is important to determine the cause of hypersplenism. The main therapeutic approach for this syndrome is management directed at the underlying primary disease, usually chronic liver disease. When chronic liver disease is advanced, additional therapeutic options may need to be adopted. After assessing the severity of impaired hepatocellular function in a patient with advanced chronic liver disease, splenectomy may

be considered if the splenic vein is thrombosed. An alternative approach is partial or total embolization of the splenic artery, which, in some recent studies, has been associated with good results, in particular, lower morbidity and mortality rates than those associated with surgery. Partial embolization preserves the immunological function of the spleen and is the preferred option for patients with cirrhosis[10].

IMPAIRED BLOOD COAGULATION

The liver plays a central role in blood coagulation. Acute and chronic hepatocellular diseases are usually associated with defective blood coagulation due to a variety of different causes. These include: decreased hepatic synthesis of factors Ⅱ, Ⅶ, Ⅸ and Ⅹ; the presence of inhibitors of these factors; decreased clearance of activated coagulation factors; thrombocytopenia; impaired platelet function; hyperfibrinolysis; and disseminated intravascular coagulation[11,12]. Coagulation defects complicating liver disease predispose to an increased bleeding tendency, which increases both morbidity and mortality [11-13].

Defective blood coagulation associated with hepatocellular disease may be monitored using global screening tests, such as the PT and the activated partial thromboplastin time. In mild hepatocellular disease, PT usually is within the normal range or only modestly prolonged. In more advanced hepatocellular disease, prolongation of PT tends to reflect the severity of hepatocellular failure. Vitamin K routinely is administered parenterally (usually only once) to patients with liver disease and a prolonged PT, to exclude vitamin K deficiency as a cause of the prolonged PT[11].

Thrombocytopenia (platelet count < 150 000/L) is common in patients with chronic liver disease; it has been reported in as many as 76% of patients with cirrhosis[4,12]. The pathogenesis of the thrombocytopenia is complex; it includes splenic pooling, and increased destruction and impaired production of platelets (Figure 1). Impaired production of platelets is caused, at least in part, by low levels of thrombopoietin. Prolonged bleeding time, and impaired aggregation, reduced adhesiveness and abnormal ultrastructure of platelets reflect abnormal platelet function; these abnormalities have been attributed to an intrinsic platelet defect. Specific treatments to attempt to reverse the effects of this defect are not usually given, but platelet transfusions or platelet-stimulating agents have been administered in some cases.

An important coagulation defect associated with chronic liver disease is low levels of factor Ⅶa. In recent years, the hemostatic agent recombinant factor Ⅶa has become available as a potentially new therapeutic agent for use in the management of coagulopathy in patients with cirrhosis. This agent may enhance initial control of acute variceal bleeding[14]. However, such therapy is associated with significant side effects, such as vascular injury and thrombosis.

Hyperfibrinolysis is another cause of impaired hemostasis in patients with liver disease. In a non-randomized trial[15], antifibrinolytic amino acids were administered to patients with acute or chronic liver disease, who had upper gastrointestinal bleeding and acquired defects of blood coagulation. However, administration of such amino acids does not have an established place in therapy.

Thrombotic events, although rare in patients with cirrhosis, may occur. They tend to involve particularly the portal and/or mesenteric veins.

A rational approach to managing disorders of blood coagulation in patients with liver disease is important because of the high risk of associated secondary hemorrhage.

APLASTIC ANEMIA

Aplastic anemia associated with liver disease is characterized by development of pancytopenia and hypocellular bone marrow in relation to the occurrence of hepatitis[16]. The main feature of this syndrome is injury to or loss of pluripotent hematopoietic stem cells, in the absence of infiltrative disease of the bone marrow[16-19].

Hepatitis-associated aplastic anemia (HAA) has been defined as a variant of aplastic anemia, which occurs concurrently with or within 6 mo of an increase in the serum level of alanine aminotransferase to at least five times the upper limit of the reference range. Severe marrow aplasia may be induced by hepatitis viruses, such as hepatitis B virus and hepatitis C virus (HCV), and also by other viruses, such as human immunodeficiency virus, Epstein-Barr virus, transfusion-transmitted virus and echovirus[16,20]. Parvovirus B19 commonly infects pro-erythroblasts and may induce transient red-cell aplasia, particularly in patients with chronic hemolytic anemia. It has been postulated that viruses and/or antigens, through the mediation of γ interferon or the cytokine cascade, induce lymphocyte activation and ultimately apoptotic death of hematopoietic cells in the bone marrow[17].

Clinical presentation includes symptoms and signs related to pancytopenia, such as pallor, fatigue, hemorrhagic manifestations, progressive anemia, and bacterial infections. The diagnosis of HAA is suggested by a complete blood count, which reveals pancytopenia (including anemia) together with absolute reticulocytopenia[16]. A bone marrow biopsy typically reveals hypocellularity that affects red and white cell precursors and megakaryocytes; residual hematopoietic cells appear morphologically normal[19].

The two major options for treating severe HAA are hematopoietic cell transplantation and immunosuppressive therapy. According to recent reviews, response rates to these approaches are 75%-88% and 75%-80%, respectively[16,18]. Blood and platelet infusions are often necessary before instituting specific treatment; before administration blood products should be irradiated to avoid sensitization.

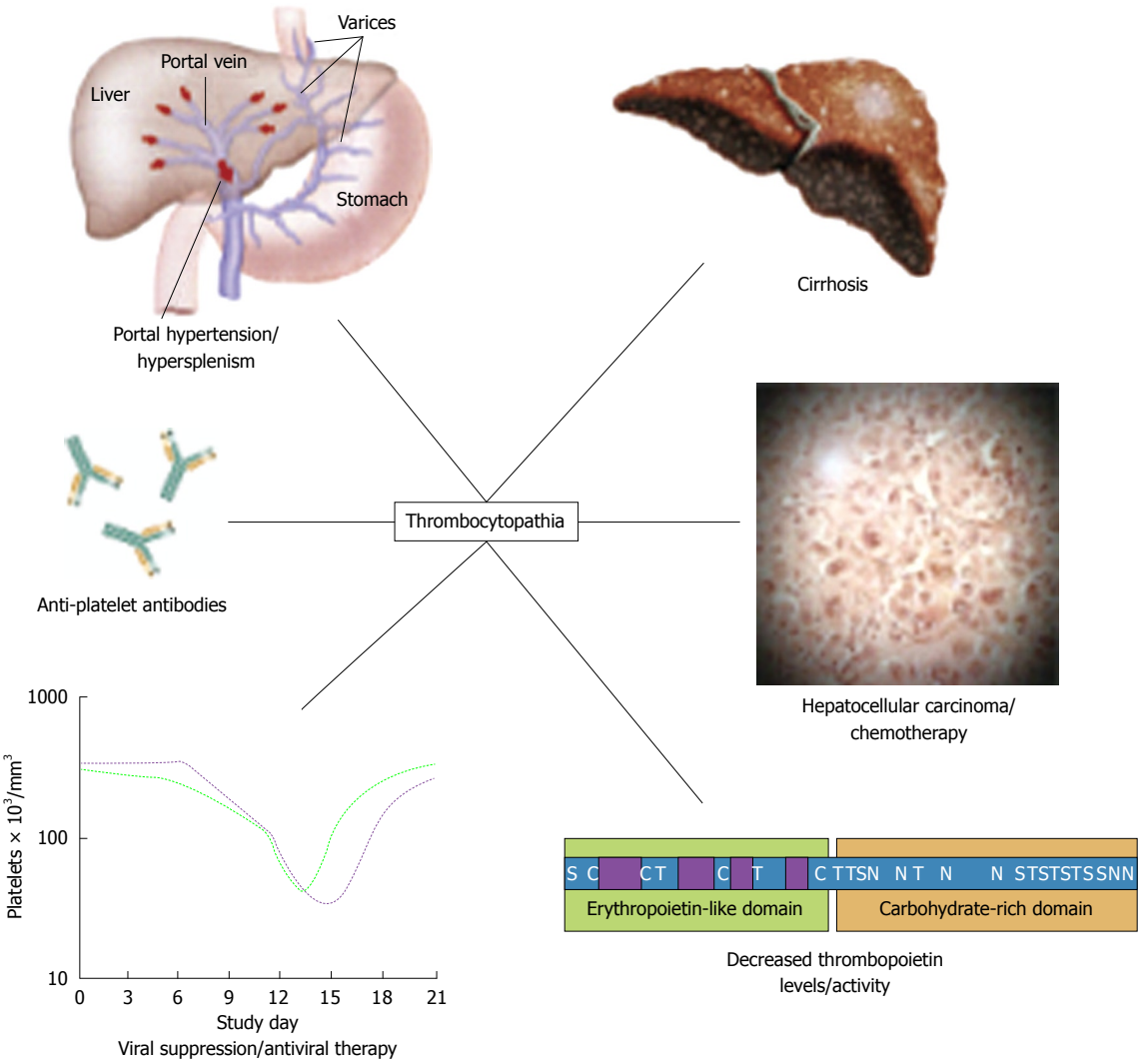

Figure 1 Development of thrombocytopenia in patients with chronic liver disease (adapted from Afdhal *et al*[12]).

ANEMIA SECONDARY TO TREATMENT OF HEPATITIS

Currently, optimal treatment for chronic infection with HCV infection is a combination of therapy with pegylated interferon and ribavirin. Of hematological abnormalities that may be associated with such combination therapy, the most common is anemia[21]. Significant anemia (hemoglobin < 10 g/dL) has been observed in 9%-13% of patients receiving interferon and ribavirin; moderate anemia (hemoglobin < 11 g/dL) occurs in about 30% of patients undergoing such treatment[20-22]. There are several mechanisms by which anemia may occur during combination therapy for HCV infection, and ribavirin and/or interferons may contribute to anemia. In this context, hemoglobin concentrations decrease mainly as a result of ribavirin-induced hemolysis[20].

Anemia caused to ribavirin leads to modifications of the dose in up to 25% of patients, and this type of anemia may be problematic in patients with HCV infection, especially those who also have renal or cardiovascular disorders. Adherence to ribavirin therapy is one factor that is critically important in the treatment of HCV infection. Although ribavirin-associated anemia can be reversed by reducing the dose of ribavirin or by discontinuing the drug altogether, this approach compromises outcomes by significantly decreasing rates of sustained virological response. A recent study reviewed the predictors of anemia in patients undergoing treatment for HCV infection[21]. Patients with impaired renal function may be at an increased risk of ribavirin-related anemia and, accordingly, should be monitored carefully. Furthermore, a decrease in hemoglobin concentration of ⩾ 1.5 g/dL by week 2 of treatment has been found to be an excellent early predictor of subsequent substantial decreases in hemoglobin. This predictor might be applied to identify candidates for early intervention for management of anemia to facilitate maintenance of the dose of ribavirin. One of the specific approaches to manage ribavirin-associated anemia is administration of recombinant human erythropoietin[21]. After 16 wk of ribavirin therapy, patients who had also been given erythropoietin alfa had significantly higher mean hemoglobin levels than patients in a control group. In patients with chronic hepatitis C, viramidine, a prodrug of ribavirin that is

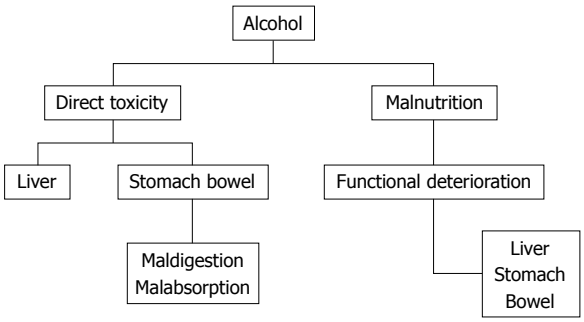

Figure 2 Scheme, in patients with alcoholic liver disease, depicting how different effects of alcohol may contribute to anemia (adapted from Moreno Otero *et al*[23]).

selectively taken up by the liver, has the potential of maintaining the antiviral efficacy of ribavirin, while decreasing the risk of hemolytic anemia[4].

Interferons may also contribute to anemia. Their main relevant action is induction of bone marrow suppression. This effect of interferon results in suppression of compensatory reticulocytosis associated with ribavirin-induced hemolytic anemia. Thus, the bone-marrow-suppressive effect of interferon may contribute to anemia, which complicates therapy with combination of interferon and ribavirin[4].

ALCOHOL, LIVER DISEASE AND ANEMIA

Alcohol is implicated in the pathogenesis of chronic liver disease; it may contribute to anemia secondary to its direct effects on the liver and also to other diverse mechanisms (Figure 2)[23].

Markers of iron overload tend to be higher among those who consume more than two alcoholic drinks per day than among non-drinkers, after adjusting for potential confounding factors[24]. Consumption of alcohol appears to be associated with an approximately 40% reduction in the risk of development of iron deficiency anemia.

Folic acid and vitamin B12 deficiencies develop frequently in patients with cirrhosis. These deficiencies may be related to inadequate food intake or intestinal malabsorption. They are suspected when examination of a blood film reveals hypersegmented cells and oval macrocytes, in addition to round macrocytes characteristic of chronic liver disease. When anemia is caused by these deficiencies, the mean corpuscular volume is increased and bone marrow shows megaloblastic erythropoiesis.

Anemia due to folic acid deficiency may result, not only from a lack of folic acid in the diet, but also the weak antifolate action of ethanol. Folic acid deficiency is the most common cause of a low hematocrit in hospitalized patients who are alcoholics[25,26]. Parenterally administered vitamin B12 not only corrects anemia caused by vitamin B12 deficiency, but may also induce improvement in the peripheral neuropathy that are associated with this deficiency[23]. Supplements of vitamins A, B and C may be administered empirically to patients with advanced alcoholic disease.

Table 2 Etiologic factors that may contribute to anemia associated with alcoholism (adapted from Lewis *et al*[26])

Cause of low hematocrit	Possible contributing factors
Hemorrhage and/or iron deficiency	Alcoholic gastritis
	Portal hypertension
	Peptic ulceration
Hemolysis	Chronic liver disease and/or cirrhosis
	Zieve syndrome
	Spur cell anemia of severe liver disease
Reduced erythropoiesis	Anemia of chronic disease
	Nutritional (e.g. folic acid deficiency)
	Sideroblastic anemia
	Alcohol toxicity
Hypersplenism	Portal hypertension
Hemodilution	Fluid retention of chronic liver disease
	Aggressive intravenous fluid therapy

Source: *Nat Clin Pract Gastroenterol Hepatol* © 2007 Nature Publishing Group.

Anemia in an alcoholic may also arise as a consequence of the direct toxic effects of alcohol on erythrocyte precursors in the bone marrow. Management of alcohol-induced suppression of erythropoiesis includes abstinence from alcohol and a nutritious diet with appropriate supplements.

Other factors that may contribute to anemia and a low hematocrit in alcoholic patients are given in Table 2.

CONCLUSION

Liver diseases are frequently associated with hematological abnormalities. Anemia of diverse etiology occurs in many of these patients. Bleeding is one of the most severe causes of anemia, with a high mortality, and defective blood coagulation contributes to the anemia. Other mechanisms of anemia include aplastic anemia secondary to previous hepatitis, or side effects of treatment of hepatitis with interferon and ribavirin. In patients with alcoholic liver disease, different effects of alcohol may contribute to anemia, such as malabsorption, malnutrition or direct toxic effect. The pathogenesis of the anemia in each case is different and it is important to begin the correct therapy.

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TOPIC HIGHLIGHT

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Anemia and inflammatory bowel diseases

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Abstract

Too often anemia is considered a rare or unimportant manifestation in inflammatory bowel disease (IBD). However, over the last 10 years a number of studies have been conducted and the most relevant conclusions obtained are: (1) anemia is quite common in IBD; (2) although in many cases anemia parallels the clinical activity of the disease, many patients in remission have anemia, and iron, vitamin B12 and/or folic acid deficiency; (3) anemia, and also iron deficiency without anemia, have important consequences in the clinical status and quality of life of the patient; (4) oral iron can lead to gastrointestinal intolerance and failure of treatment; (5) intravenous iron is an effective and safe way to treat iron deficiency; (6) erythropoietin is needed in a significant number of cases to achieve normal hemoglobin levels. Thus, the clinician caring for IBD patients should have a comprehensive knowledge of anemia, and apply recently published guidelines in clinical practice.

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Key words: Anemia; Inflammatory bowel diseases; Ferritin; Iron

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INTRODUCTION

In addition to the intestinal signs and symptoms (such as abdominal pain or diarrhea), inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are associated with a number of extraintestinal manifestations (at joints, skin, eyes, *etc*). Furthermore, systemic manifestations of IBD may also include malnutrition and anemia[1,2].

Compared with the average awareness of other extraintestinal disease complications such as arthritis or osteopathy, the topic of anemia in IBD has received little attention: perhaps it is so common that sometimes it is considered an unavoidable manifestation of the disease[2,3]. Although efficient therapeutic options have been developed for the treatment of IBD-associated anemia, treating anemia often has had a low priority for gastroenterologists[4]. However, anemia is a clinically relevant condition which may affect quality of life or the ability to work[5,6], and it is a comorbid condition which is associated with other diseases (such as transfusion-associated hepatitis C) or even death[1,7].

Anemia is a disease and should be approached as such. Anemia in IBD is not just a laboratory marker, it is a condition which needs a specific diagnostic and therapeutic approach. Moreover, it is a complex condition, because there are many factors which can cause anemia in IBD patients.

The aim of the present manuscript is to review the main clinically relevant aspects of the diagnosis and management of anemia in the patient with IBD.

PREVALENCE OF ANEMIA IN IBD

Often anemia is not even mentioned, perhaps because some authors think it is a rather uncommon problem in IBD[2]. On the contrary, anemia is very common in IBD patients, although the reported prevalence of this condition has been markedly variable, depending both on the definition and on the patient population considered (hospitalized patients *vs* outpatients)[1,8]. In a systematic review published in 2004, the prevalence of anemia in

patients with IBD ranged from 9% to 74%[8]. In a more recent systematic review[1], which included 19 studies (mainly on Crohn's disease) the figures ranged from 6% to 74%. Even more recently, we reviewed published studies evaluating the prevalence of anemia in IBD[9-30], and calculated a mean prevalence of 17%[2]. However, as the prevalence was 16% in outpatients, this figure increased up to 68% when only hospitalized patients were included. Therefore, it may be concluded that anemia could be the most common systemic complication of acute IBD[2].

PREVALENCE OF IRON DEFICIENCY IN IBD

It is also common to consider that iron deficiency is an exceptional finding in IBD[2]. On the contrary, this condition is even more common than anemia, but to demonstrate it requires active investigation. In fact, iron deficiency is the main cause of anemia in IBD patients, as a consequence of dietary restrictions, malabsorption (in part as a result of inflammation), intestinal bleeding, and/or undertreatment of anemia (achieving normal hemoglobin values does not mean normal iron stores). In a recent systematic review[1], the prevalence of iron deficiency ranged from 36% to 90% (depending on the definition of iron deficiency and on the type of cohort included)[16,21,26,31-33]. In the most recent systematic review[2], mean prevalence of iron deficiency in IBD was 45%, which underlines the fact that this condition may be considered the rule rather than the exception in these patients, especially in severe cases.

THE MULTIFACTORIAL ORIGIN OF ANEMIA IN IBD

Anemia in patients with IBD results primarily from iron deficiency because of chronic intestinal blood loss from inflamed mucosa, although in active disease more complex mechanisms involving absorption are also important[34]. However, the anemia in IBD is likely to be multifactorial in origin, frequently being the result of a combination of iron deficiency (the first cause) and anemia of chronic disease (the second major cause)[35]. In some cases, anemia may also be induced by drugs (sulfasalazine, thiopurines), hemolysis, and myelodysplastic syndrome. Finally, in some patients with Crohn's disease, impaired absorption of vitamin B12 and/or folate because of small intestinal inflammation and/or extensive bowel resection, may contribute to anemia[35], and all these conditions frequently overlap. Therefore, anemia in IBD is often complex and commonly represents a particular example of the combination of, at least, iron deficiency anemia and anemia of chronic disease, and may be a challenge even to the most astute clinician[4,24].

IRON METABOLISM PARAMETERS FOR THE DIAGNOSIS OF IRON DEFICIENCY IN IBD

The diagnosis of iron deficiency is traditionally based on a combination of parameters, including hematological and iron metabolism indices[36]. Pure iron deficiency is recognized by low iron, ferritin and transferrin saturation but increased transferrin concentrations. However, diagnosing iron deficiency in the setting of IBD may be difficult, particularly when both iron deficiency and the anemia of chronic disease are present (as previously mentioned, both conditions frequently coexist). In these circumstances, many of the laboratory measures of iron status may be unreliable, as inflammation influences parameters of iron metabolism[24,37]. For example, in the presence of chronic inflammation, the elevation in transferrin levels typical of iron deficiency may not be found, as patients with low albumin tend also to have low transferrin concentrations[38]. Similarly, iron and total iron binding capacity levels are often difficult to interpret in the presence of inflammation[35]. Finally, serum ferritin, the most accessible and well known measure of stored iron and the most powerful tests for iron deficiency[39], can be normal or even increased - in response to inflammation, as it is an acute phase reactant - even in the presence of severe iron deficiency[24]. Therefore, although at present ferritin is generally considered as the most efficient indicator of iron deficiency, this parameter may not provide adequate information about the storage compartment in the setting of inflammatory conditions such as IBD[24]. Testing for increased soluble transferrin receptor concentration distinguishes reliably between iron deficiency and anemia of chronic disease, but it is not yet widely available[40,41].

Accordingly, it has been suggested that diagnostic criteria for iron deficiency need to be adapted to the level of inflammation. Thus, in patients without biochemical (C-reactive protein, *etc*) or clinical (diarrhea, endoscopic findings, *etc*) evidence of inflammation, the cut-off point for defining a low level of serum ferritin is < 30 μg/L; however, in the presence of inflammation, the lower limit of this parameter consistent with normal iron stores should be increased up to 100 μg/L[37,42]. Some authors do suggest considering the presence of ferropenia if there are low iron values and < 16% transferrin saturation[43].

DRUG-RELATED ANEMIA IN IBD

Some drugs commonly used in the treatment of IBD can have myelosuppressive effects, both indirect (for instance the "antifolic" effect of salazopyrine) or even direct (such as azathioprine or mercaptopurine)[44]. In particular, sulfasalazine affects erythropoiesis by several mechanisms including folate absorption, hemolysis and aplasia[45]. Isolated anemia in patients on azathioprine or mercaptopurine is unlikely to be caused by these drugs; in some cases, however, a mild and asymptomatic reduction in hemoglobin may be detected in patients treated with thiopurine drugs.

THE IMPACT OF ANEMIA ON THE QUALITY OF LIFE OF IBD PATIENTS

The repercussion of anemia on quality of life in

both general patients[46,47] and specifically in patients with IBD[3,4,6,8] is substantial. Moreover, anemia may impair quality of life even in the absence of specific symptoms[5,6]. As has accurately been noted by Gasche *et al*[3,4], for a long time it was thought that the clinical symptoms of anemia (such as fatigue, headache, dizziness, shortness of breath, or tachycardia) occurred only when the hemoglobin level dropped abruptly. It had been argued that patients would adapt to low hemoglobin levels if anemia developed slowly. This has led to the concept of "asymptomatic" anemia. In truth, the term "asymptomatic" seems to reflect the fact that impairments in physical condition, quality of life, and cognitive function may be unrecognized by both patients and their doctors. Therefore, the process of adaptation to chronic anemia would, in fact, be adaptation to a lower quality of life[3,4]. These concepts have been thoroughly developed in other pathologies, especially in patients on dialysis: intravenous iron can be a key point in management of these patients[48].

Remarkably, the quality of life in IBD patients may be as low as in anemic patients with advanced cancer[49]. Moreover, chronic fatigue caused by anemia can debilitate, affect and worry these patients as much as abdominal pain or diarrhea[4]. Therefore the beneficial impact on quality of life derived from anemia correction in IBD patients can be similar to the control of diarrhea[4,6,50].

THE ROLE OF TREATMENT OF THE UNDERLYING DISEASE (IBD) ON THE CORRECTION OF ANEMIA

A general correlation exists between disease activity and the depth of the anemia[38]. Active disease can cause anemia because of multiple factors, the most recently demonstrated being anemia of chronic disease and impairment of iron absorption in active Crohn's disease. Therefore, the most important measure for IBD anemia treatment is the treatment of the underlying disease[1,24]. Although apparently obvious, sometimes this step is missed in actual clinical practice. Moreover, the long term effect to alleviate anemia depends on whether the bowel inflammation itself can be adequately treated. Every effort to accomplish this has to be undertaken in order to preclude recurrent anemia[37,51].

WHEN TO START IRON SUPPLEMENTATION IN IBD ANEMIC PATIENTS?

There may be a tendency to look upon anemia as an unavoidable accompaniment to IBD[38]. Only in recent years has correction of anemia been highlighted as a specific therapeutic aim in these patients[38]. It should not be assumed that some level of anemia is a normal finding in IBD patients and consequently need not be treated[3]. On the contrary, iron supplementation should be started as soon as anemia (hemoglobin < 13 g/dL in males, and < 12 g/dL in females[52]) is detected. Thus, the World Health Organization definitions of anemia apply to patients with IBD. In fact, it is possible that patients without anemia but with iron deficiency should be considered for treatment because even without anemia, iron deficiency can have clinical relevance. In summary, anemia in IBD patients should be aggressively diagnosed, investigated, and treated[2].

WHEN TO STOP IRON SUPPLEMENTATION IN IBD ANEMIC PATIENTS?

Apart from the correction of hemoglobin levels, the primary therapeutic goal is to improve quality of life. Therefore, the therapeutic objective of the treatment with oral iron should be to completely correct both the anemia and iron deficiency, and not only to partially increase the hemoglobin levels. Thus, our final aim should be to achieve the previously mentioned normal values (hemoglobin > 13 g/dL in males, and > 12 g/dL in females), in accord with that recommended in patients without IBD[3,4,53]. In fact, it is important to remember that the highest improvement in the quality of life is observed precisely when the hemoglobin levels increase from 11 to 13 g/dL[4,54]. Moreover, all patients should receive enough iron supplementation to correct anemia and replenish body stores[39,55]. In other words, the goals of anemia treatment, both in patients with and without IBD, are to normalize not only the hemoglobin value but also the iron stores, usually defined by the serum ferritin level[2].

DOSE OF IRON SUPPLEMENTATION IN IBD ANEMIC PATIENTS

Although conventional wisdom "says" that up to 200 mg of elemental iron (and even 400 mg in some textbooks) per day is required to correct iron deficiency anemia, this is probably incorrect[56]. Since a maximum of 10-20 mg of oral iron can be absorbed per day, very high doses and even high doses are questionable. In fact, there is no rationale to use "high" doses of iron to treat iron deficiency anemia (in IBD or in any other associated disease). There is no evidence to support high doses of iron in comparative trials[57-59]. This makes sense from a physiologic standpoint since it is well known that the iron absorptive process is very efficient yet can be saturated[56]. In this respect, a single tablet of most of the ferrous salt preparations (for example sulphate) provides more iron than the intestine is able to absorb in one day[57,58]. On the other hand, non-absorbed iron salts can be toxic to the intestinal mucosa[60-67], and perhaps could activate the disease[1,24]. In any case, high doses of iron may cause diarrhea, which in turn not only impair quality of life but also may make it difficult to differentiate from an IBD relapse[65,68]. Finally, non-absorbed iron salts may inhibit (i.e. by feedback) the intestinal iron absorption and decrease tolerance and compliance, which is difficult especially in young patients requiring several complex oral treatments.

Therefore, since absorption and efficacy of oral iron are no greater when high doses are used, and because adverse effects of this preparation are dose-related, oral iron, if used, should be recommended in low doses (e.g. 50-100 mg of elemental iron daily)[2].

RESPONSE OF ANEMIA TO ORAL IRON SUPPLEMENTATION IN IBD PATIENTS

The main factor in favor of oral iron is convenience. However, even when iron treatment is correctly prescribed, the oral route has relevant limitations, such as[2]: (1) only part of the iron is absorbed and, as previously mentioned, experimental and clinical evidence suggests that the non-absorbed iron salts can be toxic and proinflammatory, and perhaps could activate the disease[1,24]; (2) absorption of oral iron can be severely compromised because of disease activity[34], and in some Crohn's disease cases because of previous intestinal resections or involvement of the duodenum; (3) oral iron is often not well tolerated by patients. From a recent systematic review on the management of anemia in IBD which included several studies prescribing oral iron[27,35,65-67,69,70], the intolerance rate (mainly because of nausea, abdominal pain, or diarrhea) was a common finding leading to discontinuation in up to 21% of the cases[1]. Moreover, IBD patients often do need to take several oral drugs and overall compliance could be compromised by oral iron side effects[71]; (4) oral supplementation results in a slow response, and in some patients persistent blood loss exceeds the capacity of intestinal absorption of iron[72].

INTRAVENOUS IRON FOR THE TREATMENT OF IRON DEFICIENCY ANEMIA IN IBD

The efficacy of intravenous iron for the treatment of iron deficiency anemia in the general population (without IBD) has been demonstrated in numerous studies[73]. Although the experience with intravenous iron in IBD is more limited, it is similarly encouraging[6,16,50,51,66,70,74-78]. Iron sucrose was prescribed in most cases, which was effective in 50%-91% of the patients (depending on the criteria used for efficacy definition)[1]. More recently, a mean response of iron deficiency anemia to the treatment with this intravenous iron formulation was calculated to be 73%, which is a considerably high figure[2]. In summary, intravenous iron sucrose is more effective (in terms of faster and prolonged response) than oral iron supplements, and has a better safety profile which might positively influence the compliance of IBD patients. Accordingly, the inconvenience of intravenous iron is offset by the advantages in achieving better therapeutic results.

Following a widely recommended algorithm, the initial therapeutic strategy of iron deficiency anemia in IBD patients would be based on the level of hemoglobin. Patients with hemoglobin > 10/10.5 g/dL would initiate treatment with oral iron, while in those with levels < 10/10.5 g/dL, which are generally considered to denote severe anemia, the intravenous route would be of choice[1,3,8]. Intravenous iron should also be prescribed to patients with hemoglobin > 10/10.5 g/dL when intolerance to the oral formulation is present. In summary, the established indications for the use of intravenous iron are: severe anemia (generally defined as hemoglobin < 10 g/dL[1,8,37], although some authors set the cut-off point at 10.5 g/dL), need for quick recovery in mild anemia, intolerance to oral iron, and failure of oral iron.

Although in IBD iron sucrose is the most used intravenous formulation; there are other new intravenous iron preparations which theoretically could be used, with an extremely low incidence of adverse effects, and in particular severe adverse effects[1,73,79,80], but data in the specific IBD population is lacking, as reviewed by Auerbach[79]. The experience with low-molecular-weight iron dextran is rather more extensive and encouraging[79] and also a new molecule, iron carboxymaltose, merits mention because its pharmacokinetic characteristics and preliminary clinical experience seems very promising, and in this case was obtained directly in an IBD population[81].

ROLE OF ERYTHROPOIETIN IN THE TREATMENT OF ANEMIA IN IBD

As previously mentioned, anemia in IBD patients results primarily from iron deficiency because of chronic intestinal blood loss. However, intestinal inflammation is mediated by overproduction of cytokines, which may contribute to the generation of anemia in chronic disease, accompanied by inadequate erythropoietin production[16]. Thus, IBD-associated anemia is a unique example of a combination of chronic iron deficiency and anemia of chronic diseases. Since it was first used in chronic renal failure, recombinant human erythropoietin has been shown to be effective for treating the anemia that accompanies several chronic diseases[41]. During the last few years, several studies have evaluated the efficacy of erythropoietin in IBD patients, reporting encouraging results[16,20,27,50,75,82-84]. Nevertheless, as the cost of erythropoietin is much higher than the cost of intravenous iron, the latter formulation should be considered first-line therapy in patients with severe anemia, and erythropoietin therapy should be considered only for patients with low erythropoietin levels or who are unresponsive to intravenous iron[37,85]. One must not forget to exclude or correct other causes of anemia in IBD patients before administering erythropoietin[86]. Finally, erythropoietin should be reserved for patients in which aggressive management of IBD (including immunosuppressive therapy) has not suppressed inflammation, which underlines the idea that erythropoietin is an adjunct, - and not an alternative, -to appropriate treatment of IBD[38].

Erythropoietic agents should always be combined with intravenous iron supplementation, because functional iron deficiency, -defined as an inappropriate availability of iron for erythropoiesis despite normal

body iron stores, is likely to develop[76]. In the particular case of Crohn's disease, folic acid and vitamin B12 status should also be frequently checked[43] and deficiencies adequately corrected. Accordingly, erythropoietin therapy has been accompanied by iron supplementation in all trials published so far[1]. In summary, the enhancement of erythropoiesis by erythropoietin makes it mandatory to administer iron supplementation during therapy to meet the increased demand[86].

CONCLUSION

Anemia is rather common in IBD. Particularly in Crohn's disease, it can be a very difficult clinical problem because iron deficiency, vitamin B12 and/or folic acid defects, malabsorption, malnutrition, inflammation, intestinal resection, and drug effects all can be the cause or contribute to a multifactorial and complex problem. The control of inflammation is a key point, but often is not enough to treat anemia. As anemia has a considerable impact on the quality of life of patients, a thorough and complete diagnostic and therapeutic strategy should be followed to help our patients have as normal a life as possible. Very recent evidence raises a very important problem for the clinician: anemia can be a chronic or at least a recurrent problem in IBD; patients should be followed up after completing treatment, and anemia and iron deficiency actively assessed in the standard investigations[48].

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, *Series Editors*

Intravenous iron in inflammatory bowel disease

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Abstract

The prevalence of anemia across studies on patients with inflammatory bowel disease (IBD) is high (30%). Both iron deficiency (ID) and anemia of chronic disease contribute most to the development of anemia in IBD. The prevalence of ID is even higher (45%). Anemia and ID negatively impact the patient's quality of life. Therefore, together with an adequate control of disease activity, iron replacement therapy should start as soon as anemia or ID is detected to attain a normal hemoglobin (Hb) and iron status. Many patients will respond to oral iron, but compliance may be poor, whereas intravenous (IV) compounds are safe, provide a faster Hb increase and iron store repletion, and presents a lower rate of treatment discontinuation. Absolute indications for IV iron treatment should include severe anemia, intolerance or inappropriate response to oral iron, severe intestinal disease activity, or use of an erythropoietic stimulating agent. Four different products are principally used in clinical practice, which differ in their pharmacokinetic properties and safety profiles: iron gluconate and iron sucrose (lower single doses), and iron dextran and ferric carboxymaltose (higher single doses). After the initial resolution of anemia and the repletion of iron stores, the patient's hematological and iron parameters should be carefully and periodically monitored, and maintenance iron treatment should be provided as required. New IV preparations that allow for giving 1000-1500 mg in a single session, thus facilitating patient management, provide an excellent tool to prevent or treat anemia and ID in this patient population, which in turn avoids allogeneic blood transfusion and improves their quality of life.

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Key words: Inflammatory bowel disease; Anemia; Iron deficiency; Functional iron deficiency; Erythropoiesis stimulating agents; Oral iron; Intravenous iron

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INTRODUCTION

When body iron stores are depleted, iron supplementation seems beneficial, although the optimal route of administration remains controversial. Oral iron supplementation is adequate in some clinical conditions. Administration of oral iron, in the absence of inflammation or significant ongoing blood loss, can correct the anemia, provided significant doses can be tolerated. However, although conventional wisdom "says" that up to 200 mg of elemental iron per day is required to correct iron deficiency anemia (IDA), this is probably incorrect[1]. Early studies indicated that the co-administration of iron with ascorbic acid (vitamin C) might be of benefit in enhancing iron absorption, since, in theory, more ferrous iron is maintained in solution. However reports indicated that such co-administration can induce severe toxicity in the gastrointestinal tract[2]. Moreover, classically, oral iron intake separately from meals is recommended for increasing its absorption but this enhances digestive intolerance and, therefore, decreases compliance. In addition, the absorption of iron salts can be diminished by co-administration of some antibiotics (mainly quinolones, doxycycline, tetracyclines, chloramphenicol or penicillamine); proton pump inhibitors and anti-acid medication (aluminum,

bicarbonate, zinc or magnesium salts), levodopa, levothyroxine, cholestyramine, phytates (high fiber diets), soy products, ibandronate, etidronate, tannates, calcium, and phenolic compounds (coffee, tea), whereas amino acids seem to act as enhancers of iron absorption[3].

On the other hand, non-absorbed iron salts may produce a variety of highly reactive oxygen species including hypochlorous acid, superoxides and peroxides that may lead to digestive intolerance, causing nausea, flatulence, abdominal pain, diarrhea or constipation, and black or tarry stools, and perhaps could activate relapsed inflammatory bowel disease (IBD)[2].

Although preoperative oral iron has shown to be efficacious in uncomplicated IDA, in anemia of chronic disease (ACD) [e.g. Crohn's disease (CD)], as well as in that associated with acute inflammation (e.g. postoperative period after gastrointestinal surgery), the effectiveness of oral iron administration is rather limited since absorption is down-regulated, and the small amount of iron absorbed is directed to the reticulo-endothelial system (RES), where it is sequestered[4].

In these situations, intravenous (IV) iron has emerged as a safe and effective alternative for IBD anemia management. This takes into consideration factors such as intolerance of or contraindications to oral iron, severe anemia (especially if accompanied by significant ongoing bleeding), short time to surgery, need of a fast recovery or the use of erythropoiesis-stimulating agents (ESAs)[5]. Because daily iron absorption is only 1-2 mg (up to 10 mg in deep ferropenia), for patients presenting with moderate to severe anemia at least three to 4 mo of oral iron administration are needed to correct hemoglobin (Hb) levels and replenish iron stores. As IV iron can allow up to a five-fold erythropoietic response to significant blood-loss anemia in normal individuals[6], Hb starts rising in a few days, the percentage of responding patients is higher and iron stores are replenished. Boosting iron stores is an advantage, particularly for patients receiving ESAs[7].

INTRAVENOUS IRON AGENTS

All IV iron agents are colloids with spheroidal iron-carbohydrate nanoparticles. Each particle consists of an iron-oxyhydroxide core (Fe^{3+}) and a carbohydrate shell that stabilizes the iron-oxyhydroxide core. Differences in core size and carbohydrate chemistry determine pharmacological and biologic differences between the different iron complexes, including clearance after injection, iron release *in vitro*, early evidence of iron bioactivity *in vivo*, and maximum tolerated dose and rate of infusion[8,9]. Complexes can generally be classified as labile or robust (kinetic variability), and as weak or strong (thermodynamic variability), with all possible intermediates. Four different products are mostly used in clinical practice: iron gluconate, iron sucrose, iron dextran, and iron carboxymaltose[2,10,11] (Table 1).

Iron gluconate

Iron gluconate has a core tightly bound to gluconate and weakly associated with sucrose (molecular weight 38 kDa), and is a type Ⅲ iron complex (labile and weak) with fast degradation kinetics and direct release to plasma proteins (apotransferrin, apoferritin, and others). The potential for acute adverse reactions related to labile iron release after IV injection, which is caused by oversaturation of the transferrin binding capacity, is higher with iron gluconate compared to the other available IV iron preparations. Non-transferrin-bound labile iron may induce acute endothelial cell injury and a transient capillary leak syndrome. Clinical symptoms of iron acute toxicity include nausea, hypotension, tachycardia, chest pain, dyspnea (lung edema), and bilateral edema of the hands and feet, and should not be misread as anaphylaxis[9]. To avoid these side effects, the maximum recommended dose is 125 mg; whereas the administration of total dose is not recommended. The use of iron gluconate for iron deficiency (ID) in patients on dialysis has been found to be efficacious and safe[8,9].

Iron sucrose

Iron sucrose has a core tightly bound to sucrose (molecular weight 43 kDa), and is a partially stable type with medium degradation kinetics and partial uptake of released iron by plasma proteins such as (apo)-transferrin but also by the RES (Type Ⅱ: semi-robust and moderately strong). Its half life is relatively short (5-6 h) and the amount of iron transported by transferrin, calculated using the Michaelis-Menten model for a single dose containing 100 mg of iron, is around 30 mg Fe^{3+}/24 h[12]. Following a single IV injection of 100 mg iron sucrose to anemic patients, up to 95% of the injected iron was utilized within 2-4 wk. During the last few years, experience of using iron sucrose in various forms of ID has evolved. In spite of its safety profile, nowadays a test dose is still required at most European countries. Single doses of 100-200 mg as an IV injection[13] or up to 500 mg over an infusion time of 3.5 h seem to be safe[14]. The maximal recommended dosage is 600 mg/wk (200 mg iron as iron sucrose injected or infused intravenously no more than three times a week) but this amount exceeds the physiological needs of the proliferating erythroblast. If the infusion speed is too fast (above 4 mg Fe^{3+}/min) or the single total iron dose too high (above 7 mg Fe^{3+}/kg, with a maximum of 500 mg), non-transferrin bound labile iron may cause transient hypotension, tachycardia, and dyspnea, as described for iron gluconate. Paradoxically diarrhea, epigastric pain or dummy aches could appear within minutes to hours after infusion. Cases of phlebitis have been described, but they are probably secondary to longer lasting administration (rather than larger doses), the need to keep a venous access, or the use of solutions that are too dilute (iron concentration must be at least 1 mg/mL). Overall, iron sucrose is currently considered as the safest IV iron preparation[15].

Iron dextran

Iron dextran is a stable parenteral iron product with

Table 1 Some characteristics of the different intravenous iron formulations

	Iron gluconate	Iron sucrose	Iron dextran (LMWID)	Ferric carboxymaltose
Complex type	Type Ⅲ Labile and weak	Type Ⅱ Semi-robust and moderately strong	Type Ⅰ Robust and strong	Type Ⅰ Robust and strong
Molecular weight (kDa)	38	43	73	150
Initial distribution volume (L)	6	3.4	3.5	3.5
Plasma half-life (h)	1	6	30	16
Labile iron release	+++	±[1]	-	-
Direct iron donation to transferrin (% injected dose)	5-6	4-5	1-2	1-2
Test dose required	No	Yes/No[3]	Yes	No
Maximal single dose (mg)	125	300	TDI	1000
Premedication	No	No	TDI only	No
Life-threatening ADE[2] (× 10^6 doses)	0.9	0.6	11.3	??
Death rate (× 10^6 doses)[2]	0.25	0.11	0.78	??

[1]If the infusion speed > 4 mg Fe^{3+}/min or dose > 7 mg Fe^{3+}/kg; [2]Data from patients with chronic kidney disease; [3]Not required at USA and UK. ADE: Adverse drug events; LMWID: Low molecular weight iron dextran; TDI: Total dose infusion.

a molecular weight of 73 kDa (Low molecular weight iron dextran, LMWID) or 156 kDa (High molecular weight iron dextran, HMWID). This type I iron complex (robust and strong) shows high structural homogeneity and only slow and competitive delivery to endogenous iron binding proteins. Complexes are actively phagocytosed by macrophages of the RES before they are released and become available for Hb synthesis. Although the plasma half life of LMWID is 30 h (3 d for HMWID), the full process of iron release from the dextran complex in the RES, storage in ferritin and delivery as TBI to the bone marrow or other tissues may take several months[16]. Iron dextran can be administered as intramuscular (i.m.) or IV injections and as IV infusion, but a test dose is always required before the first administration. The stability of the dextran complex allows administration of high single doses (so called "total dose infusion" which may be given over 4-6 h). In contrast, the bioavailability of iron following i.m. administration has not been studied extensively. There seems to be a risk of incomplete and variable absorption of the iron from the injection site, and a considerable amount (30%-50%) of iron can remain at the i.m. injection site for many months. Therefore i.m. injections are no longer recommended[17]. However, these iron complexes may cause well know dextran-induced anaphylactic reactions, especially in patients receiving HMWID (not commercially available in Europe and considered as an obsolete and dangerous IV iron agent). The exact mechanism of the anaphylactic reaction to iron dextran has not been clarified yet, but it seems to be related to the antibody-mediated release of mediators by mast cells[15].

Ferric carboxymaltose (FCM)

FCM is another stable parenteral iron product with a molecular weight of 150 kDa very similar to iron dextran in terms of stability and structure (Type I , robust and strong). The pharmacokinetic characteristics of FCM are similar but not identical to iron dextran. The distribution volume of both preparations corresponds nearly to that of plasma, but half life is approximately 16 h for FCM as compared to 30 h for LMWID. It seems that FCM is broken down quicker than iron dextran because α-amylase does not affect dextran, or acts at a very slow rate[2]. A study using positron emission tomography has shown that iron from FCM accumulates in the liver, spleen and bone marrow and substantial amounts were found in these organs within minutes. In addition, FCM is able to exchange iron rapidly with transferrin[18]. As a result, the utilization of iron for RBC increased rapidly up to days 6 to 9, after which the utilization increased at a much lower rate. Patients with IDA showed iron utilization over 90% after 24 d compared to 60%-80% utilization for patients with renal anemia[18]. FCM is designed to mimic physiologically-occurring ferritin, providing high iron utilization, without the disadvantageous characteristics associated with iron dextran (anaphylaxis) and iron sucrose (high pH, high osmolarity, dosage limitations, and the long duration of administration). Up to 100-200 mg FCM can be administered as IV injection and up to 1000 mg iron can be infused in at least 15 min and no test dose is required (Table 1). In comparison, the European Union (EU) prescribing information for other IV iron preparations indicates they can be administered only in low doses (e.g. usual recommended dose of LMW iron dextran is 100-200 mg of iron and the maximum EU dose of iron sucrose is 200 mg of iron) over a period of greater than 30 min, which results in the need for frequent infusions to administer the total calculated iron replacement dose[19]. No serious adverse effects, including deaths, were considered related or likely related to FCM by trial investigators; however, the US Food and Drugs Administration has raised concerns about a potential mortality safety signal based on a increased of deaths in comparison to other arms across clinical trials[20]. In contrast, since 2007 the use of FCM corresponds to over 17 000 patient-years (one patient corresponds to 2000 mg iron), and up to September 2008 no anaphylactoid reactions or death have been reported, suggesting a good safety profile for FCM[2]. Therefore, information regarding

FCM safety in the clinical setting is somehow conflictive and further post authorization trials to confirm its benefit and safety are needed.

EFFICACY OF PARENTERAL IRON AGENTS IN IBD

Experience with the use of IV iron therapy is extensive in different clinical settings over the last 60 years. In the late 1980s, the introduction of recombinant human erythropoietin (rHuEPO) led to a revitalized interest in the use of iron therapy, either in combination with rHuEPO therapy, or alone. Intravenous iron therapy can be used in a variety of clinical settings, as long as iron parameters are carefully monitored. In a number of studies, IV iron was shown to be useful for the treatment of anemia associated with a variety of medical [IBD, chronic kidney disease (CKD), chronic inflammatory arthritis, congestive cardiac failure, pregnancy and postpartum, or cancer] and surgical conditions (orthopedic, cardiac, colorectal cancer, and gynecological surgical procedures)[21,22]. Interestingly, in the settings of CKD or cancer related anemia, the use of IV iron resulted not only in a more rapid and complete response to rHuEPO, but also in a reduction of rHuEPO dose, and probably in a reduction of rHuEPO side effects, such as thrombosis[23,24].

Approximately, one third of IBD patients suffer from recurrent anemia across different studies (ranging from 6% to 73%, depending on Hb cut-off for the definition of anemia; patient selection, IBD phenotype and year of publication), and the prevalence of ID is even higher [mean prevalence: 45%, 95% confidence interval (CI): 40%-50%]. A retrospective study found that the prevalence of mild to moderate anemia significantly decreased in the IBD population between 1993 and 2003 (33.8% *vs* 16.7%, $P = 0.013$), although the prevalence of severe anemia was similar (6.3% *vs* 5.6%, P = NS), and the only difference detected between the two cohorts was the increased use of immunosuppressive drugs (mainly azathioprine)[25]. Both ID (due to intestinal blood loss that cannot be matched by duodenal iron absorption, creating a negative iron balance) and ACD (due to the inflammatory nature of the disease) contribute most to the development of anemia in IBD, whereas cobalamin or folate deficiency and various other causes of anemia such as hemolysis occur infrequently. Whatever the underlying mechanism, anemia is universally accepted as a condition having a significant impact on the affected patient's quality of life[1,26].

Anemia control and recovery in patients with IBD has a beneficial impact on quality of life indices. Our goal is to attain Hb levels above 13 g/dL in males and 12 g/dL in females by the administration of iron supplements, with or without erythropoietin. However, it is worth noting that without an appropriate control of disease activity, the management of anemia associated to IBD is much more difficult. Thus, it is desirable to initiate the pharmacological treatment after adequate inflammation control[27].

According to the recommendations of the Guidelines on the Diagnosis and Management of Iron Deficiency and Anemia in Inflammatory Bowel Diseases (Statement 2B), iron supplementation should be initiated when IDA is present (Grade A). For ID without anemia, different approaches to iron replacement should be considered and discussed with the patient. If patients are likely to develop IDA the monitoring frequency should be increased (Grade D)[26], although it is unknown how often monitoring should be performed.

When body iron stores are depleted, iron supplementation seems beneficial, although the optimal route of administration remains controversial. Total iron deficit (TID) can be calculated using the Ganzoni's formula: TID (mg) = Weight (kg) × (Ideal Hb - Actual Hb) (g/dL) × 0.24 + depot iron (500 mg).

According to this formula, a person weighing 70 kg with an Hb level of 9 g/dL would have a body iron deficit of about 1400 mg. Nevertheless, Ganzoni's formula may underestimate iron depot in males, as in them it has been consistently reported to be 700-900 mg[28]. Thus, a TID of 1600-1800 mg may be a more realistic estimation for this subject.

Following the administration of oral iron to a patient with uncomplicated IDA, it takes 2-2.5 wk for the Hb to start rising, 2 mo for it to reach normal levels and 6 mo for iron stores to be replete[7]. However, the efficacy of oral iron therapy in IBD patients may be hindered by some IBD specific factors, such as reduced absorption of iron due to inflammation and gastrointestinal side effects of oral ferrous iron (due to the release of activated hydroxyl radicals that may lead to digestive intolerance, causing nausea, flatulence, abdominal pain, diarrhea or constipation, and black or tarry stools)[2]. In addition, oral iron compounds are not all alike, as they may vary in composition, elemental iron concentration, absorption profile, efficiency or tolerance. As a non-written rule, the best tolerated oral agent is usually the one that contains or delivers less iron.

In a crossover study of 19 IBD patients randomly assigned to start treatment with ferrous fumarate 120 mg orally once daily or iron sucrose 200 mg IV three times during a period of 14 d, oral ferrous fumarate, but not IV iron sucrose, increased clinical disease activity in IBD patients[29]. In contrast, iron sucrose, but not ferrous fumarate, increased intravascular oxidative stress[29]. However, a prospective study comparing usage, tolerance, and efficacy of 4 wk therapy with oral iron therapy in patients with ID and IBD and patients with ID of non-inflammatory cause, intolerance to iron was reported in 24% of the patients who had IBD (non-active) and 29% of the patients who did not (P = NS), and only a tiny minority of IBD patients relapse in association with use of oral iron therapy[30]. This data suggest that patients with IBD are no more intolerant to oral iron than other patients and have similar rates of repletion, but the low number of evaluable patients ($n = 47$) precluded the drawing of definite conclusions. Nevertheless, to avoid the risk of poisoning,

Table 2 Characteristics of the clinical trials involving IBD patients that compared IV iron administration with oral iron or no intervention included in this review

Study (yr)	*n*	Study design	Compound	Baseline Hb (g/dL)	Total dose, mg (schedule)	Duration (wk)	Response (%)	DCT (%)
Gasche *et al*[31] (2001)	103	Multicentre, open-label	Iron sucrose	≤ 10.5	1200 mg (6 × 200 mg)	4	65	0
Bodemar *et al*[32] (2004)	59	Retrospective	Iron sucrose	< 12	Mean 1400 mg (1-2 × 200 mg/wk)	8 12	60 91	0
Schröder *et al*[14] (2005)	46	Multicentre randomized open-label	Iron sucrose (22)	< 10.5 (F)	Mean 1418 mg (7 mg/kg + 5 × 200 mg)	6	55	4.5
			Ferrous sulfate (24)	< 11 (M)	Mean 5600 mg (100-200 mg/d)		53	20.8
García-López *et al*[35] (2006)	70	Single centre prospective observational	Iron sucrose	< 10.5[1]	Mean 920 mg (200-1800 mg) (200 mg/1-3 times a week)	Mean 5 (1-9)	67	0
Kulnigg *et al*[37] (2008)	200	Multicentre randomized open-label	Ferric carboxymaltose (137)	≤ 10	1000-1500 mg (1-2 infusion of 500-1000 mg)	12	77	1.5
			Ferrous sulfate (63)		16800 mg (200 mg/d)		68	7.9
Lindgren *et al*[33] (2009)	91	Multicentre randomized investigator-blinded	Iron sucrose (45)	< 11.5	Mean 1700 mg (200 mg/1-2 wk)	20	66	7
			Ferrous sulfate (46)		Mean 38400 mg (200-400 mg/d)		47	22
Gisbert *et al*[34] (2009)	100	Multicentre, open-label	Iron sucrose (22)	< 10	Not reported (2 × 200 mg/wk if Hb < 10)	26	77	0
			Ferrous sulfate (78)	> 10	19000 mg (106 mg/d)		89	5.1

[1]Also no response or intolerance to oral iron or clinical need of quick recovery of anemia. ΔHb ≥ 2 g/dL or normal Hb; DCT: Discontinuation due to serious adverse events; F: Female; M: Male.

other oral iron compounds (such as iron polymaltose which has very low toxicity and meets the requirements for a food supplement) might be used instead of ferrous salt preparations[2] and lower doses (e.g. 50-100 mg of elemental iron) should be recommended[1]. Additionally, the response and tolerance should be monitored and treatment changed to IV iron if necessary (Grade C)[26].

Is this statement supported by the information reviewed above? Because of the limitations of oral iron therapy in IBD patients, parenteral routes of iron administration should be preferred, even though many patients will respond to oral iron. Intravenous iron is more effective, better tolerated, and improves quality of life to a greater extent than oral iron supplements (Grade A)[26]. Absolute indications for IV iron include severe anemia (Hb < 10 g/dL), intolerance or inappropriate response to oral iron (once iron therapy has been initiated the response may be: "complete", if Hb increases ≥ 2 g/dL; "partial", if Hb increases 1-1.9 g/dL; or "no-response", if Hb increases < 1 g/dL), severe intestinal disease activity, concomitant therapy with an erythropoiesis stimulating agent, or patient preference[26]. In a prospective study of 103 patients with severe IBD-associated anemia who received IV iron sucrose for 4 wk (total dose 1200 mg), Gasche *et al*[31] investigated the parameters that can predict effectiveness. Overall, a complete response at the end of the fourth week was observed in 67 (65%) patients, and the variables significantly associated with response were serum erythropoietin, soluble transferring receptor, transferrin, and IL-6 levels. Once again, these data emphasized the need for an adequate inflammation control in IBD patients. We will review some studies assessing the efficacy of IV iron in anemic IBD patients (Table 2).

In this regard, a retrospective observational study in IBD patients with poor response or intolerance to oral iron, the administration of iron sucrose (200 mg once or twice per week to reach total ID) resulted in a "complete" response (Hb increment ≥ 2 g/dL or correction of anemia) in 60% of patients within 8 wk and in 90% of patients within 12 wk[32]. However, a randomized, controlled, open-label, multicenter trial performed in 46 patients with anemia and transferrin saturation ≤ 20% and/or serum ferritin concentrations ≤ 20 μg/L found no differences in Hb increment within 6 wk between patients receiving IV iron sucrose and those receiving iron sulfate, but resulted in building up iron stores (about ferritin = 200 ng/mL, after 6 wk)[14]. In addition, intractable gastrointestinal adverse events caused permanent study drug discontinuation in five patients (20.8%) receiving iron sulfate, whereas only one patient (4.5%) was withdrawn because of side effects due to IV iron sucrose[14]. Thus, although being equal in short-term efficacy, these results suggest a better gastrointestinal tolerability for iron sucrose.

In a very recent study, 91 patients with IBD and anemia (Hb < 11.5 g/dL) were randomized to oral iron sulfate (n = 46) or IV iron sucrose (n = 45) treatment for 20 wk. More patients in the IV iron group completed the study (93% *vs* 78%, P = 0.001), increased their Hb ≥ 2 g/dL (66% *vs* 47%, P = 0.07), raised their ferritin levels to normal (74% *vs* 48%, P = 0.013), and recovered from anemia (84% *vs* 59%, P = 0.007) compared to patients in the oral iron group. In addition, treatment with IV iron sucrose improved iron stores faster and more effectively than oral iron (P = 0.002). Only 22 patients (48%) tolerated the prescribed oral dose, and 52% reduced the dose or withdrew from treatment because of poor tolerance[33].

Finally, in a prospective multicenter study of 100 IBD patients with IDA [59 CD, 41 ulcerative colitis (UC)], those with Hb > 10 g/dL were prescribed oral ferrous sulphate (n = 78) and those with Hb < 10 g/dL received IV iron sucrose (n = 22). Hb normalization was achieved in 89% with oral and 77% with IV iron, and was associated with a relevant improvement in the patients' quality of life. IBD activity increase was not demonstrated in any patient. Four patients (5.1%) showed oral iron intolerance leading to discontinuation of treatment, whereas no adverse events were reported for IV iron[34]. Thus, oral iron treatment was effective and well tolerated in most IBD patients, and did not exacerbate the symptoms of the underlying IBD, whereas IV iron was an effective and safe treatment in more severely anemic or intolerant patients.

At one author's centre, the safety and efficacy of IV iron sucrose therapy was evaluated in a preliminary study of 70 patients with digestive pathology (54 IBD: 27 CD, 18 UC, nine pouchitis)[34]. IV iron sucrose in an "outpatient regimen" was used for patients with IDA due to digestive disorders with at least one of the following criteria: (1) no response or intolerance to oral iron; (2) IBD with severe anemia (Hb < 10.5 g/dL) and/or (3) clinical need of quick recovery of anemia. Average baseline Hb was 9.8 ± 1.7 g/dL, and 11.7 ± 1.5 g/dL at the end of treatment (mean increase 1.9 g/dL, range -2 to 5.5 g/dL), Hb increase exceeded 2 g/dL in 47% of treatments, and anemia was corrected in 67.8% of patients. No severe adverse events were witnessed. The authors concluded that IV iron sucrose should become the standard of care in IBD patients with ID[35].

Therefore, treatment with IV iron sucrose is effective, safe, and well tolerated in correcting Hb and iron stores in patients with IBD, especially in those with severe anemia. The main disadvantage of IV iron sucrose is the need for multiple infusions as the maximun weekly dose should not exceed 600 mg. The availability of stable parenteral iron compounds allowing for TDI infusion may greatly facilitate iron replacement therapy in IBD patients.

As for children, the safety and efficacy of IV iron therapy was retrospectively evaluated in 70 pediatric patients with IBD (50 CD, 20 UC) who received a total of 119 TDI iron dextran infusions between February 1994 and February 2000. The average increase in Hb concentration was 2.9 g/dL. The authors concluded that TDI infusion of iron dextran, when appropriately used, is a safe and potentially efficacious treatment for children with IBD and IDA who are unresponsive to, or noncompliant with, oral iron therapy[36]. However, as mentioned above, iron dextran, especially HMWID, has the disadvantage of potentially life-threatening dextran-associated anaphylactic reactions.

More recently, Kulnigg *et al*[37] randomized 200 anemic IBD patients (about Hb = 9 g/dL) to receive IV FCM (FCM, n = 173; maximum 1000 mg iron per infusion) at 1-wk intervals until the patients' calculated TID was reached or oral ferrous sulfate (100 mg *bid*) for 12 wk. There were no differences between groups in Hb improvement at week 12 (3.8 ± 2.0 g/dL in both groups) or treatment-related adverse events, but response (defined as Hb increase of > 2.0 g/dL) was higher for FCM at week 2 (P = 0.0051) and week 4 (P = 0.0346), with a lower rate of discontinuation of study medication due to adverse events (1.5% and 7.9%, respectively), than for oral iron. Thus, FCM seems to be effective and safe in IBD-associated anemia,

Overall, from data depicted in Table 2, the mean response of IBD-associated anemia to treatment with IV iron (weighted mean) was (281/382) 73.6% and (140/215) 65.1% with oral iron, [odds ratio (OR): 1.49, 95% CI: 1.02-2.17, P = 0.02]. When the analysis was performed for data extracted from prospective randomized trials only, the response to IV iron was 72.5% (143/198) *vs* 58.2% (71/122) (OR: 1.87, 95% CI: 1.13-3.09, P = 0.0097). In addition, reviewed data strongly suggest that for patients with IBD, treatment with IV iron is effective, safe, well tolerated, provides a fast Hb increase and a sufficient refill of iron stores, and presents a lower rate of treatment discontinuation than oral iron. However, further research is needed to ascertain what is the appropriate timing to start treatment, which are the target Hb and ferritin levels to reach, and how IV iron may affect IBD clinical time course.

A TENTATIVE ALGORITHM FOR IRON REPLACEMENT IN IBD

Although further research is needed to ascertain what is the appropriate time to start treatment, which are the target Hb and ferritin levels to reach, or how IV iron may affect the IBD clinical time course, a tentative, easy to follow algorithm for iron replacement in IBD patients is depicted in Figure 1.

According to this algorithm, in which we assume that the severity of ID and anemia correlates with disease activity, the total iron dose and the route of administration rely upon baseline Hb, serum ferritin level and transferrin saturation. For male IBD patients (70-90 kg) with Hb > 13 g/dL and ferritin < 30 ng/mL, TID is estimated to be around 800-1000 mg. The corresponding value for TID in women (60-80 kg) with Hb > 12 g/dL would be 600-800 mg. If there are no contraindications, oral iron would be the simplest replacement therapy (50-100 mg/d, for 2-3 mo), and iron complexes are preferred to iron salts. If there is a contraindication for, or a non adequate response (ferritin < 100 ng/mL after 6-8 wk) to, oral iron, an IV iron preparation should be administered: iron sucrose (200 mg IV, 1-2 times/wk), LMWID (up to 1000 mg IV, single dose), or FCM (up to 1000 mg IV, single dose).

As for patients with Hb between 10 g/dL and 12/13 g/dL, ferritin < 100 ng/mL and transferrin saturation < 20%, TID is estimated to be 1300-1800 mg. Oral iron supplements could still be indicated (100 mg/d for 4-6 mo) but the IV route is preferred, as it will pro-

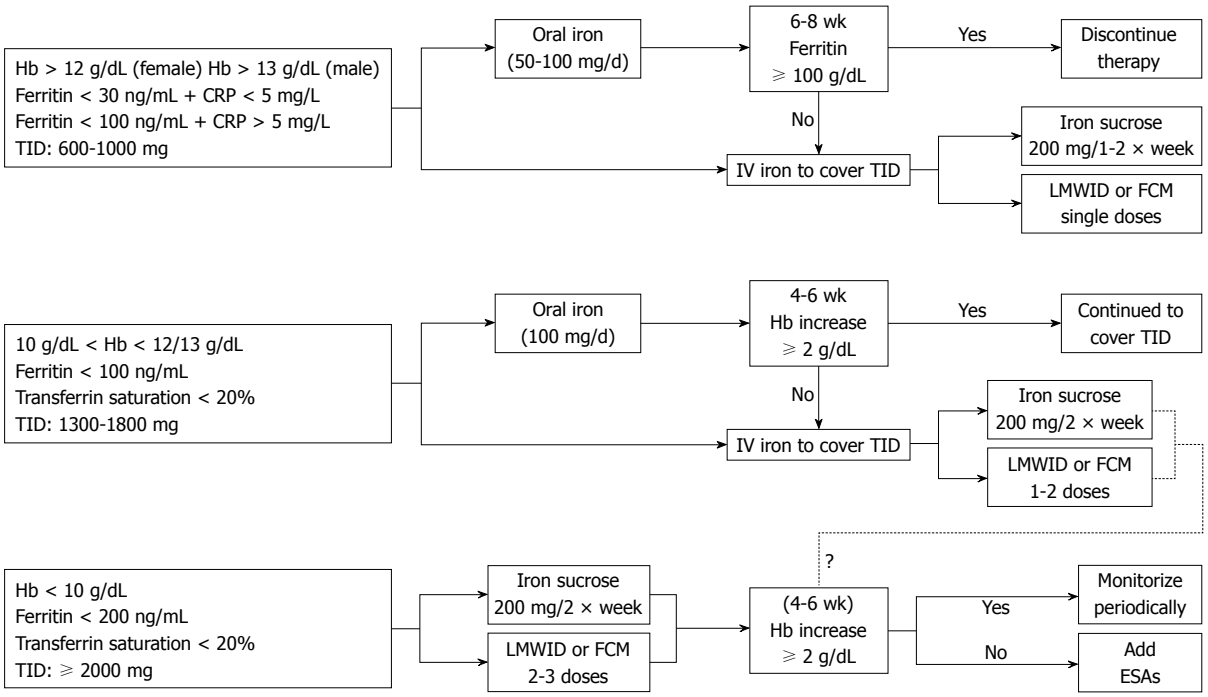

Figure 1 A tentative algorithm for iron replacement therapy in patients with inflammatory bowel disease. Hb: Hemoglobin; TID: Total iron deficit; CRP: C-reactive protein; LMWID: Low molecular weight iron dextran; FCM: Ferric carboxymaltose; ESA: Erythropoiesis stimulating agent.

vide a faster Hb recovery (iron sucrose 200 mg IV, twice a week; LMWID or FCM, two doses one week apart). Nevertheless, IV iron should be administered when there is intolerance to, or a non complete response to, oral iron, as defined by an Hb increase ⩾ 2 g/dL or Hb normalization.

For patients with Hb < 10 g/dL and transferrin saturation < 20%, a higher ferritin trigger is selected (< 200 ng/mL), and a TID ⩾ 2000 mg is estimated. These patients should receive IV iron (iron sucrose 200 mg IV, twice a week; LMWID or FCM, 2-3 doses). Patients should be re-evaluated after 4-6 wk; if there is not a complete Hb response, adjuvant treatment with erythropoiesis stimulating agents (e.g. epoetin, darbepoetin) should be considered.

SAFETY OF PARENTERAL IRON AGENTS

Nausea, abdominal pain, constipation, diarrhea, injection site reactions (pain, superficial phlebitis), metallic taste, headache, dizziness and rash may occur with all IV preparations, and were observed in clinical trials with an incidence of 1%-3%. However, the incidence of life-threatening adverse drug events (ADEs) associated with parenteral iron is much smaller.

Allergic and anaphylactic reactions

The numbers of non-CKD patients receiving IV iron are not large enough to draw definitive conclusions regarding the safety of IV iron agents in these clinical settings. Therefore, we will focus on ADEs associated with parenteral iron in CKD patients, as they are the largest collective receiving these drugs. According to data from the United States Food and Drug Administration (FDA) on ADEs attributed to the provision of four formulations of IV iron (HMWID, LMWID, iron gluconate and iron sucrose) during 2001-2003, the total number of reported parenteral iron-related ADEs was 1141 amongst approximately 30 million doses administered (approx. 38 ADEs per million), with 11 deaths (seven iron dextran, three iron gluconate, one iron sucrose)[15]. Relative to lower molecular weight iron dextran, total and life-threatening ADEs were significantly more frequent among recipients of higher molecular weight iron dextran and significantly less frequent among recipients of sodium ferric gluconate complex and iron sucrose. The absolute rates of life-threatening ADEs were 0.6, 0.9, 3.3 and 11.3 per million for iron sucrose, sodium ferric gluconate complex, lower molecular weight iron dextran and higher molecular weight iron dextran, respectively, whereas absolute rates of death were 0.11, 0.25, 0.75 and 0.78 per million, respectively (Table 1). However, there were no significant differences in mortality rates between LMWID and iron gluconate (OR: 0.3, 95% CI: 0.1-1.3) or iron sucrose (OR: 0.2, 95% CI: 0.1-1.0), and there are no conclusive data available regarding the safety of FCM. Therefore, the frequency of IV iron-related ADEs reported to the FDA has decreased, and overall, the rates are extremely low (Table 1). In addition, the rates of ADES associated with IV iron, including iron-related deaths, are much lower than that of ABT-related severe side effects (10 per million) and ABT-related deaths (four per million)[38].

IV iron and infection

Current information on the relationship between IV iron and infection, and between IV iron and oxidative

stress deserves special consideration. Elemental iron is an essential growth factor for bacteria with many species expressing iron transport proteins that compete with transferrin, and it has long been suggested that patients with iron overload are at increased risk of infection[39]. In contrast, in the peritoneal dialysis population, no increased risk of peritonitis was found in patients receiving IV iron with respect to those not receiving IV iron[40]. In addition, a meta-analysis of six observational studies (807 patients) revealed that the administration of IV iron to patients undergoing major orthopedic surgery led to a significant decrease in both transfusion rate [relative risk (RR): 0.60, 95% CI: 0.50-0.72, $P < 0.001$] and infection rate (RR: 0.45, 95% CI: 0.32-0.63, $P < 0.001$)[41]. Nevertheless, despite this absence of definitive clinical data, it seems sensible to avoid IV iron administration in the setting of acute infection, and to withhold IV iron in patients with pre-treatment ferritin values > 500 ng/mL[5].

IV iron and oxidant damage

Biologically active iron, which is released by all IV iron agents, also plays a role in inflammation, oxidative stress and the propensity for accelerated atherosclerosis. Persistent oxidative stress in CKD patients promotes inflammation and, in turn, atherogenesis, and increased cardiovascular morbidity and mortality. However, available evidence relating IV iron administration to atherogenesis is indirect, and there is little evidence that IV iron adversely affects survival in patients with dialysis-dependent CKD. Nevertheless, the evidence argues for caution, not complacency, in prescribing IV iron[9].

IV iron and cancer development

The association between iron overload with cancer risk in humans has been under increased scrutiny in recent decades, although epidemiological studies on the association of iron with cancer remain inconclusive. The concerns are mostly focused on a possible risk associated with dietary iron in colorectal cancer, the increased risk of developing hepatocellular carcinoma in hereditary hemochromatosis and related hepatic iron overload and cirrhosis, and association between occupational exposure to iron and kidney, lung and stomach cancers. The risk of iron-induced sarcoma by repeated i.m. injections of iron dextran has also been raised. However, IV iron therapy has not been associated with an increase in tumor incidence[42].

CONCLUSION

The prevalence of anemia across the studies on patients with IBD is high (30%) and that of ID is even higher (45%). However, the prevalence of anemia is decreasing, and this seems to be related with the availability and use of IV iron[43].

Iron replacement therapy should start as soon as anemia or ID is detected (Grade D), and its goal is to attain a normal level of Hb, ferritin and transferrin saturation (Grade D)[26]. Importantly, our efforts to correct anemia should rely on adequate inflammation control, in the absence of which no proper approach to this condition is feasible[27].

Although many IBD patients will respond to oral iron, IV iron is more effective, better tolerated, and improves the quality of life to a greater extent than oral iron supplements (Grade A)[26]. Absolute indications for IV iron include severe anemia, intolerance or inappropriate response to oral iron, severe intestinal disease activity, use of ESAs, or patient preference[26].

The use of ESAs should be restricted to those patients presenting with Hb < 10 g/dL and who do not appropriately respond to IV iron replacement for 4 wk (Grade B)[26].

After the initial resolution of anemia and the repletion of iron stores, patients should be closely monitored, and maintenance iron treatment should be provided as required. New IV preparations that allows for giving up to 1000-1500 mg in a single session, provide an excellent tool to avoid or treat anemia and ID in the IBD patient population.

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, *Series Editors*

Use of agents stimulating erythropoiesis in digestive diseases

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Abstract

Anemia is the most common complication of inflammatory bowel disease (IBD). Control and inadequate treatment leads to a worse quality of life and increased morbidity and hospitalization. Blood loss, and to a lesser extent, malabsorption of iron are the main causes of iron deficiency in IBD. There is also a variable component of anemia related to chronic inflammation. The anemia of chronic renal failure has been treated for many years with recombinant human erythropoietin (rHuEPO), which significantly improves quality of life and survival. Subsequently, rHuEPO has been used progressively in other conditions that occur with anemia of chronic processes such as cancer, rheumatoid arthritis or IBD, and anemia associated with the treatment of hepatitis C virus. Erythropoietic agents complete the range of available therapeutic options for treatment of anemia associated with IBD, which begins by treating the basis of the inflammatory disease, along with intravenous iron therapy as first choice. In cases of resistance to treatment with iron, combined therapy with erythropoietic agents aims to achieve near-normal levels of hemoglobin/hematocrit (11-12 g/dL). New formulations of intravenous iron (iron carboxymaltose) and the new generation of erythropoietic agents (darbepoetin and continuous erythropoietin receptor activator) will allow better dosing with the same efficacy and safety.

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Key words: Erythropoiesis-stimulating agents; Recombinant human erythropoietin; Darbepoetin; Continuous erythropoietin receptor activator; Inflammatory bowel disease; Anemia

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INTRODUCTION

Anemia is a frequent complication in patients with digestive diseases, especially chronic processes such as inflammatory bowel disease (IBD)[1,2], and is associated with a higher rate of hospitalization and worse quality of life[3,4]. The main cause of anemia associated with IBD is iron deficiency, but there is also a component of anemia that is associated with chronic diseases. Therefore, when there is no good response to intravenous iron therapy, combination with erythropoietic agents, erythropoietin (EPO) or darbepoetin, may assist in the management of anemia[3,5-7]. The other subgroup of patients with digestive diseases in whom we can use erythropoiesis-stimulating agents (ESAs) are those being treated for chronic hepatitis C, whose anemia is caused by the use of rivabirin[8,9].

Human EPO is a glycoprotein hormone of 30.4 kDa, which is composed of 165 amino acids, and is the main regulator of erythropoiesis. It inhibits the apoptosis of erythroid progenitors and induces the clonal proliferation of normoblasts[10-12]. In adults, it is synthesized mainly in the kidney, which produces 90%-95% of the total circulating EPO; the remainder, about 10%, is synthesized by liver[13,14]. In addition to kidney and liver, it has been shown that other organs can secrete EPO, including peripheral endothelial cells, vascular smooth

muscle cells, neurons, astrocytes, cardiomyocytes and microglia[15].

The basal production maintains a relatively constant level of EPO in plasma, within a range between 5 and 29 U/L, which can increase up to 100 times in the presence of serious anemia[16]. Tissue hypoxia is the most important trigger for increased synthesis of EPO[10,17].

In 1977, Miyake *et al*[18] purified EPO molecule, which enabled cloning of the gene in 1983, and the subsequent development of recombinant EPO in mammalian cell lines[19]. The clinical use of EPO was started in 1986[20] for the treatment of anemia of chronic kidney disease (CKD)[21]. Treatment with EPO was able to eliminate almost completely transfusion dependence in hemodialysis patients, who in turn achieved a significant improvement in quality of life[22-24]. Treatment of anemia with EPO has helped to reduce the need for transfusions and the associated risks: transmission of infection, allogeneic iron overload and immunological damage[25].

There is a general consensus, as stated by the recommendations of panels of international experts (The National Kidney Foundation Dialysis Outcomes Quality Initiative[26], the Canadian Society of Nephrology[27], the European Renal Association/European Dialysis and Transplantation Association[28,29], Kidney Disease: Improving Global Outcomes[30] and the Japanese Society for Dialysis Therapy[31,32]), that the partial correction of anemia in patients with CKD improves clinical parameters and quality of life compared with the lowest levels of hemoglobin[26-32]. At present, for instance, in the United States, > 90% of patients on chronic dialysis and 30% on pre-dialysis are treated with EPO, with an average hemoglobin level of 12.0 g/dL[33-35]. Subsequently, its use has been extended to the treatment of anemia associated with other diseases such as cancer[36-38], IBD[3,5-7], heart failure[39,40] and the anemia associated with hepatitis C virus treatment[8]. In addition to its role as an erythropoietic agent, EPO has other potential therapeutic uses currently under extensive investigation[41,42], for use as a cytoprotective agent in the central nervous system[43,44] and intestinal mucosa[45-47]. Other possible applications include are cytoprotection in peripheral neuropathy, retinopathy and myocardial ischemia[48,49].

TYPES OF EPO

In clinical practice there are several forms of EPO available for the treatment of anemia (Table 1). The first generation are two forms of recombinant human EPO (rHuEPO): epoetin α (Epogen, Amgen and Procrit/Eprex; Johnson & Johnson/Janssen-Cilag) and EPO β (NeoRecormon; Roche), which are administered three times a week[50]. The development during the last decade of ESAs with a higher degree of glycosylation and prolonged half-life has allowed less frequent administration. A preparation called long-acting darbepoetin α (Aranesp; Amgen) produces a similar physiological response compared with rHuEPO[51]. It has more chains of carbohydrates and sialic acidic residues, which gives it a different pharmacokinetic profile from that of rHuEPO, with a half-life approximately three times higher (25.3 h *vs* 8.5 h by iv administration), and a plasma clearance four times slower. This allows a frequency of administration of once weekly, or even every 2-4 wk[52,53]. The equivalence relationship between rHuEPO and darbepoetin α from the molecular weight of both proteins is 1:200[54]. In clinical practice, the multiplication factor is not so simple, as the required dose of rHuEPO can be higher, which probably is related to an increase in resistance to EPO[55]. A pegylated derivative, the continuous EPO receptor activator (CERA) (epoetin β-methoxy polyethylene glycol, CERA; Roche) is another erythropoietic agent that activates repeatedly the EPO receptor. It has an elimination half-life in humans of about 130 h, and so can be administered every 3-4 wk[56-59]. Several studies have shown an effect similar to EPO in maintaining hemoglobin levels[60-65]. CERA is not available in United States, but is currently used in Europe. An additional advantage is that CERA can be kept out of the fridge and used for up to 1 mo (at < 25℃).

There is a new generation of erythropoietic analogues: synthetic erythropoiesis protein and peptide mimetics of EPO. These new agents stimulate erythropoiesis through activation of EPO receptors[66-69]. Hematide (developed by Affymax) is a synthetic peptide agonist of the EPO receptor, and although it has no structural homology with EPO, it is able to activate the EPO receptor and stimulate erythropoiesis over a 1-mo period, with good tolerance and stability at room temperature[66].

SIDE EFFECTS OF ESAs

The use of ESAs is related to several common side effects that should be well known by clinicians. Hypertension is the most common side effect, with an overall incidence of 5%-24%[70]. Vascular access thrombosis occurs with higher incidence[71,72], although it is not found in other small studies[73,74]. The risk of serious cardiovascular events, such as thromboembolism and death, increases when hemoglobin levels increase rapidly (> 1 g/dL every 2 wk)[75]. The development of anti-EPO antibodies is a very rare but serious complication of treatment with erythropoietic agents. From 1998 to 2004, it was apparent that there was a significant increase in the number of patients who developed pure red-cell aplasia (PRCA) secondary to the appearance of neutralizing anti-EPO antibodies[76,77]. PRCA is characterized by severe anemia, high transfusion requirement and a total lack of response to increasing doses of rHuEPO. Most of the reported cases have been patients with chronic renal failure who received rHuEpo α (Eprex) subcutaneously. PRCA was related to a change of formulation of Eprex (replacement of human serum albumin by polysorbate 80, because of the risk of bovine spongiform encephalopathy) and formation of immunogenic micelles. Alternatively, it has been suggested that leachates released by the uncoated rubber stoppers of the pre-filled syringes may interact with polysorbate 80 and

Table 1 Erythropoietic agents available

	Half-life (h)		Periodicity	Initial dose	Target levels
	iv	sc			
Epoetin β	9	24	1-3 times per week	100-150 IU/kg per week, (max 300 IU /kg)	
Epoetin α	7	20	1-3 times per week	100-150 IU/kg per week, (max 300 IU /kg)	Hemoglobin: 11-12 g/dL, Hematocrit: 33%-36%,
Darbepoetin α	25	48	Every 1-2 wk	0.45 μg/kg every 2 wk	↑ hemoglobin every 2 wk: 0.5-1 g/dL
CERA	133	137	Every 2-4 wk	0.6 μg/kg every 2 wk	

act as an adjuvant to the immune response. Failure in the cold chain is also a potential factor involved[78-80]. The number of cases reported has dropped significantly from 2003, with none in 2007. This may have resulted from a change in the route of administration (at present the iv route is used in most patients on dialysis), maintenance of the cold chain, or elimination of uncoated rubber syringe stoppers[75]. Should this complication occur, clinical guidelines for diagnosis and treatment are readily available[81-84]. It has been suggested that the use of the new analogue hematide in these cases may be effective[85].

Other possible side-effects are edema, fever, dizziness, insomnia, headache, pruritus, constipation, among other.

USEFULNESS OF ESAs IN DIGESTIVE DISEASES

IBD

Anemia is the most common complication of IBD[86,87]. Inadequate monitoring and treatment leads to a worse quality of life[88,89] and increased morbidity and hospitalization[90-92]. Repeated loss of blood, and to a lesser extent malabsorption of iron are the main causes of iron deficiency in IBD[86]. There is also a variable component of anemia that is related to chronic inflammation[3,4,7,87,93]. This involves failure of iron transport that is mediated by inflammatory cytokines, such as hepcidin, which is the main negative regulator of iron absorption in the small intestine and of iron sequestration by macrophages[94-101], and an inappropriately low production of EPO for the degree of anemia[16,90,101-103]. The management of anemia in IBD should focus on proper control of the inflammatory process, as well as iron supplementation, and in cases of resistance, to assess iron therapy in combination with erythropoietic agents[3,87,91,104]. Up to 25%-30% of patients with anemia associated with IBD combination therapy may require iron and erythropoietic agents to correct anemia[3,105,106]. In other diseases (cancer, rheumatoid arthritis, AIDS), EPO levels < 500 mU/mL (some authors suggest < 100 mU/mL) may respond to administration of rHuEPO[107-112].

In patients with anemia associated with IBD, high levels of transferrin (iron deficit indicators) as well as high levels of serum EPO (an indicator of a correct response to anemia) may predict a good response to treatment with iv iron. In contrast, low levels of serum EPO indicate the need to associate agents erythropoietic in addition to iron treatment[3].

Since the increased production of hepcidin in anemia of chronic diseases may limit the oral absorption of iron, it should be given by the iv route. The iv administration of iron has proven its efficacy, safety and tolerability, with iron sucrose[7,89] as the new formulation iron carboxymaltose, that allows the administration of 1 g of iron in 15 min[112,113]. The use of erythropoietic agents in the treatment of anemia associated with IBD is useful for patients who do not respond to treatment with iv iron, and in whom the aggressive treatment of IBD (including immunosuppressive therapy) has not abolished the mucosal inflammation, and who require additional blood transfusions[5,7,114-116].

Hemoglobin target

Previous studies on the use of EPO in patients with CKD have found different results concerning the desirable target level of hemoglobin and its effect on cardiovascular prognosis. In 1997, the KDOQI guidelines[117] recommended target levels for hemoglobin/hematocrit between 33% (11 g/dL) and 36% (12 g/dL). A similar recommendation was made in the 2006 update of the KDOQI guidelines[26] although on that occasion, an upper limit for hemoglobin was set, because there was no evidence to maintain a target ⩾ 13 g/dL. The European Best Practice Guidelines Working Group did not recommend the complete correction of hemoglobin levels in patients with diabetes or cardiovascular disease[29].

The clinical benefits and adverse effects associated with normal or near normal hemoglobin values were evaluated in multiple randomized studies that assessed mortality and morbidity from cardiovascular or cerebrovascular events, good control of blood pressure, quality of life, functional status and vascular access thrombosis[71,118-126]. The results of these studies have not suggested any improvements after correction of anemia, except in quality of life. Despite differences in their populations, two large randomized studies published in November 2006, the CHOIR[127] and CREATE[128] studies have shown that attempts to correct anemia completely did not reduce mortality or cardiovascular disease in CKD patients, compared with partial correction. A meta-analysis that included these two studies concluded that patients with a higher target hemoglobin have a significantly higher risk of all-cause mortality and vascular access thrombosis[129].

In light of these data, the KDOQI guidelines were reviewed and updated in 2007, with a recommended hemoglobin level of 11-12 g/dL, and not exceeding 13 g/dL[130]. Also the European Best Practice Guidelines Working Group[75] has concluded that hemoglobin

> 13 g/dL may be associated with cardiovascular events in these patients.

Factors that influence the increase in mortality with higher hemoglobin targets may include the impossibility of achieving the target hemoglobin, a too high hemoglobin target, the toxic effects of high doses of ESAs, the presence of comorbidity and other features[131-135]. These factors were evaluated in a second analysis of the CHOIR study[136] that found no clinical factor associated with risk, after adjustment and multivariate analysis, except for high dose of epoetin (> 20000 U/wk), which was an independent risk factor for death, myocardial infarction, heart failure, or stroke. This increased risk was observed in the high and low hemoglobin groups, particularly among those who did not reach the target hemoglobin. These results suggest that increased mortality is due to high doses of ESAs rather than higher hemoglobin targets[137]. Epoetin α dose should not exceed 20000 U/wk in patients with CKD, and probably in other diseases. These patients should be assessed for other causes of poor response to treatment with EPO. Some studies have suggested the possible relationship between the variability of the hemoglobin level and the patient[138,139]. Although there is variability in the results of different studies[120,131,140-143], the most consistent observation is that there are better results in terms of quality of life, with no increase in adverse reactions of hemoglobin in the range of 11-12 g/dL (hematocrit 33%) compared with lower levels[139-144]. There is a large study under way in relation to the normalization of hemoglobin. The Trial to Reduce Cardiovascular Events with Aranesp Therapy study is a randomized, placebo-controlled trial in pre-dialysis CKD patients with type 2 diabetes mellitus[145,146], which is due to end in 2011.

In clinical practice, given the difficulty of maintaining standards within the narrow target range of 11-12 g/dL[147-149], it's accepted the range 10-12 g/dL, particularly in patients with good tolerance.

The target haemoglobin level in patients with IBD is still to be determined. Data from recent studies in CKD patients indicate increased morbidity and mortality in relation to high levels of hemoglobin (normal)[130-133], as in patients with cancer[103,104]. It seems appropriate to establish a target hemoglobin level of 11-12 g/dL, which demonstrates greater benefit in quality of life and cost-effectiveness in renal patients[120,133,139-145].

Dosage, monitoring and control

To maintain adequate levels of hemoglobin with erythropoietic agents iron stores must be normalized. Iron should be administered in sufficient quantity to achieve a transferrin saturation ⩾ 20% and a ferritin level ⩾ 100 ng/mL[7,87,150]. The response to EPO is dose dependent, but varies from patient to patient, depending on the frequency and route of administration (iv or sc), although to a lesser extent with darbepoetin[151,152]. The hypertension may complicate treatment, particularly if hemoglobin level rises quickly (> 1 g/dL every 2 wk)[153]. With the present preparations, sc and iv administration are indistinguishable; however, sc administration presents some advantages over iv, such as a lower incidence of hypertension, and a 25%-50% reduction in dose compared with iv administration[154-157]. Another important advantage of iv administration is a longer half-life (24 h *vs* 9 h)[158,159]. Daily sc EPO is more effective than administration 2-3 times weekly[160], although administration less frequently than every 2 wk is also effective[161-163]. Discomfort at the injection site is minimal. It is important to remember to maintain the cold chain at 4℃ to preserve a high effectiveness[164].

EPO can be started at a dose of 100-150 U/kg per week sc with iron supplements. The dose can be increased by 25% every 2-4 wk to reach 300 U/kg per week. It is not worthwhile to continue increasing the dose in patients who do not respond after 12 wk, and in these patients, the dose should be kept to the minimum effective dose to avoid transfusion[97,131]. The appropriate response should increase the hemoglobin level at least 0.5 g/dL at 2-4 wk. It is recommended analytical control in this period of time and if the hemoglobin increase is over target (> 10-12 g/dL) or > 1 g/dL in 2 wk, we must reduce dose by 25%. In contrast, if the hemoglobin level is < 10 g/dL (with adequate iron deposits) or hemoglobin increase < 1 g/dL in 4 wk, then we must increase dose by 25%[101,104,165]. Children usually require higher doses than adults to achieve a similar response[29,166]. In practice, most patients are dosed per unit dose (syringe) rather than kg. There are wide range of doses in pre-filled syringes.

Darbepoetin α can be administered iv and sc, with the main advantage of the half-life three times longer than epoetin. Effectiveness of Darbepoetin has been proved with administration weekly, every 2 wk[167] and monthly[117,168,169]. The initial dose of darbepoetin is 0.45 μg/kg every 2 wk and that of CERA is 0.6 μg/kg every 2 wk. Once the patient has been stabilized, the monthly dose may be doubled. Several well-designed prospective studies in renal patients have demonstrated the safety and effectiveness of weekly or even monthly treatment with epoetin α[170-174] in a similar way to new formulations of ESAs with long half-life[175].

Resistance

Evidence of EPO resistance stems from studies with an inadequate response EPO in 5%-10% of patients[176] or in patients who develop EPO resistance after a good initial response. There is a resistance to EPO when a sufficient dose of it, equal to or greater than 300 U/kg per week is not reached the desired concentration of hemoglobin[28].

It has been shown that the most frequent and important cause of resistance to EPO is iron deficiency, but there are other less frequent factors[28,177].

Blood loss is the most frequent cause of absolute iron deficiency, which is defined by ferritin levels < 100 ng/mL, transferrin saturation < 20%, and/or hypochromic red cells increased by 10%. Relative or functional iron deficiency results from difficulty in transferring stored iron to red blood cells and is defined by the existence of transferrin saturation < 20% while

maintaining high levels of ferritin > 100 ng/mL. The main causes of relative iron deficiency are acute and chronic inflammatory processes and chronic liver diseases[178]. Elevated levels of parathyroid hormone in CKD patients are another cause of non-negligible resistance to EPO[179]. The presence of an inflammatory disorder increases the resistance to treatment with erythropoietic agents, including the production of inflammatory cytokines that interfere with iron metabolism, reducing their availability in the bone marrow and causing functional iron deficiency. The process of dialysis may be associated with an increase in the induction of cytokines and the appearance of an inflammatory response syndrome[180].

In patients undergoing hemodialysis, carnitine metabolism is altered and carnitine deficiency is more likely in patients with a protein-deficient diet and high dialysis dose[181]. Carnitine may improve anemia in hemodialysis patients with EPO resistance[182] by mechanisms not yet known[183], or stabilizing the membrane as an antioxidant agent, or by stimulating erythropoiesis, by the increase in the number of reticulocytes demonstrated in some patients on hemodialysis treated with L-carnitine[184]. The dose used in patients with EPO resistence is 1 g iv post-hemodialysis[185].

Folic acid is involved in the process of regeneration and maturation of hematopoietic precursors. Folic acid deficiency is associated an ineffective erythropoiesis, and a megaloblastic anemia. Malnutrition, malabsorption, alcoholism, and various drugs that lower intestinal absorption, such as diphenylhydantoin, contraceptives and barbiturates, can cause folic acid deficiency. In dialysis patients with resistance to EPO, with normal ferritin levels, adjuvant treatment with folic acid may be attempted. It has been shown that the use of folic acid (10 mg/d) in hemodialysis patients improves the response to EPO, especially when presented high mean corpuscular volume, even with normal levels of folic acid[186].

The effectiveness of erythropoietic response to stimulation can be assessed by resistance index of ESAs. This expresses the relationship between the dose of erythropoietic agents and hemoglobin concentrations maintained (IU/kg per week divided by hemoglobin). Resistance index of ESAs varies from one patient to another and also in the same patient over time. These values range from 0 in patients able to maintain adequate hemoglobin level through the endogenous production of EPO, to > 50 IU/kg per week and per g/dL of hemoglobin in patients who cannot maintain adequate hemoglobin, even after high-dose EPO therapy (> 300 IU/kg per week)[187-189].

Anemia following treatment with ribavirin in HCV patients

Hemolytic anemia is a frequent side effect of early use of ribavirin in the treatment of hepatitis C. It has a negative impact on quality of life, and it can, in extreme cases, cause deterioration in brain function and even death. Furthermore it is a common reason for reduction or discontinuation of antiviral therapy, which compromises the effectiveness and reduces the sustained viral response. The administration of EPO can improve anemia, without the need to reduce the dose of ribavirin[190-194].

FUTURE EXPECTATIONS WITH THE USE OF EPO

It has been discovered in recent years that the EPO is also synthesized locally by many tissues, especially in response to metabolic stress. EPO functions as a protective molecule that inhibits apoptosis in a wide variety of cell types, and reduces inflammation and local edema, as well as improving tissue regeneration[195]. The mechanisms of tissue protection are mediated by a receptor. This receptor is different other than that mediates the effects erithropoyetics of EPO[196]. The future of EPO therapy in ischemic diseases, and as protective cytotoxic therapies appears promising. Of course clinical trials comparing its efficacy with other conventional therapies are needed[197]. Also being evaluated is the effect of EPO on intestinal endothelial cells[45], and in the maintenance and repair of the mucosa[46].

SUMMARY AND PROSPECTS

For nearly 25 years, the anemia of chronic renal failure has been treated with rHuEPO, which has resulted in a significant improvement in quality of life and survival and avoidance of dependence on repeated transfusions[22-24]. Subsequently, rHuEPO has been used in other conditions that occur with anemia of chronic diseases such as cancer, rheumatoid arthritis[109] or IBD[3,5,114-116], and anemia associated with the treatment of hepatitis C[190-194]. It has also been investigated for its protective effects in other acute diseases such as myocardial ischemia and brain and kidney diseases[198]. The classical dosing schedule of three times weekly has been simplified with the advent of new generations of erythropoietic agents such as darbepoetin α, which allows administration every 2 wk, and CERA, which has a longer half-life that allows prolonged administration monthly. Research continues to develop new biologically similar molecules such as agonists of the EPO receptor as hematide, which allows the treatment of patients with PRCA and monthly administration[85]. As well as searching for orally active formulations that will simplify the management of anemia[199-202]. There have also been negative aspects of treatment with EPO, as the cases of pure red cell aplasia[64,80], or poor outcomes in relation to death and cardio vascular events in study of normalization of hemoglobin (in connection especially with high doses of EPO in patients with poor response)[127-129,137] and worse survival in patients with cancer[103,104]. However, EPO has been shown to be effective and safe in the treatment of anemia of chronic renal failure and has improved significantly quality of life of patients with chronic anemia[22-25].

Similarly, the development of iv iron therapy was a major breakthrough in the management of chronic anemia. Iron sucrose has demonstrated its safety, efficacy and tolerance[7,86,87]. Other new formulations of iron

that allow rapid iv administration of large doses with good efficacy and tolerance will make administration easier[5,110,202].

CONCLUSION

To summarize we can say that erythropoietic agents come to complete the range available therapeutic for treatment of anemia associated with IBD that begins by treating the inflammatory disease basis, as well as intravenous ferroterapia first choice. In cases of resistance to treatment with iron may raise a combined therapy with erythropoietic agents to try to achieve near-normal levels of hemoglobia/hematocrit (11-12 g/dL). The new formulations of iv iron (iron carboxymaltose) and the new generation of erythropoietic agents (darbepoetin and CERA) will allow a more comfortable and spaced dosage schedule, with the same efficacy and safety.

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, *Series Editors*

Blood transfusion for the treatment of acute anaemia in inflammatory bowel disease and other digestive diseases

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Abstract

Allogeneic blood transfusion (ABT) is frequently used as the first therapeutic option for the treatment of acute anaemia in patients with inflammatory bowel disease (IBD), especially when it developed due to gastrointestinal or perioperative blood loss, but is not risk-free. Adverse effects of ABT include, but are not limited to, acute hemolytic reaction (wrong blood or wrong patient), febrile non-hemolytic transfusional reaction, bacterial contamination, transfusion-related acute lung injury, transfusion associated circulatory overload, transfusion-related immuno-modulation, and transmission of almost all infectious diseases (bacteria, virus, protozoa and prion), which might result in increased risk of morbidity and mortality. Unfortunately, the main physiological goal of ABT, i.e. to increase oxygen consumption by the hypoxic tissues, has not been well documented. In contrast, the ABT is usually misused only to increase the haemoglobin level within a fixed protocol [mostly two by two packed red blood cell (PRC) units] independently of the patient's tolerance to normovolemic anaemia or his clinical response to the transfusion of PRC units according to a "one-by-one" administration schedule. Evidence-based clinical guidelines may promote best transfusion practices by implementing restrictive transfusion protocols, thus reducing variability and minimizing the avoidable risks of transfusion, and the use of autologous blood and pharmacologic alternatives. In this regard, preoperative autologous blood donation (PABD) consistently diminished the frequency of ABT, although its contribution to ABT avoidance is reduced when performed under a transfusion protocol. In addition, interpretation of utility of PABD in surgical IBD patients is hampered by scarcity of published data. However, the role of autologous red blood cells as drug carriers is promising. Finally, it must be stressed that a combination of methods used within well-constructed protocols will offer better prospects for blood conservation in selected IBD patients undergoing elective surgery.

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Key words: Anaemia; Blood transfusion; Autologous blood transfusion; Inflammatory bowel diseases; Risk assessment

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INTRODUCTION

In surgical, medical and critically ill patients, allogeneic blood transfusion (ABT) is frequently used as the first therapeutic option for the treatment of acute anaemia, especially when it developed due to traumatic or acute blood loss or when the patient's compensatory mechanisms have a limited capacity of response[1-4]. As a result, a high proportion of patients receive at least one ABT unit during their hospital stays.

However, many reports show variation in transfusion practice for comparable groups of patients between hospitals, and even between different departments

within a hospital. Variations in rates of transfusion may be due to many factors, but still differing opinions on the threshold level of haemoglobin (Hb) and the implementation of transfusion alternatives are the most important ones. The first may reflect uncertainty about the benefits and risks of ABT, and the second different perceptions of the value of stimulation of erythropoiesis and minimising blood loss and subsequent transfusion[5].

Of note, the main physiological goal of ABT, i.e. to increase oxygen consumption by the hypoxic tissues, has not been well documented[6-8]. In contrast, the ABT is usually misused only to increase the Hb level within a fixed protocol [mostly two by two packed red blood cell (PRC) units] independently of the patient's tolerance to normovolemic anaemia or his clinical response to the transfusion of each PRC unit according to a "one-by-one" administration schedule.

Unfortunately, good clinical studies and outcome data establishing the benefits and risks of ABT for a patient in different clinical settings are not available[9]. To the best of our knowledge, there are very few situations or pathological entities where ABT and/or liberal transfusion criteria have shown to benefit our patients (drepanocytosis, thalassemic or low-weight premature infants)[10,11]. Thus, to promote best transfusion practices and reduce variability, minimizing the avoidable risks of transfusion, and to help clinicians in choosing the appropriate treatment options, a number of clinical guidelines have been issued by different medical societies[12-17]. In this article, we review the indications, benefits and risks of blood transfusion of the different blood components available, both allogeneic and autologous, with special emphasis on patients suffering from inflammatory bowel disease (IBD), either ulcerative colitis (UC) or Crohn's disease (CD).

INDICATIONS FOR ABT

Transfusion of PRC is indicated in order to achieve a fast increase in the supply of oxygen to the tissues, when the concentration of Hb is low and/or the oxygen carrying capacity is reduced, in the presence of inadequate physiological mechanisms of compensation. Tissue oxygenation depends on various factors: (1) the concentration of Hb; (2) the saturation of Hb, which, in turn, depends on the O_2 tension and the affinity of the Hb for O_2; (3) the O_2 requirements, that is, the volume of oxygen needed by the tissues to carry out their aerobic function. But, there are a number of clinical factors that affect the physiological mechanisms of adaptation to anaemia: (1) a reduced increase in cardiac output: hypovolaemia, coronary artery disease, disorders of heart valves, congestive heart disease, negative inotropic drugs; (2) decreased capacity to increase the extraction of O_2: acute respiratory distress syndrome (ARDS), sepsis, systemic inflammatory response syndrome (SIRS), traumatic ischemia-reperfusion syndrome; three, altered gas exchange: chronic obstructive pulmonary disease (COPD), ARDS; and four, increased consumption of O_2: fever, pain, stress, sepsis, SIRS or hyperventilation syndromes[17].

When there is an indication to correct anaemia, but the situation is not urgent, strategies other than transfusion are preferred, such as the use of haematopoietic drugs (iron, vitamin B12, folic acid, recombinant erythropoietin) (see another chapter).

Non-surgical IBD patients

Nowadays the administration of oral or IV iron supplements, with or without erythropoiesis-stimulating agents, but not ABT, is the standard therapy for the anaemia of the medical IBD patient (see corresponding article in this issue). According to the recommendations of the Guidelines on the Diagnosis and Management of Iron Deficiency and Anemia in Inflammatory Bowel Diseases (Statement 3A), the goals of anaemia treatment are to increase Hb, and iron studies above the lower threshold of normal, to prevent a further fall in Hb, to avoid the use of ABT, to relieve symptoms related to anaemia, and to improve the quality of life (Grade D)[18]. Nevertheless, some IBD patients are still being transfused, mostly because of acute gastrointestinal haemorrhage, although the Guidelines do not provided any recommendations in this regard. In our modest opinion, these ABT are some times administered without strict criteria, just to raise a low Hb figure ("cosmetic" transfusion) or to treat a hypovolemic situation, but not to correct transitorily hypoxic symptoms or signs.

Fortunately, severe acute gastrointestinal haemorrhage is uncommon in IBD, although among children 0-5 years of age patients presented with more rectal bleeding than patients aged 6-17 years, despite the later having a more complicated disease course[19].

Belaiche *et al*[20] reviewed 34 cases of acute gastrointestinal haemorrhage in CD patients (defined as acute rectal bleeding originating in diseased bowel and requiring an ABT of at least 2 U of PCR within 24 h). When the bleeding source was identified, the bleeding lesion was an ulcer in 95% of cases, most often in the left colon. The treatment was surgical (20.6%), endoscopic (20.6%), or medical (58.8%), and there were no deaths. The authors concluded that given the potential efficacy of endoscopic or medical treatment, as well as the absence of mortality, a conservative approach may be suggested as first-line therapy in the majority of patients.

Similarly, Pardi *et al*[21] characterized the clinical features and course of such haemorrhage in patients at their institution from 1989 to 1996. Thirty-one patients had acute lower gastrointestinal bleeding from IBD (three UC, 28 CD), representing approximately 1% of admissions for IBD. Again, the bleeding lesion presented with an ileocolonic or colonic localization in most cases (68%), and surgery was required in less than half of cases. However, in both patient series, recurrent haemorrhage was not rare, and for these cases surgery may be the most appropriate treatment.

More recently, Kostka *et al*[22] characterized the clinical features and course of 11 separate episodes of severe haemorrhage in six of 156 patients with CD (3.8%),

treated between 1985 and 2003 at their institution. Emergency surgery was necessary to stop the primary or recurrent haemorrhage in four patients. The authors concluded that, although a conservative approach may be first-line therapy, surgery is inevitable in patients suffering from massive bleeding and in patients with recurrent bleeding. Similar conclusions were reached by Veroux *et al*[23] in a previous series of five CD patients with severe gastrointestinal bleeding.

In a recent review of five years, Campos *et al*[24] reported 301 episodes of massive transfusion occurred at a university hospital, and found that 51 out of these 301 episodes were due to upper gastrointestinal haemorrhage (17%), with a mortality rate of 52%. It is recommended that the Transfusion Services have a special protocol to manage severe bleeding emergency, however the blood component administration schedules are neither clear nor uniform. In the last few years, there has been a general review of the blood component schedule during massive transfusion[25]. Nowadays, data from several large observational studies show that early beginning of plasma and platelet administration, including the use of the so-called "transfusion packs", could improve mortality of patients with severe bleeding[26].

Surgical IBD patients

As for acute gastrointestinal haemorrhage, other emergency complications of IBD are rare, but may be life-threatening, require surgery, and result in permanent end organ damage. The most common non-bleeding complications associated with UC are fulminant colitis and toxic megacolon, and often result in a total proctocolectomy. The most common non-bleeding complications associated with CD are abscesses and intestinal obstruction, and usually also require surgical intervention and intestinal resection[27].

Despite pharmacological advancements, the management of IBD has become increasingly complicated. In a study of 2892 adults with CD and 5895 with UC who received care between 1998 and 2005, Herrinton *et al*[28] found: a shift in gastroenterology-related visits from the gastroenterology division to primary care; an increased use of IBD-related drugs; a 36% decline in the prevalence of prolonged steroid exposure for CD patients, with a 27% increase for UC patients; a 30% decline in the hospitalization rates for CD and UC patients; and a 50% decline in the surgery rate for UC, but no significant change for CD. In this regard, it is worth noting that in UC patients, surgery remains an important part of the overall treatment plan, especially for prophylactic total colectomy in certain patients at high risk for colorectal cancer[29].

As for other patients undergoing major surgery, in the surgical IBD patient, intraoperative and postoperative management of potential or actual blood loss should include (ASA)[16].

Monitoring the amount of blood loss: A periodic visual assessment of the surgical field and communication with the surgical team should be done to assess the presence of excessive microvascular bleeding (i.e. coagulopathy).

Monitoring for the presence of inadequate perfusion and oxygenation of vital organs: By using conventional (e.g. blood pressure, heart rate, oxygen saturation, urine output, electrocardiography) and/or special monitoring systems (e.g. echocardiography, mixed venous oxygen saturation, blood gasses, National Institute of Radiological Sciences).

Monitoring for transfusion indications: Measure Hb or haematocrit when there is substantial blood loss or there is any indication of organ ischemia. However, as the haematocrit value is subjected to a variety of bias, especially in hypovolemic anaemic patients, Hb concentration should be preferred[30]. In this regard, it's worth noting that in most of the available hematological analyzers the "haematocrit" is an indirectly calculated parameter.

Transfusion of allogeneic red blood cells (RBCs): Maintain adequate intravascular volume and blood pressure with crystalloids or colloids until the criteria for ABT are met (see below). Adequate quantities of RBCs should be transfused to maintain organ perfusion (PCR units should be transfused one-by-one, and anaemia symptoms reassessed after each transfusion). When appropriate, pre-deposited autologous blood donation and other means to decrease blood loss (e.g. deliberate hypotension, antifibrinolytics) may be beneficial (see below).

Hb transfusion triggers

The only indication for the transfusion of PRC is to correct or prevent tissue hypoxia; thus, the parameter "of choice" for making decisions should be intracellular pO_2. However, this parameter is not useful for clinical purposes and it is, therefore, necessary to rely on "surrogate" parameters, such as Hb and the haematocrit. The indication for and the degree of urgency of PRC transfusions cannot, however, be defined only on the basis of the values of Hb or the Htc, but must be based on a complete evaluation of the patient's clinical condition and the possible presence of mechanisms compensating for the anaemia (see above)[17].

According to ASA guideline on blood transfusion[16], PRC should usually be administered when the Hb level is less than 6 g/dL, whereas they are usually unnecessary when the level is more than 10 g/dL. These conclusions may be altered in the presence of anticipated blood loss. The determination of whether intermediate Hb concentrations (i.e. 6-10 g/dL) justify or require PRC transfusion should be individualised. This indication should be based on any ongoing indication of organ ischaemia, potential or actual ongoing bleeding (rate and magnitude), the patient's intravascular volume status, and the patient's risk factors for complications of inadequate oxygenation. These risk factors include a low cardiopulmonary reserve and high oxygen consumption[16,17].

Table 1 Transfusion haemoglobin threshold according to patient's age and characteristics and type of anaemia

Haemoglobin threshold (g/dL)	Patients characteristics and type of anaemia
< 5	Patients with chronic anaemia and without risk factors[1]
< 6	Patients with symptomatic[2] chronic anaemia and without risk factors
	Acute anaemia in younger patients
< 7	Asymptomatic chronic anaemia in patients with risk factors
	Acute anaemia in surgical and critically ill patients
< 8	Acute anaemia in surgical patients older than 65 yr
< 9	Acute anaemia in patients with organ dysfunction[3]
< 10	Patients with massive transfusion
> 10	Do not transfuse

[1]Risk factors; [2]Symptomatic anaemia; [3]Organ dysfunction.

These indications are in agreement with those proposed by most international clinical guidelines and documents issued by several medical societies, such as the American Consensus Conference on perioperative red blood cell transfusion (Hb < 8 g/dL)[12], British Haematological Society (Hb < 8 g/dL)[13], the Scottish Guideline on Perioperative Blood Transfusion (Hb < 7 g/dL)[4], or the Spanish Society for Blood Transfusion (Hb < 7 g/dL and Hb < 5 g/dL in chronic anemia)[15]. Recently, the Italian Transfusion Society[17] proposed the same recommendations as ASA[16], but giving a Grade of recommendation 1A. A tentative table of Hb transfusion thresholds as a function patient's age and characteristics and type of anaemia is given in Table 1.

Transfusion yield

As a rough guide, in adults one unit of PRC increases the Hb concentration by 1 g/dL and the haematocrit by about 3%. In children, the transfusion of 5 mL/kg increases the Hb concentration by about 1 g/dL. In the case of a lower than expected transfusion yield, conditions causing the loss, sequestration or destruction of RBCs should be looked for. Such conditions include: occult bleeding, repeated blood sampling (particularly in children), fever, hypersplenism, primary and secondary immunological causes, mechanical or other type of hemolysis[17].

BENEFITS OF ABT

The experience with Jehovah's Witness patients suggests that, overall, postoperative anaemia is well tolerated if Hb > 7 g/dL, but it increases the risk of mortality when Hb falls below 5 g/dL, this effect being magnified by blood loss and cardiac disease (Hb < 10 g/dL), and indirectly suggests that ABT is life-saving in this context[9]. Also, ABT may be life-saving in children with severe anaemia and signs of respiratory distress and, possibly, in very low birth weight infants[9]. In contrast, for critically ill patients, severe anaemia (Hb < 8 g/dL) increases the risk of mortality by 1.54, whereas the transfusion of 1-2, 3-4, or more than 4 U increases this risk by 1.48, 2.68, and 4.01, respectively[31]. However, ABT may be life-saving in extremely ill patients with cardiovascular disease[11]. Theoretical reasons for using a "liberal" or "restrictive" transfusion protocol are given in Table 2.

Table 2 Theoretical reasons supporting the restrictive or the liberal use of allogeneic red cells in normovolemic patients

Rationale supporting the liberal use of red cells
Augmenting O_2 delivery may improve patient survival and functional recovery
Increased risk of coronary ischaemia due to increased demand
Reduces respiratory work
Age, disease severity and drugs may interfere adaptation to anaemia
Improved safety margin if further blood loss
Increased safety of donor blood products
Rationale supporting the restrictive use of red cells
Moderate anaemia has not proved to increased mortality
Red cell transfusions impair microcirculatory flow
Progressive loss of red cell functionality during storage
Pathologic supply dependency is rare
Risk of pathogen transmission
Immunodepression causing increased infections and tumor relapse following transfusion
Risk of TRALI and TACO
Blood products are increasingly scarce and expensive

TRALI: Transfusion-related acute lung injury; TACO: Transfusion associated circulatory overload.

On reviewing the literature, it can not be concluded that ABT is beneficial for less sick patients and those without cardiovascular disease when Hb is above 7 g/dL[11,32]. On the other hand, the effects of anaemia below 7 g/dL and the subsequent beneficial effects of ABT, if any, can also not be determined from published studies since patients either refuse to receive ABT for religious reasons or are transfused systematically, except in the case of chronically anaemic patients like those with thalassemia or drepanocytosis[10].

As for patients with cardiovascular diseases, there appears to be some evidence that ABT, in small amounts, can reverse ischaemic changes and restore normal myocardial function. However, the randomized controlled trials conducted so far have offered contradictory results regarding the safety of restrictive transfusion triggers in older patients. Whilst four of them reported that a restrictive transfusion protocol resulted in the transfusion of appreciably fewer units of RBC, with no differences between groups regarding postoperative morbidity or mortality[11,32-34], the last one which was designed to find differences in postoperative quality of life, found that in elderly patients undergoing surgery for hip fracture repair a restrictive transfusion threshold (Hb < 8 g/dL) may result in a higher incidence of postoperative cardiovascular complications (10% *vs* 2%, $P = 0.05$) and 30 d mortality (8% *vs* 0%, $P = 0.02$) when compared with a liberal transfusion threshold (Hb < 10 g/dL)[35]. The Transfusion Trigger Trial for Functional Outcome in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair[36], which has been planned to be a 2600 patient, multicentre clinical trial, will most probably address the question of whether patients with cardiovascular disease

or cardiovascular risk factors undergoing surgical repair of hip fracture benefit from a higher or lower transfusion trigger[15]. While data from randomized trials are available, patients with suspected or proven myocardial ischaemia must be closely monitored and ABT should be given unit-by-unit until cardiac function is normalized, rather than to adopt a fixed transfusion trigger for all such patients[15].

Packed red cell transfusion to a predeterminated Hb in view of optimizing oxygen transport is definitely not supported by a large trial in critically ill patients [Transfusion Requirements In Critical Care (TRICC) trial][11]. In fact, in the study by Hébert *et al*[11], patients submitted to a restrictive transfusion strategy seem to have better outcomes that those submitted to a liberal transfusion strategy, especially those who are younger (< 55 years old) or less ill (APACHE score < 20). However, the analysis by Deans *et al*[37] suggests that the results of the TRICC trial were strongly influenced by non-comparable subgroups with different practice misalignments in each arm of the study. The excess risk incurred by each of these subgroups makes the comparison of mortality rates between the two treatment arms in the overall study difficult to interpret. Furthermore, as publications before this trial indicated that clinicians used higher transfusion thresholds in patients with ischaemic heart disease compared with younger, healthier patients, neither arm fully represented or was compared with current practices, and it remains unclear if the use of an absolute transfusion threshold is superior to adjusting therapy based on individual patient characteristics or implementing consensus guidelines for transfusion practices[37].

The rationale behind ABT is to restore oxygen delivery and provide a reserve should further blood loss occurs. After PRC transfusion, an increase in Hb levels is readily observed, but controversial results were found when evaluating the influence of stored PRCs on tissue oxygenation, assessed by surrogate markers of oxygenation, such as gastric pH and CO_2[7,8]. Very recently, Leal-Noval *et al*[6] and Smith *et al*[38] studied the effect of ABT on cerebral oxygenation in patients with severe traumatic brain injury and found erythrocyte transfusion to be associated with a variable and prolonged increment of cerebral tissue oxygenation. However, no relationship was observed between brain tissue partial pressure of oxygen ($PtiO_2$), cerebral perfusion pressure and Hb concentration. In addition, 3 h after transfusion, all patients with basal $PtiO_2$ < 15 mmHg showed an increment in $PtiO_2$, *vs* 74.5% of patients with $PtiO_2 \geqslant$ 15 mmHg, $P < 0.01$)[6]. Moreover, in a subsequent study, this group demonstrated that an increment in $PtiO_2$ was only observed in patients receiving blood stored for less than 19 d[39]. Thus, low baseline $PtiO_2$ levels could define those patients who benefit the most from ABT.

However, ABT providing Hb of 10-11 g/dL might be required for patients with COPD, as it may reduce minute ventilation and the work of breathing, for patients bleeding to improve haemostasis in massive transfusion[25,26], and for surgical patients with sickle cell disease, reducing HbS levels by direct transfusion or by RBC exchange (partial or total) to avoid sickling crisis[10,14].

In conclusion, while ABTs save lives, they save far fewer lives than we have been taught to believe. As ABT will never be a risk free therapy (see below), each and every unit transfused unnecessarily is, potentially, a noxious unit. In other words, each ABT which is not strictly indicated is severely contraindicated!

RISKS OF ABT

Nowadays, due to a careful donor selection and the introduction of highly sophisticated tests for pathogen detection, ABT is safer but more scarce and expensive than ever, and is still not risk-free. Transfusion therapy with PRCs can cause adverse reactions, which are classified on the basis of their etiopathogenesis and the time of occurrence with respect to the transfusion. Adverse effects of ABT (Table 3) include a broad panoply including, but not limited to, incorrect blood transfusion ("wrong blood"), acute or delayed haemolytic transfusion reaction, allergic reaction (from urticaria to anaphylactic reaction), bacterial contamination, transfusion-related acute lung injury (TRALI), transfusion associated circulatory overload (TACO), transfusion-related immuno-modulation (TRIM), and transmission of infectious diseases (viruses, protozoas and prions)[40], which result in increased risk of morbidity and mortality. As the risks of ABT-transmitted viruses were reduced to exceedingly low levels in the US and Europe, TRALI, haemolytic transfusion reactions (HTRs), and transfusion-associated sepsis (bacterial contamination) emerged as the leading causes of ABT-related deaths[41]. Since 2004, preventive measures for TRALI and bacterial contamination have been implemented, but their implementation remains incomplete[41]. Infectious causes of ABT-related deaths currently account for less than 15% of all transfusion-related mortality, but the possibility remains that a new transfusion-transmitted agent causing a fatal infectious disease may emerge in the future[40,41]. These, together with the possible advantages of implementing restrictive transfusion protocols, strongly indicate that transfusion practice in surgery and intensive care should be (and must be) modified, especially in terms of the level of pretransfusion Hb ("restrictive" *vs* "liberal" use of ABT; "acceptable Hb" *vs* "optimal Hb") (Table 2). Briefly, we will describe several of the most important adverse reaction to transfusion.

Transfusion reaction: According to Serious Hazards of Transfusion report 1994-2004, 1832 out of 2628 reported incidents corresponded to episodes of incorrect blood component transfused (IBCT) (wrong blood product or wrong patient)[42]. Thus, the incidence of reported IBCT is about 1:20 000-25 000 and the consequences can be disastrous. In Spain[43], 246 "near-miss administration errors" and 134 of IBCT were reported during 2007. Of those, 49 led to HTRs (33 due to ABO incompatibility), with at least three deaths reported and confirmed[4]. In a Spanish region (Catalunya) the incidences were estimated as 1:11 000 of IBCT, 1:77 000 of "ABO-mistakes" and 1:310 950 of death secondary to transfusion[44]. As for

Table 3 Risks or hazards of allogeneic blood transfusion

Acute transfusion reactions
Immunologic reactions
Acute haemolytic reaction (or THRs)
Febrile non-haemolytic reaction
Allergic reactions: Urticaria and anaphylaxis
Acute non-cardiogenic pulmonary edema: TRALI
Alloimmunization with acute platelet destruction
Non-immunologic reactions
Bacterial contamination
TACO
Hypotensive reaction
Non-immunologic haemolysis
Others: Hypocalcemia, hyperkalemia (cardiac arrest), hypothermia, hyperglycemia, *etc*
Delayed transfusion reactions
Immunologic reactions
Delayed haemolytic reaction
Alloimmunization against blood cell antigens (also platelets and leukocytes)
Graft *vs* host disease
Transfusion-related immunomodulation
Post-transfusion purpura
Non-immunologic reaction
Transfusion-transmitted infection: viruses (Hepatitis A, B, C, E, VIH 1-2, West Nile virus, HTLV Ⅰ-Ⅱ, Citomegalovirus, Virus Herpes viridae, TTV, SEN-1, SARS, *etc*), protozoa (malaria, babe biosis, Chagas disease, *etc*), prion (new variant Creutzfeldt Jacob disease)
Post-transfusion hemosiderosis (iron overload)

TRALI: Transfusion-related acute lung injury; TACO: Transfusion associated circulatory overload.

the surgical patient, general anaesthesia may mask the symptoms of both HTRs and non-HTRs. Signs of HTRs include hypotension, tachycardia, haemoglobinuria and microvascular bleeding, but these may be erroneously attributed to other causes in the anaesthetized patient. The most common signs of a non-haemolytic transfusion reaction in awake patients include fever, chills, or urticaria. However, these signs may not be detectable during anaesthesia. Thus, checking for signs and symptoms of a THR should periodically be done in the anaesthetized patient, including urine output and colour and peak airway pressure[16].

Bacterial contamination: Bacterial contamination of blood products, most frequently platelets, is one of the leading causes of death from ABT[40,41] (seven deaths in UK from 1996 to 2004)[42]. The increased risk of bacterial overgrowth is related to a storage temperature of > 20-24℃. Many blood banks are now culturing their platelet concentrates, or inactivating them, although this leads to a decreased functionality. If a patient develops a fever within 6 h of receiving platelets, sepsis from contaminated platelets may be a possibility. Between 2001 and 2003, an average of 11.7 years in the United States were reported to the Food and Drug Administration, whereas 7.5 per year were reported in 2004 and 2005 - a decrease attributable in part to the mandating of bacterial screening of platelets beginning in 2004[40]. In Spain, during 2007 at least 17 cases, with one death, have been reported and confirmed[43].

TRALI: TRALI is a well-characterized and serious adverse consequence of blood product transfusion; its overall occurrence is almost certainly more common than the quoted estimate of one case in 4000 U of blood transfused (as TRALI is generally unrecognized or misdiagnosed, its actual incidence is unknown)[45]. TRALI is non-cardiogenic pulmonary edema resulting from immune reactivity of certain leukocyte antibodies a few hours after transfusion. Signs and symptoms will appear 1-2 h after transfusion and are in maximum force within 6 h. Hypoxia, fever, dyspnea, and even fluid in the endotracheal tube may occur. There is no specific therapy other than stopping transfusion and instituting critical care supportive measures. Most patients recover in 96 h, although TRALI is one of the top three most common causes of transfusion related deaths[45]. In Spain, 32 cases have been reported and confirmed during 2007, with at least two deaths[43]. The estimated rate was 1/32 000 (at Catalunya), but the authors suspected a great underreporting[44]. For example, during preparation of this manuscript one of the authors diagnosed one highly probable case of TRALI during a plasma exchange, but no physician at the intensive care unit knew what TRALI meant.

TACO: TACO is a cause of hydrostatic pulmonary oedema with clinical and radiologic manifestations similar to those of TRALI. In fact, the distinction between TRALI and TACO after transfusion is difficult, in part because the two conditions may coexist[44,46]. During 2007, in Spain at least 39 cases had been reported and confirmed, with at least one death confirmed[43].

TRIM: TRIM is associated with increased risk of postoperative infection[2,3,47-49]. The combined data from three studies including over 1700 patients undergoing elective cardiac surgery with cardiopulmonary bypass showed that (1) transfusion of RBC concentrates was independently associated with increased rates of postoperative pneumonia, mediastinitis and sepsis; (2) these effects were dose-dependent and storage-time-dependent; and (3) other blood components might be also involved[50-52]. As for patients undergoing elective surgery for gastrointestinal cancer resection or urgent surgery for hip fracture repair, similar data have been reported[4,49,53].

Infectious diseases: Another major adverse effect of transfusion therapy is the transmission of infectious agents[40,41]. For the past 20 years, transfusion induced hepatitis and acquired immunodeficiency syndrome (AIDS) have been dominant concerns regarding ABT. These infectious risks are now very rare. One of the major reasons for the decrease in blood borne infections has been the use of nucleic acid technology (NAT). The human immunodeficiency virus (HIV), C hepatitis virus (CHV), and West Nile virus can now be detected by this technology. To date, malaria, Chagas disease, severe acute respiratory syndrome (SARS), and variant Creutzfeldt-Jakob disease (vCJD) cannot be detected[16]. The possible safety intervention that might further

reduce the risk of transfusion-transmitted infection is not static, as new agents continue to emerge, old ones change their properties and epidemiologic patterns, and new information and technology become available to change our understanding of that risk[40,41]. During 2007 in Spain, twenty five cases of possible hepatitis B or C were reported, but none of them were confirmed[43]. Between 2005-2007 five cases of Chagas disease were diagnosed, although four of them retrospectively after a "look back"[43].

PREOPERATIVE AUTOLOGOUS BLOOD DONATION (PABD)

PABD consists of obtaining the patient's own blood prior to surgery in order to administer it if necessary afterwards[1,54]. Extraction frequency, type of blood component or bag for conservation, and volume of blood extracted are established by each Local Blood Bank on an individual basis[1,54-56]. In the elective surgical setting, PABD is a convenient, predictable, safe and widely practised form of transfusion support[4]. However, PABD cannot be made available to all patients (minimal Hb ⩾ 11 g/dL), and it is contraindicated in the presence of several infectious, cardiac, oncologic or neurologic pathologies. Hospital admission and operative dates must be guaranteed, as donated blood has a limited storage life of 35 d (up to 42 d in additive SAG-mannitol solution). In addition, it used to carry some of the risks of ABT, especially IBCT, although in the European Union there are regulations aimed to reduce these risks (e.g. personalized unit identification, separate conservation and transport, de-referral for allogeneic use, *etc*), and these can often present logistical difficulties[1,54,55]. Nonetheless, PABD avoids immunological and viral hazards of ABT. In spite these benefits, PABD use is decreasing worldwide[56].

In accordance with the conclusion of the Spanish Consensus Statement on Alternatives to Allogeneic Blood Transfusion (Seville's Document)[55]: PABD would be indicated in elective surgery if the risk of ABT > 20%-30%, and in patients with difficulties receiving ABT; PABD can be used safely in children and elderly populations[54,57]; the administration of rHuEPO in patients with moderate anaemia, facilitates PABD[57]; PABD contribution to ABT reduction is decreased when a transfusion protocol is adopted; PABD may have problems of over-collection and over-transfusion (in fact, PABD increases the total number of transfusion episodes). It is not without infectious risks; PABD erythrocytes undergo "storage lesion". PABD has been classically associated with higher rates of "clerical errors", and ABT may still be required (break-through transfusion).

In this regard, it is well known that surgery in IBD is frequently associated with a need for perioperative blood transfusions, but PABD is often limited by IBD-associated anaemia, although it is reversible by intravenous iron and rHuEPO. Consequently there is a paucity of studies on the use of PADB in surgical IBD patients (and none in the use of perioperative cell salvage or acute normovolemic haemodilution). Mittermaier *et al*[58] tested the feasibility of PABD (2-4 U; 350-450 mL blood per unit; 1 U/wk) in six patients (five CD, one UC) with indications for elective bowel resection IBD. Patients received 200 mg of iron sucrose IV after each donation, plus concomitant rHuEPO if there was preexisting anaemia or C-reactive protein > 2 mg/dL. Four patients received PABD transfusions intra- or postoperatively, and no patient needed ABT. No serious adverse events were observed during blood donations, perioperatively, and during the one year follow-up period. Thus, when appropriately indicated and supplemented with IV iron and rHuEPO, PABD seems to be safe and useful for surgical IBD patients. Since 2003, we have treated 2668 PABD patients in our hospital, only three of them with IBD (two CU and one EC) scheduled to prostatectomy, coxarthrosis and avascular femoral necrosis surgeries, donating two units each patient. The first patient required two doses of β-epoetin 30 000 IU plus iron sucrose iv 400 mg, and the second was transfused with two PABD units plus two ABT units.

Recently, PABD has been used, not as an alternative to ABT, but as a carrier of drugs, owing to the ability of the RBC membrane to be opened and resealed under appropriate conditions. In an uncontrolled pilot study, Annese *et al*[59] investigated efficacy and safety of dexamethasone-encapsulated erythrocytes in 10 steroid-dependent adults IBD patients (five UC, five CD). Fifty milliliters of blood were drawn from each subject; dexamethasone 21-phosphate (Dex 21-P) was encapsulated into erythrocytes by means of specially designed equipment, and drug-loaded erythrocytes were infused into the original donors (5.5 ± 2.4 mg Dex 21-P). The procedure was repeated after 4 and 8 wk, and patients were instructed to withdraw corticosteroids. After the third infusion, all patients were in clinical remission. After a mean follow-up of 12 ± 3 mo, six patients relapsed, and the remaining four patients remained in remission. Pre-existing steroid-related adverse effects disappeared during the follow-up. The authors concluded that Dex 21-P loaded autologous RBC is a feasible technique, which is safe, maintains patients in clinical remission and allows steroid withdrawal.

More recently, infusions of autologous RBCs loaded with Dex 21-P, performed every 4 wk for 24 mo, to 18 consecutive paediatric patients with steroid-dependent CD resulted in a reduction of the CD activity index, 78% of patients discontinued steroids and endoscopic findings showed remission in 44% of patients, whereas none of the patients experienced serious side effects[60]. Therefore, infusions of autologous RBCs loaded with Dex 21-P seem to be safe and useful for maintaining long-term remission in paediatric patients with moderately active CD. However, large randomized controlled trials are needed to confirm these promising results.

CONCLUSION

ABT used to be the first therapeutic option for the treatment of acute anaemia in IBD patients, especially

when it developed due to gastrointestinal or perioperative blood loss, but was not risk-free. Adverse effects of ABT include, but are not limited to, IBCT (wrong blood), THRs, bacterial contamination, TRALI, TACO, TRIM and transmission of infectious diseases, which might result in increased risk of morbidity and mortality. Evidence-based clinical guidelines may promote best transfusion practices and reduce variability, minimizing the avoidable risks of transfusion, and help clinicians in choosing the most appropriate treatment for the patient-ABT, PABD or pharmacologic alternatives. The acceptance of normovolemic anaemia is indeed one of the most effective measures to reduce ABT requirements. Thus, the adoption of restrictive transfusion criteria (lower threshold level of Hb) must be the first measure to implement in order to reduce both the number of transfused units and transfused patients. Obviously, the second measure must be the pharmacological treatment of anaemia. PABD consistently reduces the frequency of ABT, with a small incidence of adverse effects, although its contribution to ABT avoidance is reduced when performed under a transfusion protocol. In addition, interpretation of utility of PABD in surgical IBD patients is hampered by scarcity of published data. However, the role of autologous RBCs as drug carriers is promising. Finally, for selected patients, a combination of methods used within well-constructed protocols will offer better prospects for blood conservation in IBD patients undergoing elective surgery.

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ORIGINAL ARTICLES

Clonality and allelotype analyses of focal nodular hyperplasia compared with hepatocellular adenoma and carcinoma

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Abstract

AIM: To identify clonality and genetic alterations in focal nodular hyperplasia (FNH) and the nodules derived from it.

METHODS: Twelve FNH lesions were examined. Twelve hepatocellular adenomas (HCAs) and 22 hepatocellular carcinomas (HCCs) were used as references. Nodules of different types were identified and isolated from FNH by microdissection. An X-chromosome inactivation assay was employed to describe their clonality status. Loss of heterozygosity (LOH) was detected, using 57 markers, for genetic alterations.

RESULTS: Nodules of altered hepatocytes (NAH), the putative precursors of HCA and HCC, were found in all the FNH lesions. Polyclonality was revealed in 10 FNH lesions from female patients, and LOH was not detected in any of the six FNH lesions examined, the results apparently showing their polyclonal nature. In contrast, monoclonality was demonstrated in all the eight HCAs and in four of the HCCs from females, and allelic imbalances were found in the HCAs (9/9) and HCCs (15/18), with chromosomal arms 11p, 13q and 17p affected in the former, and 6q, 8p, 11p, 16q and 17p affected in the latter lesions in high frequencies (≥ 30%). Monoclonality was revealed in 21 (40%) of the 52 microdissected NAH, but was not found in any of the five ordinary nodules. LOH was found in all of the 13 NAH tested, being highly frequent at six loci on 8p, 11p, 13q and 17p.

CONCLUSION: FNH, as a whole, is polyclonal, but some of the NAH lesions derived from it are already neoplastic and harbor similar allelic imbalances as HCAs.

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Key words: Clonality analysis; Focal nodular hyperplasia; Hepatocellular adenoma; Liver tumorigenesis; Loss of heterozygosity; Nodules of altered hepatocytes

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Cai YR, Gong L, Teng XY, Zhang HT, Wang CF, Wei GL, Guo L, Ding F, Liu ZH, Pan QJ, Su Q. Clonality and allelotype analyses of focal nodular hyperplasia compared with hepatocellular adenoma and carcinoma. *World J Gastroenterol* 2009; 15(37): 4695-4708 Available from: URL: http://www.wjgnet.com/1007-9327/15/4695.asp DOI: http://dx.doi.org/10.3748/wjg.15.4695

INTRODUCTION

Focal nodular hyperplasia (FNH) is defined as a lesion composed of hyperplastic hepatic parenchyma, subdivided into nodules by fibrous septa that may form stellate scars[1,2]. As dystrophic vessels are observed in

the fibrous septa and an arteriole is present within most of the nodules, it is considered parenchyma overgrowth responsive to increased blood flow secondary to vascular malformations[3]. Morphologically, FNH is classified into two categories, classical and nonclassical lesions[4]. The latter includes telangiectatic, mixed hyperplastic/adenomatous types and lesions with cytologic atypia. The non-neoplastic nature appears to be supported by data from X-chromosome inactivation (XCI) analyses, which demonstrated polyclonality in all FNH lesions in most of the series[5,6]. However, monoclonality was also observed in some of the lesions[7], including telangiectatic FNH[8,9]. FNH, as a descriptive term when proposed[10], may represent a group of focal lesions with different morphologic phenotypes and genetic alterations, and may develop in different pathways. Chromosomal gains and losses have been observed in several lesions by comparative genomic hybridization, allelotyping or karyotyping[9,11], but the molecular mechanism of FNH development remains unclear.

Hepatocellular adenoma (HCA) is a benign hepatic neoplasm, and its development is associated with the long-term use of steroids, mainly oral contraceptives[2,12]. While HCA was proven to develop from foci of altered hepatocytes (FAH) in the liver of rodents[13], the neoplastic features of human HCA have been confirmed by clonality analyses[5,6,9]. Some HCA lesions tend to progress to hepatocellular carcinoma (HCC)[2,13], and the malignant transformation has been linked to β-catenin activation[14]. In contrast to HCA, FNH is a stable lesion in clinical phenotypes with a very low risk of malignant transformation, although FAH can be detected in FNH[15] and HCC was identified in the liver harboring FNH as described in several case reports[16-18].

The majority of FNH lesions are distinguishable from HCA by their morphologic features. However, the differential diagnosis is difficult for some lesions. A central scar is regarded as the most characteristic change, but it is detectable in less than half of FNH lesions. Even for classical FNH, its incidence was shown to be 62% (153/245)[4,19]. More reliable procedures remain to be established for its distinction from HCA.

In the present study, we examined the clonality status of FNH, and compared its clonality with that of HCAs and HCCs, using assays based on XCI and polymorphism at the androgen receptor (AR) and phosphoglycerate kinase (PGK) loci. Secondly, these lesions were examined for loss of heterozygosity (LOH). In addition, 57 nodules were microdissected from FNH in female patients and used for the clonality analysis. Twenty-five nodules were also isolated from an FNH lesion and were used for LOH analysis.

MATERIALS AND METHODS

Tissue samples and histological examination

A total of 46 hepatic lesions were used, including 12 FNH lesions from 10 patients (Table 1), 12 HCAs from 11 patients and 22 well-differentiated HCCs (Table 2). These patients were admitted to the Cancer Hospital, Chinese Academy of Medical Sciences in Beijing and Tangdu Hospital, the Fourth Military Medical University in Xi'an during the period from 1998 to 2007. All the lesions were resected by an operation. Representative formalin-fixed, paraffin-embedded tissue samples were retrieved from the archives for both tumors and the surrounding liver parenchyma. Sections of 4 μm in thickness were prepared and stained by hematoxylin and eosin (HE).

All slides were reexamined independently by three pathologists (Su Q, Cai YR and Gong L), and their histologic features were reevaluated. FNH of the classical form was considered when the lesions showed characteristic features as proposed by other authors[3,4,10]. All the HCA lesions were identified from livers without evidence of cirrhosis or diffuse fibrosis by their macroscopical and histological features using well-established criteria[2]. FAH and nodules of altered hepatocytes (NAH) were identified on HE-stained sections[20], and the lesions were further highlighted by a marked reduction or even absence of CK18-immunoreactivity in glycogenotic clear cells as described previously[21]. Dysplasia, also designated as "small-cell change (SCC)"[20,22] and "small-cell dysplasia"[23], was recognized and graded into low-grade and high-grade lesions. The histological grades of HCCs were assessed as described by Hamilton and Aaltonen[1], with the well-differentiated HCCs corresponding to all of the grade Ⅰ and some of the grade Ⅱ lesions by the criteria of Edmondson and Steiner[24].

Immunohistochemical staining was performed using a streptavidin-labeled peroxidase (S-P) kit as in our previous study[20]. The primary antibodies used in this study included those against cytokeratin (CK) 18, CK19, CD34, hepatitis B virus (HBV) surface antigen (HBsAg; Clone 3E7), p53 protein (DO-7) and Ki-67 antigen. All reagents were purchased from Dako (Glostrup, Denmark). Levels of Ki-67 antigen expression were expressed as Ki-67-labeling indices (Ki-67-LI). The nuclear accumulation of p53 protein was evaluated as many (3+, > 30%), moderate (2+, 5%-30%), few (+, < 5%) and absent (-, 0%), as described previously[25].

Extraction of genomic DNA

Sections of 8 μm in thickness were prepared. After staining with HE, the lesions of FNH, HCA and HCC were identified by microscopic examination. Then the lesional tissues, each covering an area of at least 1 cm × 1 cm, were collected by a rubber policeman, and pooled into 1.5-mL tubes. The surrounding liver parenchyma and fibrous tissue were collected and used as controls. Previous XCI tests have demonstrated that monoclonality can be determined provided a given cell population contains at least 75% monoclonal cells[26]. To guarantee reliability of the following assays, mesenchymal tissue areas containing inflammatory cell clusters were eliminated to enhance purity of target cells to at least 80%. Genomic DNA was then isolated with

Table 1 Clinicopathological features of 12 FNH lesions and data of immunohistochemistry, clonality and LOH assays

Case numbers	Age (yr)/gender	Lesion codes	Lesion sizes (cm)	Ki-67-LI (%)	p53-LI (%)	Clonality by XCIA	No. of LOH
01	40/F	01[1]	5.0	1.6	0	PC[2]	0
02	30/F	02	2.5	0.5	0	PC[2]	0
		03	7.0	0.8	0	PC[2]	0
03	22/M	04[3]	5.2	0.7	0	NT	0
04	44/M	05[1]	1.2	0.5	0	NT	0
05	31/F	06[1]	4.7	0.3	0	PC[2]	0
06	43/F	07	3.2	1.0	0	PC[2]	NT
07	44/F	08[1]	2.0	0.8	0	PC[4]	NT
08	53/F	09[5]	4.8	1.0	0	PC[2]	NT
09	23/F	10[1,5]	4.5	1.2	0	PC[4]	NT
10	46/F	11[1,5]	5.3	0.8	0	PC[3]	NT
		12[1]	1.5	0.9	0	PC[4]	NT

[1]Some small preneoplastic foci, composed of clear cells, identified in surrounding liver parenchyma; [2]Tested on phosphoglycerate kinase (PGK) locus; [3]Nodules of altered hepatocytes (NAH) isolated from FNH by microdissection for LOH detection; [4]Noninformative at PGK locus and the data obtained by the assay on androgen receptor (AR) locus; [5]NAH isolated from FNH by microdissection for clonality assessment by XCIA. FNH: Focal nodular hyperplasia; Ki-67-LI: Ki-67 antigen-labeling indices; p53-LI: p53 protein-labeling indices by DO-7; XCIA: X-chromosomal inactivation assay; LOH: Loss of heterozygosity; F: Female; M: male; PC: Polyclonality; NT: Not tested for its origin from a male patient.

Table 2 Clinicopathological features of 12 HCAs and 22 HCCs, with data of clonality and LOH assays

Case numbers	Age (yr)/ gender	Lesion codes	Lesion sizes (cm)	Lesion types	Ki-67-LI (%)	p53-LI (%)	Clonality by XCIA	No. of LOH	Chromosomal arms affected by LOH
11	36/F	HCA01	3.5	HCA	1.3	0	MC[1]	3	8p, 11p, 16q
12	57/F	HCA02	3.0	HCA[2]	1.3	0	MC[1]	1	17p
13	28/M	HCA03	7.3	HCA, SCC[2]	2.5	0	NT	4	11p, 13q, 17p
14	52/M	HCA04	8.5	HCA	1.1	0	NT	3	11p, 17p
15	31/F	HCA05	7.0	HCA	3.1	0	MC[1]	4	11p, 13q
16	29/F	HCA06	1.0	HCA[2]	2.0	0	MC[1]	NT	NT
		HCA07	12.0	HCA[2]	1.8	0	MC[1]	5	1p, 13q, 17p
17	30/F	HCA08	1.0	HCA, SCC[2]	1.5	0	MC[3]	1	13q
18	29/F	HCA09	5.0	HCA	0.9	0	MC[1]	4	6q, 11p, 13q, 17p
19	37/F	HCA10	5.0	HCA	1.3	0	MC[3]	2	13q, 17p
20	33/F	HCA11	2.0	HCA[2]	2.3	0	MC[1]	NT	NT
21	40/F	HCA12	1.5	HCA[2]	2.0	0	MC[3]	NT	NT
22	31/F	HCC01	2.2	HCC, G1	2.0	10	NT	11	8p, 11p, 13q, 16q, 17p
23	46/M	HCC02	4.1	HCC, G1	65.0	0	NT	7	6q, 13q, 16q, 17p
24	42/M	HCC03	3.0	HCC, G1	2.5	0	NT	10	6q, 8p, 13q, 17p
25	51/M	HCC04	5.3	HCC, G1	8.2	80	NT	5	8p, 11p, 13q, 16q, 17p
26	49/M	HCC05	2.0	HCC, G1	2.0	0	NT	2	1p, 11p
27	27/M	HCC06	2.5	HCC, G1	1.2	0	NT	2	8p, 11p
28	56/M	HCC07	3.3	HCC, G1	1.5	0	NT	0	None
29	38/F	HCC08	8.3	HCC, G1	18.0	0	MC[1]	NT	NT
30	64/F	HCC09	3.6	HCC, G1	5.5	0	MC[3]	NT	NT
31	53/F	HCC10	9.5	HCC, G2	8.5	0	NT	3	8p
32	33/M	HCC11	8.0	HCC, G2	5.2	0	NT	5	6q, 11p, 17p
33	48/F	HCC12	5.2	HCC, G2	35.0	0	NT	4	6q, 8p, 16q
34	44/F	HCC13	4.2	HCC, G2	65.0	80	NT	12	8p, 11p, 13q, 16q, 17p
35	29/F	HCC14	3.6	HCC, G2	0.9	0	NT	2	1p, 11p
36	61/F	HCC15	4.5	HCC, G2	3.7	0	NT	6	6q, 13q, 16q, 17p
37	48/M	HCC16	5.0	HCC, G2	1.4	0	NT	3	6q, 11p, 17p
38	39/M	HCC17	4.0	HCC, G2	1.5	0	NT	0	None
39	39/M	HCC18	5.0	HCC, G2	1.2	0	NT	0	None
40	50/M	HCC19	2.0	HCC, G2	2.0	0	NT	8	8p, 11p, 13q, 17p
41	51/M	HCC20	7.4	HCC, G2	12.2	0	NT	1	11p
42	55/F	HCC21	2.5	HCC, G2	28.5	0	MC[3]	NT	NT
43	77/F	HCC22	5.6	HCC, G2	16.2	0	MC[3]	NT	NT

[1]Noninformative at PGK locus and the data obtained by the assay on AR locus; [2]Some small preneoplastic foci, composed of clear cells, identified in surrounding liver parenchyma; [3]Tested on PGK locus. HCA: Hepatocellular adenoma; HCC: Hepatocellular carcinoma; MC: Monoclonality; NT: Not tested for its origin from a male patient (for HCA) or its evidently malignant phenotypes; SCC: Small-cell change.

an extraction kit (QIAGEN, Chatsworth, CA, USA) following the manufacturer's instructions. The DNA samples of 50 μL each were stored at -20℃ until use.

Microdissection of preneoplastic nodules from FNH

Selected FNH specimens with well demarcated NAH were subjected to microdissection as described previously[27].

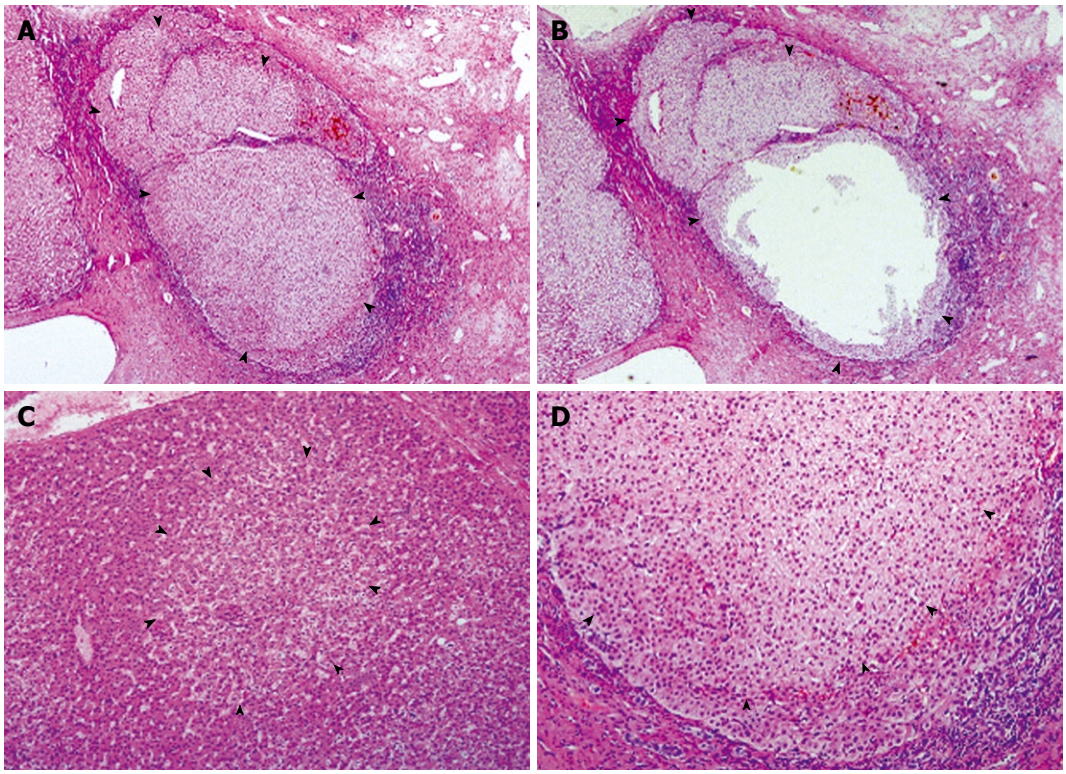

Figure 1 Preneoplastic lesions in classical FNH. A and B: FNH04 from Case 03, showing a nodule largely occupied by an expanding NAH (arrowheads), before (A) and after microdissection (B); C: Portion of FNH06, showing an FAH (arrowheads), in which the altered hepatocytes integrate well with the surrounding hepatic plate; D: Portion of an NAH composed mainly of clear hepatocytes (arrowheads), showing compression to surrounding liver parenchyma. HE, A and B, × 40; C and D, × 100.

Ten consecutive sections were deparaffinized with xylene, rehydrated by rinsing through graded alcohol, stained with HE and immersed in glycerol. Larger nodules, 2.5-5.0 mm in diameter, from these sections were identified and correlated. Target tissues were outlined and collected with a clean 4½ needle under a microscope (Figure 1A and B). The tissues of the same nodules from the consecutive sections were pooled together and stored in 1.5-mL tubes. Using the same procedure, tissues of the same size were also collected from the surrounding liver parenchyma and were used as controls. Genomic DNA was isolated as described above.

Clonality assays based on XCI mosaicism in somatic tissues in female patients

The principles of XCI assays have been described previously[28]. Briefly, there are two X chromosomes within each somatic cell in females, one from the father and the other from the mother. Both PGK and AR genes are located on X chromosomes, showing readily demonstrable polymorphism in different frequencies. The former carries a *Bst*XI restriction-polymorphic site, reflecting the G/A single-nucleotide polymorphism at exon 1, while the latter is polymorphic at the CAG short tandem repeat (STR). These allelic differences and the XCI mosaicism enable us to identify monoclonal, neoplastic lesions from normal or regenerating tissues composed of polyclonal cell populations[28,29].

The DNA templates extracted from tumors and the non-neoplastic tissues were incubated with 5 U of *Hpa*II (Promega, Madison, WI, USA) at 37℃ for 12 h in a volume of 20 μL containing 0.2 μL of 10 mg/mL bovine serum albumin and 2 μL of 10 × reaction buffer, and heated at 95℃ for 5 min to inactivate the enzyme. The digested DNA samples, 5 μL each, were then subjected to nested PCR for amplification of exon 1 of PGK and AR genes, as described previously[28]. The PGK products, 10 μL each, were incubated with 5 U of *Bst*XI (Promega) at 47℃ for 8-10 h. The digested products were then resolved in 2% (g/mL) agarose gel containing ethidium bromide (0.2 μg/mL), and visualized under ultraviolet light. The fluorescence intensities of the products of these two alleles, at the positions of 530 and 433 bp separately, were assessed using LabWork 3.0 (UVP). Efficacy of the reaction was demonstrated by including a sample from a male patient with FNH (Case 03) who had been demonstrated to harbor a *Bst*XI restriction site.

The amplification products of AR exon 1, 3 μL each, were mixed with the same volume of sample buffer (99% formamide, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol), resolved on 10% polyacrylamide gel containing urea (8.0 mol/L), and visualized by silver staining. Sizes of the products were determined using 100-bp and 10-bp DNA ladders (Gibco BRL).

LOH assays by conventional and multiplex PCR

A total of 57 loci, whose allelic loss has been frequently observed in HCC samples from various regions[30-38], were chosen for the LOH assay. Amplification of the DNA samples from the whole FNH lesions, HCAs and HCCs was performed by conventional PCR. Sequences of the primer pairs (Table 3) were obtained from Genbank

Table 3 Sequences of primers for nested PCR-based LOH assays

Markers (locations)	External primer pairs[1]	Internal primer pairs[1]
D1S199 (1p36.13)	CCTGGGCAACATAGCAG	GGTGACAGAGTGAGACCCTG
	TGACATCTTCCTCCACCTC	CAAAGACCATGTGCTCCGTA
D1S2843 (1p36.12)	AGAAGGTCGGGCACAAA	GGGCTGGGCATTACACAAC
	TTCATCACCCACCCAAAA	ATCAAATTGGCTTCTCACCG
D1S513 (1p35.2)	AGCTGAGACCCTGCCTTG	AGCTGAGACCCTGCCTTG
	AATGCCTGCTGTATGAAA	AGCCTTCAGAGCCTGCC
D4S406 (4q31.21)	TCCCAGTTTACAACCACA	CTGGTTTTAAGGCATGTTTG
	GAAGGTGGCTAACTAAAA	TCCTCAGGGAGGTCTAAT
D4S426 (4q35.2)	CAAGTAATACAATATGGTAA	ATACACTGCATCCATATATACAAGG
	ATACACTGCATCCATATATACAAGG	ACATTGTGAAATGACCACAG
D6S437 (6q25.3)	TTACTGACTTCTTGAAAGGGTG	TGTCCTGGTGGAGGCA
	ACACGCCATCACAGGAAC	GGTACAGTGTTTGACCCTAAGA
D6S305 (6q26)	AAAGCTGAGAAGCCATTCA	CACCAGCGTTAGAGACTGC
	GCAAATGGAGCATGTCACT	GCAAATGGAGCATGTCACT
D6S1008 (6q27)	GCTACTTCAGCAGGGTT	AAGAAAGACTAGAGAGACAGACAGC
	AATGACCACGAGTTCTTC	ATCATTTGCCCATTTACCAA
D6S297 (6q27)	CAAGAAGTGTCTCTAAAGATAAGG	CAAGAAGTGTCTCTAAAGATAAGG
	GCAAGATGTAGAAGGAGA	CAACCAACCACGGTATATG
D8S277 (8p23.1)	GTTCCCTATGGCTAAAG	CCAGGTGAGTTTATCAATTCCTGAG
	AGTTTGCCTCCAGAAA	TGAGAGGTCTGAGTGACATCCG
D8S1754 (8p22)	TTTGGGTAAGATACTTTAAGCG	CAGGGAAGTCTCGGTTTG
	AAATAGTCGGGTCGGGAG	TCAGGGACACGATTCAGC
D8S1827 (8p22)	AGAAATAGCCAAAGAAGC	GACAGAATCATGTGGCCTTT
	CTGGGAAGGTCAGCAGATTG	TTTTGTAAAATGTAAAATTGGCTTT
D8S261 (8p22)	GAGGCTACTAACAACGTG	TGCCACTGTCTTGAAAATCC
	TATTACAGTGGTTATGGC	TATGGCCCAGCAATGTGTAT
D8S258 (8p21.3)	ACAATAATGATGAGGCCAGAA	CTGCCAGGAATCAACTGAG
	CAGCCAATACGCAGGAGC	TTGACAGGGACCCACG
D8S298 (8p21.3)	CTAGTTATTATGCAGAGGTGACATC	AGGCTTACACCCATGGACC
	GAGGCTGGTTAGCATCACG	ACGCAGCACACAACATCAT
D8S1771 (8p21.2)	GGGGTGGGTGTAGATATT	TTTACAAGAACCACCTGCC
	TTGATTCTTTGCTGATGC	GATATAAAACATGACTTTGCTACCC
D8S1772 (8q22.1)	TTGATGCTCCTCTGTTGC	GATCTGAGCCTTCCTACTGTC
	AAGTCCTCCCTTTGCTGA	TGCCTTTTGGTGAATGG
D8S522 (8q24.12)	TATAGCAAATATGTTCACG	GAAAACAAAACAGGCTCC
	CCATCTTGGCTCCTC	TGACCAAACAACTAGACACC
D11S1301 (11p13)	GCCACAGCACTCCAG	GGCAACAGAGTGAGACTCA
	GACAACCTCCCTCACC	GTGTTCTTTATGTGTAGTTC
D11S2008 (11p13)	ATTCAGGAGCACAGAACA	CATCCATCTCATCCCATCAT
	GCATCCATACGGAAAGTC	TTCACCCTACTGCCAACTTC
D11S907 (11p13)	GCTTATTGTCCATACCCAAA	GCTTATTGTCCATACCCAAA
	GAAAAGCAAAGGCATCGT	AAAGNACCTTAATTTCAGGC
D13S164 (13q12.3)	GGCGATCCACCCACCTT	GCTGTGATTGCACCACC
	TGCCACGCACCTGTAGTCC	ATTACAGGCGTGACACACC
D13S289 (13q12.3)	AGGGCGTCACCGTGTT	CTGGTTGAGCGGCATT
	GACTGAGGCAGGAGGATTG	TGCAGCCTGGATGACA
D13S260 (13q12.3)	AATGGATCTGCTTGC	AGATATTGTCTCCGTTCCATGA
	ATCACTCCAAATGTAAAT	CCCAGATATAAGGACCTGGCTA
D13S171 (13q12.3)	CAGATACAGACATTTTGGAA	CCTACCATTGACACTCTCAG
	GCTCTACAGCATTGACCT	TAGGGCCATCCATTCT
D13S267 (13q13.1)	AGCTAATGGCCTGAAAGG	GGCCTGAAAGGTATCCTC
	AGAGGTCAAAGAGGAAGA	TCCCACCATAAGCACAAG
D13S220 (13q13.2)	AATCACCTCCCACCAG	CCAACATCGGGAACTG
	AGGGGTTCCTTCATCC	TGCATTCTTTAAGTCCATGTC
D13S219 (13q13.3)	CTGGATGAAAAGGAAC	AAGCAAATATGCAAAATTGC
	TTATCTCATTCAGTTGCT	TCCTTCTGTTTCTTGACTTAACA
D13S218 (13q13.3)	TTCTCATAAGAAATCCCC	GATTTGAAAATGAGCAGTCC
	TTTCATATCCCTGTTCAA	GTCGGGCACTACGTTTATCT
D13S325 (13q14.11)	ATGCAGCTTAAGTCCTTT	TCCTTTAAGTGTCTAGAGAGGAGG
	CTGTGCTATCTCCTCCAA	TCTCTCTCAGAAGTTTGGAAGC
D13S291 (13q14.11)	GTCTGACGGGAAACAGC	ATGGCCAGACTTCCCACT
	CACAAACAGAATCAACCCT	CCAGGCTCACATGCTAACA
D13S126 (13q14.2)	AGCCTCCCAAAGTGCTA	TCACCAGTAAAATGCTATTGG
	AGTCATCTGGTCCCTCAAT	GTGATTTTCAAATTTGCTCTG
D13S118 (13q14.2)	TGTAAATAGCTTAGTTG	GAAATAGTATTTGGACCTGGG
	CTACTGACATTTGCTC	CCACAGACATCAGAGTCCTT
D13S153 (13q14.2)	AACTTGGCTGCGATGATAAGAA	AGCATTGTTTCATGTTGGTG
	CCTGAGGTATTGACGAAGGGTC	CAGCAGTGAAGGTCTAAGCC
D13S284 (13q14.3)	CCAGCTCGTGTTCATTT	AAAATCAGGTGGAAACAGAAT

	GTACATTTTATAGATTCATAGAGTC	AAAGGCTAACATCGAAGGGA
D13S137 (13q14.3)	GATGGTGGGTGGGACTT	CAGGAGGGATGGACTCACTTC
	GGAATATGTGGAGGATTTATCTCTG	TTTCCTCATTCTTTCCCAATTG
D13S321 (13q21.1)	TAAATGCAATCTGAAT	TACCAACATGTTCATTGTAGATAGA
	CATAGCAAGACTCTGTC	CATACACCTGTGGACCCATC
D13S119 (13q21.1)	TCTCATTCCCATAACAT	TTATTGCCTTTGTAGATCATTG
	5' AGAGGAAAGATAGAACAA	AAGACTTTGAATGAAATTCCC
D13S170 (13q31.1)	AGCTATTATGTAACCAAT	TTGCACTGTGGAGATAAACACATAG
	TGTTGTTCTAAGCCAC	TCACATTGTCTTTTAAGGCAGGAG
D13S71 (13q31.3)	ACGCTCCTTCGTGGTG	GTATTTTTGGTATGCTTGTGC
	GTGCCTCTGTTTCTCCTATT	CTATTTTGGAATATATGTGCCT
D16S514 (16q21)	TGACACGCAATTTACCCT	CTATCCACTCACTTTCCAGG
	AATCACATTTTCCCACTG	TCCCACTGATCATCTTCTC
D16S402 (16q22.1)	GAGGCAAAGAGGTATCCA	TTTTGTAACCATGTACCCCC
	CCCACTCATTCCTGTCCC	ATTTATAGGGCCATGACCAG
D16S3029 (16q23.1)	AGGGTGTTGAGGTGTCTG	ATAGAGTTGGGCTGCATAGA
	ATAGGGTCTGCTGGGTT	CTTTCCTGAAATTGGAAGTGA
D16S3040 (16q23.2)	TATGTTGGTGGATGATTT	TACTCCGGCAAGGACG
	5' GTCAAGGAACACTGAACT	GCTGCCTAGCACATGG
D16S422 (16q24.3)	ACAGCCACCTCTATTCA	CAGTGTAACCTGGGGGC
	AACATTTTACTATATCTTACTTTCG	CTTTCGATTAGTTTAGCAGAATGAG
D16S3121 (16q24.3)	AAGTCACTGGGCTAACAAGG	CATGTTGTACATCGTGATGC
	TGGGTCCTGAGGTCACAAA	AGCTTTTATTTCCCAGGGGT
D16S303 (16q24.3)	GCAGGTTATGGATGTGAGTTTGTT	GATCAGTGCTCGTTTTTTTTGGTTTGG
	TTCGGAAGGCTGAGGCAGTA	CAACAAGAGCGAAACTCGGTCTCAA
D17S643 (17p13.3)	TGCCGTTCTCAGGTGGTT	CTTCCTTGTCTCTAAACAGTCCTTT
	TTCCTCCCTGCATGGATT	GTAGTCCCAGGGAGCTGGAAGT
D17S849 (17p13.3)	TACAATACTGCTGCAATAAG	CAATTCTGTTCTAAGATTATTTTGG
	TACAATAACATCAGGAAACA	CTCTGGCTGAGGAGGC
D17S926 (17p13.3)	GAAGTGGGAAGATTGCTT	GCAGTGGGCCATCATCA
	ATCCTCTGAACCGTATTT	CCGCAGAAGGCTGTTGT
D17S695 (17p13.3)	AGCCTGGGCAACAAGAGC	CTGGGCAACAAGAGCAAAATTC
	CAGCCAACGGGCGTCATT	TTTGTTGTTGTTCATTGACTTCAGTCT
D17S1840 (17p13.3)	GGCAACGTGGTGAAACCC	GCCTGGGCGACAGAGTGA
	ACCAGGACCGGCTGCTCT	TGGGGCAGACTTGGTCCTT
D17S1529 (17p13.3)	GTTAGCCTCTTCCTTGGACATTC	TTGTTTCTATCCACGCAGGC
	ATCATTCCGTTTCACCTTTGG	GATGGCTGTGCTTGCTGGTA
D17S1574 (17p13.3)	CTTCTTCTGCGTCAGGTATG	TCACTTATCGGCATCTGATCC
	CCAAAGTGCTGGGATTA	CTGATGTGCCTTTTGTGTGTG
D17S831 (17p13.3)	GCCCAAATCAGAAGCAAG	CGCCTTTCCTCATACTCCAG
	GACATCCATTGGCACCAC	GCCAGACGGGACTTGAATTA
D17S654 (17p13.3)	GGGGTACGCCTTTCCTCA	GACCTAGGCCATGTTCACAGCC
	GGTTGGCAAGACCACTGTTAGA	GACATCCATTGGCACCACCCCAA
D17S796 (17p13.2)	GAGCAGCTAGGATCAAGGG	CAATGGAACCAAATGTGGTC
	AGAACGGCAAACAACGAA	AGTCCGATAATGCCAGGATG
β-actin[2]	CTGTGCCCATCTACGAGG	AGAGATGGCCACGGCTGCTT
	AAAGGGTGTAACGCAACTAA	ATTTGCGGTGGACGATGGAG

[1]Nucleotide sequences were written in a 5' to 3' direction, with the forward and reverse primers for each reaction presented at the upper and lower lines, respectively; [2]Amplified as an internal control with a product of 406 bp.

(http://www.ncbi.nlm.nih.gov and www.gdb.org). The reaction mixture was 50 μL in volume, containing 50 ng of DNA templates, 4 μL of dNTP (2.5 mmol/L each; Gibco BRL), 1 μL of 20 μmol/L primers each, 5 μL of 10 × buffer (100 mmol/L Tris-HCl, pH 8.3, containing 500 mmol/L KCl and 25 mmol/L $MgCl_2$) and 1.25 U of *Taq* DNA polymerase (Gibco BRL). Amplification was conducted for 35 cycles (94℃, 40 s; 46℃, 50 s; 72℃, 1 min) following the initial denaturation at 94℃ for 5 min. The final elongation was at 72℃ for 15 min. The efficacy and reliability of the reactions were ensured by amplification of the β-actin gene in a parallel reaction.

For microdissected samples, the amounts of DNA extracted from the minute tissues were too small to complete all the reactions, and a highly efficient amplification system was needed. Nested PCR was employed for this purpose. A multiplex PCR was performed, according to the principle of Henegariu *et al*[39], followed by a second-round reaction in separate tubes. For the first-round PCR, 57 external primer pairs, as listed in Table 3, were designed based on the genomic sequences (Genbank). They were classified into 11 groups according to calculated annealing temperatures and compatibility with the assistance of Beacon Designer (Version 5.0) software, allowing amplification of four to seven STR sequences in the same mixture. The reaction mixture was 50 μL in volume as described above, but it contained 20 ng of DNA templates. The amplification was performed for 35 cycles, and the annealing temperatures varied according to the primer groups. The second-round amplification was followed using the internal primer pairs (Table 3), with the 57 reactions in separate tubes. After initial denaturation at 94℃ for 5 min, amplification was conducted for 15 cycles (94℃, 40 s; 50℃, 50 s; 72℃, 1 min) at first,

followed by 25 cycles with the annealing temperature adjusted to 46℃. The amplification products, 3 μL each, were mixed with the same volume of sample buffer, and then electrophoresed on a 15% polyacrylamide gel containing urea (8 mol/L). Gels were fixed in 10% acetic acid for 30 min, and the resolved products were visualized by silver staining.

Evaluation of data and statistical analyses

The images of the gels were subjected to analysis with a densitometer (Applied Syngene, Cambridge, UK). For clonality analysis based on polymorphism at the PGK and AR loci, a reduction of ⩾ 50% in their signal intensities for the products of either allele, as compared to the samples not treated with *Hpa*Ⅱ or *Hha*Ⅰ, is regarded as loss of XCI mosaicism[28]. Each reaction was repeated at least once to guarantee reproducibility. Moreover, parallel assays were carried out using the surrounding liver parenchyma or fibrous tissue as controls. For the tests on microdissected lesions, special attention was paid to avoid interference from the XCI skewing patches[28]. For each FNH lesion, four tissue samples of similar sizes as microdissected nodules were taken from the surrounding liver parenchyma and examined as controls.

For the heterozygosity test, normal tissue samples showing signals from two alleles migrating at expected locations and with similar intensities were considered informative, and those showing only one signal, were considered noninformative. Allelic imbalance was determined by comparing the intensity ratio between the signals for the two alleles. For a given informative marker, LOH was identified when one of two signals was absent or the allelic ratio was greater than 3 or less than 0.33. Reliability of the assays was guaranteed by including control tissue samples for all FNH lesions and microdissected nodules in a parallel way. Interpretation of LOH results of the lesions was considered reasonable when all of the control reactions from the same case gave rise to consistent allelic ratios.

Statistical computation was carried out using SPSS 13.0 software (SPSS, Inc, Chicago, IL, USA). Student and Kruskal-Wallis tests were employed to assess cell kinetic features of different lesion types reflected by Ki-67-LI, and the χ^2 test was used to compare LOH frequencies in HCA, FNH and HCC groups. $P < 0.05$ was regarded as statistically significant.

RESULTS

Clinical and pathological features

Twelve FNH lesions (FNH 01-12) were identified from 10 patients (Cases 01-10), eight patients had solitary lesions and separate lesions were identified in two liver specimens (Table 1). The patients included eight females and two males, aged 22-53 years (mean, 37.6 years). Grossly, the resected FNH lesions were 1.2-7.0 cm in diameter (mean, 3.8 cm), and were invariably described as circumscribed and solid lesions. On cut surfaces, a multinodular appearance was observed, and a fibrous stellate scar was not recorded in any of the lesions. Histologically, all the lesions were classified as the classical form[4], being well demarcated from the surrounding liver parenchyma but free of a fibrotic capsule. The lesions were subdivided incompletely into nodules with fibrous septa that contained malformed blood vessels, proliferating ductules, and scattered lymphocytes.

Most of the hepatocyte nodules appeared similar, about 1-5 mm in diameter, and histologically resembling regenerative nodules from cirrhotic livers. FAH, frequently of the clear cell type and clear/amphophilic mixed cell type, were identified in some of the nodules from all the lesions. The altered hepatocytes were arranged in plates frequently of two cells in thickness, with the sinusoids appearing crowded, but were integrated well with the surrounding hepatocytes in the majority of the lesions (Figure 1C). In some nodules, nearly all the parenchymal cells were replaced by the altered hepatocytes. These lesions became larger than other nodules, 2.5-5 mm in diameter, showing marked compression to the surrounding tissue (Figure 1D) and fulfilling the criteria for NAH[20,22]. In all the FNH lesions, including the FAH and NAH areas, the thickness of hepatic plates did not exceed two cells and no SCC was detected. All the lesions were negative for p53 protein. Ki-67-LI ranged from 0.3% to 1.6%, with a mean of 0.8%. While this value was shown to be lower than those of HCAs ($P < 0.01$) and the well-differentiated HCCs ($P < 0.01$), these three lesion types showed overlap in their Ki-67-LI, with 6 (50%) of the 12 HCAs and 6 (27%) of the 22 HCCs within the range of FNH (Tables 1 and 2). FAH, mainly small-sized, and clear-cell lesions, were also identified in the surrounding liver parenchyma in six of the 10 livers (Table 1).

As listed in Table 2, 12 HCAs (HCAs 01-12) were resected from 11 patients (Cases 11-21), including nine females and two males. The ages of the patients ranged from 28 to 57 years (mean, 36.6 years), and none had a history of using oral contraceptives or other steroid hormones. Among the 12 lesions, 10 were solitary, and two were resected from the same liver (Case 16). The lesions ranged in size from 1 to 12 cm in diameter (mean, 4.8 cm). Histologically, the lesions were well demarcated from the surrounding liver parenchyma, but an intact fibrotic capsule was absent in all the lesions. The tumor was composed of hepatocytes arranged in plates usually of two cells in thickness. Low-grade SCC was identified in HCAs 03 and 08. The portal tract was not visible within the lesions. All these lesions were negative for p53 protein. Ki-67-LI ranged from 0.9% to 3.1%, with a mean of 1.8%. Phenotypic change of the sinusoid cells, as highlighted by emergence of CD34-immunoreactivity, was observed in all the HCA lesions, being diffuse in four and patchy in eight of the lesions. FAH, mainly of the clear cell type, were also identified in the surrounding liver parenchyma in six of the 11 livers.

A total of 22 well-differentiated HCCs were collected from 12 males and 10 females (Table 2). The age of the patients ranged from 27 to 77 years, with a mean of

Table 4 Nonrandom X-chromosomal inactivation revealed in NAH microdissected from 3 FNH and their inactivation patterns

Origin of lesions	No. of NAH tested	No. with signals	No. with MC (%)	No. of lesions with	
				Upper band	Lower band
FNH09	38	34	14 (41.2)	12	2
FNH10	6	6	2 (33.3)	0	2
FNH11	12	12	5 (41.7)	0	5
Total	56	52	21 (40.4)	12	9

46.6 years. All the patients were shown to be seropositive for HBsAg. Of the 22 HCC cases, 20 appeared solitary and well demarcated and two were multifocal. The lesions ranged in size from 2.0 to 9.5 cm, with a mean of 4.6 cm. Histologically, nine of them were classified into grade 1, and 13 into grade 2. Ki-67-LI ranged from 0.9% to 65%, with the mean and median being 13.1% and 5.4%, respectively. This value was shown to be higher than that of HCAs ($P < 0.05$), while the Ki-67-LI in 10 (45%) of the 22 HCCs were within the range of HCAs. Immunoreactivity for AFP was demonstrated in four of the HCC samples (HCC 01, 11, 21 and 22). Overexpression of p53 protein was observed only in three HCC lesions, with the percentages of positive tumor cells as high as 10% in Case 21 and 80% in Cases 25 and 34. HBV infection was confirmed by immunohistochemical demonstration of HBsAg in all of the cases. Cirrhotic changes were evident in surrounding liver tissues in all the cases, where numerous NAH were identified.

Nonrandom XCI revealed in HCCs and HCAs, but not in the FNH lesions

XCI analysis was performed using tissues from 4 of the female patients with HCC (Cases 29, 30, 42 and 43) and all the nine females with HCA (Table 2). The G/A polymorphism at the PGK locus was demonstrated in three of the HCC patients (Cases 30, 42 and 43) and in three of the nine HCA patients (Cases 17, 19 and 21), showing two bands at positions 530 and 433 bp. Pretreatment with *Hpa*Ⅱ resulted in loss, or marked intensity reduction, of one band in all the tumor samples, while the allelic ratio was retained in the samples from surrounding liver parenchyma (Figure 2A). One HCC (Case 29) and the remaining HCA patients were not polymorphic at the PGK locus, but the AR locus showed CAG STR length-polymorphism. Pretreatment with *Hha* Ⅰ resulted in loss, or marked intensity reduction, of one band in these two tumor samples, while the allelic ratios were not changed in the corresponding peritumorous liver parenchyma (Figure 2B and C). These results demonstrated monoclonality and confirmed neoplastic nature in all the HCC and HCA lesions.

Of the eight female patients with FNH, 5 (Cases 1, 2, 5, 6 and 8) showed G/A polymorphism at the PGK locus, and the remaining three (Cases 7, 9 and 10) were shown to be AR-polymorphic at the CAG STR. Pretreatment with *Hpa*Ⅱ (for PGK locus) or *Hha* Ⅰ (for AR locus) did not cause a remarkable allelic ratio change in any of the 10 FNH samples (Figure 3A). These results demonstrated polyclonality of classical FNH lesions.

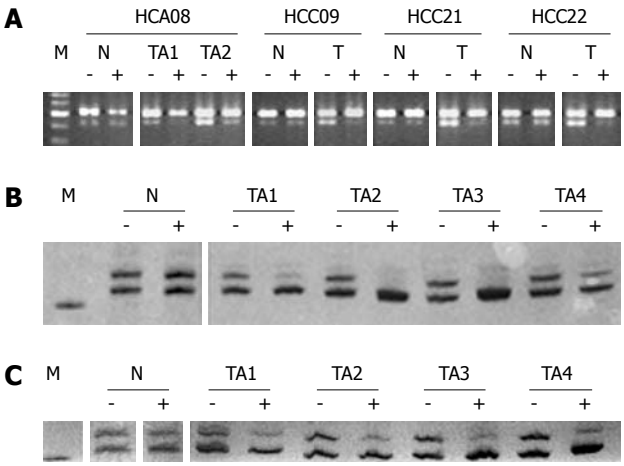

Figure 2 Representative data of X-chromosome inactivation (XCI) assays at PGK (A) and AR loci (B and C). Nonrandom XCI are present in HCAs (08 in A, 20 in B) and HCCs (09, 21 and 22 in A, 08 in C), revealing their monoclonality. A: The G/A single-nucleotide polymorphism at PGK exon 1 was demonstrated by *Bst*XI-digestion and electrophoresis on an agarose gel. The intact and cleaved amplification products migrate at positions of 530 (upper band) and 433 bp (lower band), respectively. Pretreatment with *Hpa*Ⅱ (-: Before; +: After) resulted in marked reduction of the lower band in single tumor samples (T) and separate tumor areas (TA), but not in the surrounding liver parenchyma (N). M: DNA markers, with five bands at locations of 700, 600, 500, 400 and 300 bp; B and C: The length polymorphism of AR gene was resolved on a 10% polyacrylamide gel containing urea (8 mol/L) and visualized by silver staining, with one product migrating faster than the other. Pretreatment with *Hha* Ⅰ (-: Before; +: After) resulted in loss or marked reduction of one band in tumor samples, but not in the non-neoplastic tissue. M: DNA marker at the location of 200 bp.

It appears that FNH is different in clonal composition from hepatocellular neoplasms including HCC and HCA.

Nonrandom XCI revealed in some NAH lesions microdissected from FNH

Three FNH lesions (FNH 09-11) with well-demarcated NAH from three women were chosen for microdissection. A total of 56 NAH lesions (NAH 01-56), ranging from 2.5 to 5 mm in diameter, and 5 ordinary regenerative nodules, of similar sizes and without involvement of FAH, were isolated from the FNH lesions for clonality analysis. Genomic DNA samples from 52 of the 56 dissected NAH were amplified successfully and subjected to AR (FNH 09 and 10) and PGK gene analysis (FNH11). Twenty-one (40%) of the 52 NAH showed monoclonality, including 14 (41%) of the 34 lesions in FNH09 (Figure 3B), 2 (33%) of the six in FNH10 and 5 (42%) of the 12 from FNH11 (Table 4). On average, the cross sectional areas of the 21 monoclonal

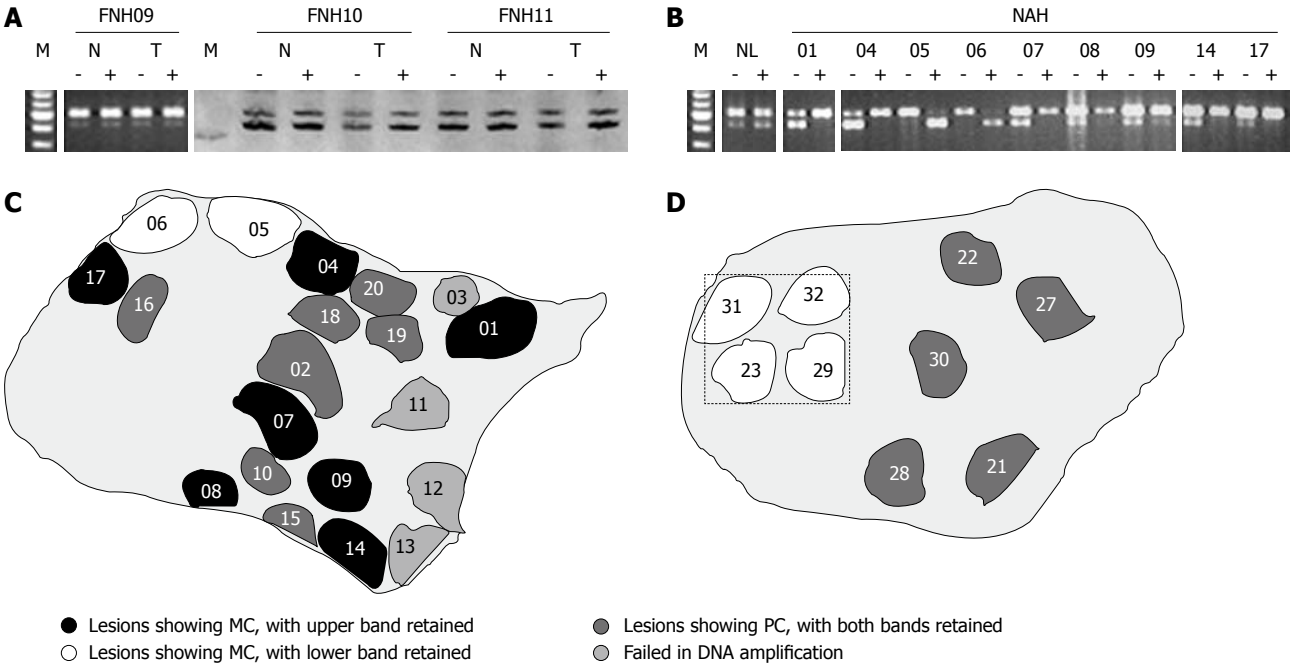

Figure 3 Clonality status of FNH lesions (A) and NAH (B), with XCI patterns revealed in different NAH (C and D). A: Polyclonality (PC) revealed in 3 FNH lesions, with FNH09 assayed at PGK locus and 10 and 11 at AR locus. *Hpa* II or *Hha* I pretreatment (-: Before; +: After) did not significantly change intensity ratios between the two bands of lesional tissues (T) compared to those of the surrounding liver parenchyma (N). M: DNA markers, with five bands at locations of 700, 600, 500, 400 and 300 bp for PGK assay, and a single band at the location of 200 bp for AR reaction; B: The PGK assay shows monoclonality (MC) in all of the nine NAH from FNH09, with the isolated reference tissue sample (NL), of a similar size to NAH, revealing polyclonality. XCI patterns of the NAH are different, with the upper band retained in NAH 01, 04, 07-09, 14 and 17, and the lower band retained in NAH 05 and 06; C: Sketch of a tissue section from FNH09, as illustrated in B, showing occurrence of monoclonal NAH with different XCI patterns and polyclonal lesions in the same section; D: Sketch of a tissue section from FNH11, showing four monoclonal and five polyclonal NAH. The clustered monoclonal lesions show the same XCI pattern, the XCI assays with small samples, as framed by hatch lines, may result in misinterpretation as monoclonality for the whole FNH lesion.

and 31 polyclonal lesions were 9.8 ± 7.0 and 9.5 ± 6.7 mm^2, respectively, the difference being insignificant ($P > 0.05$). Different alleles were retained among the 14 monoclonal NAH from FNH09 (Figure 3B and C), while identical inactivation patterns were observed in those NAH from FNH 10 and 11 (Figure 3D). All of the five ordinary nodules, four dissected from FNH09 and one from FNH11, were shown to be polyclonal in cell composition.

LOH detected in NAH, HCAs and HCCs, but not in whole FNH lesions

A total of 57 loci were examined in this study, located at 1p, 4q, 6q, 8p, 8q, 11p, 13q, 16q and 17p (Table 3). As shown in Table 2, the assay showed LOH, at least at one of the loci, in 15 (83%) of the 18 well-differentiated HCCs (01-07 and 10-20) and in all of the 9 HCAs examined (01-05 and 07-10, Figure 4A). Six of the FNH lesions (01-06), covering areas of at least 1.0 cm × 1.0 cm, were also examined, and no LOH was detected in any of them (Figure 4B).

Demonstration of clonal growth in some of the NAH from FNH, as described above, indicated that some genomic alterations may have occurred in these minute lesions. Twenty-five NAH were microdissected from an FNH lesion (FNH04) for LOH analysis. Genomic DNA samples from 13 of the microdissected lesions (NAH 57-69) were amplified successfully for the majority of the reactions, but for other samples

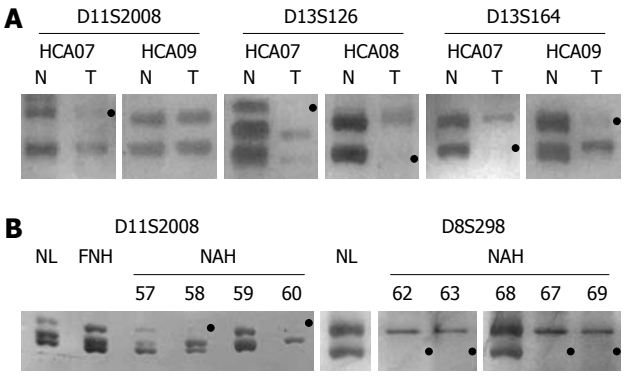

Figure 4 Representative data of amplification products at three length-polymorphic loci in HCAs 07-09 (A), FNH04 and NAH microdissected from the FNH lesion (B), with loss or marked reduction of the product from one allele (black dot) defined as LOH. A: Gels show the allelic imbalance at D11S2008, D13S164 and D13S164 in HCA07, at D13S126 in HCA08, at D13S164, but not in D11S2008, in HCA09 (T: Tumor; N: Peritumorous normal liver); B: The microdissected lesions NAH58 and NAH60, but not NAH57 and NAH59, show LOH at D11S2008. The lesions NAH62, NAH63, NAH67 and NAH69, but not NAH68, show LOH at D8S298. Both alleles are preserved in FNH04 as tested as a whole (FNH) and compared to those of the surrounding normal liver parenchyma (NL).

(NAH 70-81), the amounts of the extracted DNA were too small to complete all the reactions and the data obtained were not included. Allelic imbalance, at least at one of the loci, was demonstrated in all of the 13 NAH (Figure 4B, Table 5). The reliability of the assay was confirmed by full consistence between the results

Table 5 LOH profiles of 13 NAH microdissected from an FNH lesion (FNH04)

Lesion codes	Diameters (mm)[1]	Numbers of LOH	Loci affected[2]
NAH57	3.8	6	D6S437, D8S298, D13S118, D13S153, D16S3029, D16S3040
NAH58	3.5	5	D8S298, D11S1301, D11S2008, D13S321, D16S3121
NAH59	1.8	2	D13S164, D17S1840
NAH60	2.7	2	D11S1301, D13S321
NAH61	3.8	6	D11S2008, D13S118
NAH62	3.2	4	D8S298, D13S164, D13S321, D17S1840
NAH63	2.8	5	D1S513, D8S298, D13S118, D13S153, D17S695
NAH64	3.8	5	D6S1008, D13S218, D16S514, D16S3040, D17S926
NAH65	3.2	5	D1S513, D8S298, D11S907, D13S164, D13S126
NAH66	2.0	1	D6S1008
NAH67	2.1	4	D8S298, D11S1301, D11S907, D16S422
NAH68	2.4	3	D13S164, D13S126, D13S321
NAH69	2.8	5	D6S437, D8S298, D13S118, D13S321, D17S926

[1]The largest diameters as measured in cross sections of the lesions under a microscope; [2]The markers written in underlined characters represent loci affected in high frequencies (≥ 30%) in NAH.

obtained by the multiplex reactions and those obtained through conventional PCR.

Of the 57 loci examined, 39 (68.4%) showed LOH in at least one of the lesions tested. At the remaining loci, allelic imbalance was not detected in any of the samples examined. LOH was demonstrated in NAH, HCAs and HCCs at 21 (37%), 16 (28%) and 33 loci (58%), respectively. The allelic imbalance was more extensive among the locations tested in HCCs than in HCAs and NAH ($P < 0.01$), with their average numbers of LOH being 4.5 (0-12), 3.1 (1-5) and 4.1 (1-6), respectively. As shown in Figure 5, the most frequent LOH in NAH, HCA and HCC were at D8S298 (70%), D11S1301 (75%) and D6S1008 (50%), respectively. Several markers showed frequent LOH in the HCC samples but not in HCAs, including D6S1008, D8S1754, D8S261, D8S277, D16S3029, D16S303 and D17S796.

While LOH was demonstrated in NAH, HCAs and HCCs, its frequencies were found to vary with locations in the genome and lesion types (Figure 5), with those up to 20% and 30% considered frequent and highly frequent events, respectively. Of the 39 loci showing LOH, eight were found to be highly frequent in HCAs, clustering at 11p, 13q and 17p, six in the HCCs, located at 6q, 8p, 11p, 16q and 17p, and six in NAH, located at 8p, 11p, 13q and 17p.

Differences between these types of lesions were further demonstrated by their LOH frequencies at different locations at 8p, 11p and 17p (Figure 5). Among the four loci affected at 8p, D8S298 was the only one showing LOH in a high frequency (70%) in NAH. Its frequencies were lower in other clinically detectable hepatocellular neoplasms, being 20% in HCAs and 12% in the HCC samples. The difference between NAH and HCC was significant ($P < 0.01$), and that between NAH and HCAs did not reach statistical significance ($P > 0.05$). Among the three loci at 11p, D11S1301 and D11S2008 were affected more frequently in NAH, HCA and HCC.

As shown in Figure 5, allelic imbalances were found to affect more chromosomal locations in HCC specimens, with LOH demonstrated preferentially at D8S261 (3/8, 38%), D8S1754 (4/13, 31%), D8S277 (3/15, 20%) and D17S796 (38%). Among the loci, D17S796 did not show allelic imbalance in any of the six NAH and 10 HCAs examined. The region 17p13.3, as shown using nine markers, was affected in 7 (39%) of the 18 HCCs, 6 (67%) of the nine HCAs and 6 (46%) of the 13 NAH examined, with the locus D17S926 involved frequently in all of the three lesions. The region 16q21-24, as tested using seven markers, was affected in 6 (33%) of the 18 HCCs, 1 (11%) of the nine HCAs and 5 (38%) of the 13 NAH examined, with D16S3029 and D16S3040 affected frequently in HCC and NAH, but not in HCA specimens.

DISCUSSION

FNH occurs within an otherwise normal liver, and is detected more frequently between the ages of 20 and 50 years. A careful survey of 168 patients in France revealed a marked female predominance, with the male to female ratio being 1/8 (18/150)[4]. However, a male predilection was observed in a report from China involving 86 FNH cases, with the male to female ratio being 1.7:1 (54:32)[19]. This may reflect the differences in lifestyle and eco-environmental factors between these two countries, as a similar tendency has also been noticed for HCA[6]. The role of oral contraceptive use in its development remains a matter of debate[40,41], while its progression was observed in women using oral contraceptives[42-44]. The lesion is frequently solitary. A minority of cases ranging from 7% (6/86)[19] to 24% (40/168)[4] have been shown to have multiple (2 to 30) lesions.

The distinction between FNH and HCA may be difficult in some cases during pathological practice. A central stellate scar, when present, is helpful in establishing the diagnosis of FNH. However, this feature is undetectable in about half of FNH lesions[4,19]. Moreover, a similar change was also observed in some well-differentiated HCCs[45]. In our study, this hallmark feature was not recorded in any of the 12 lesions examined. Clearly, more reliable approaches are needed for the differential diagnosis.

The pathogenesis of FNH remains to be established. Wanless and collaborators proposed that FNH is a

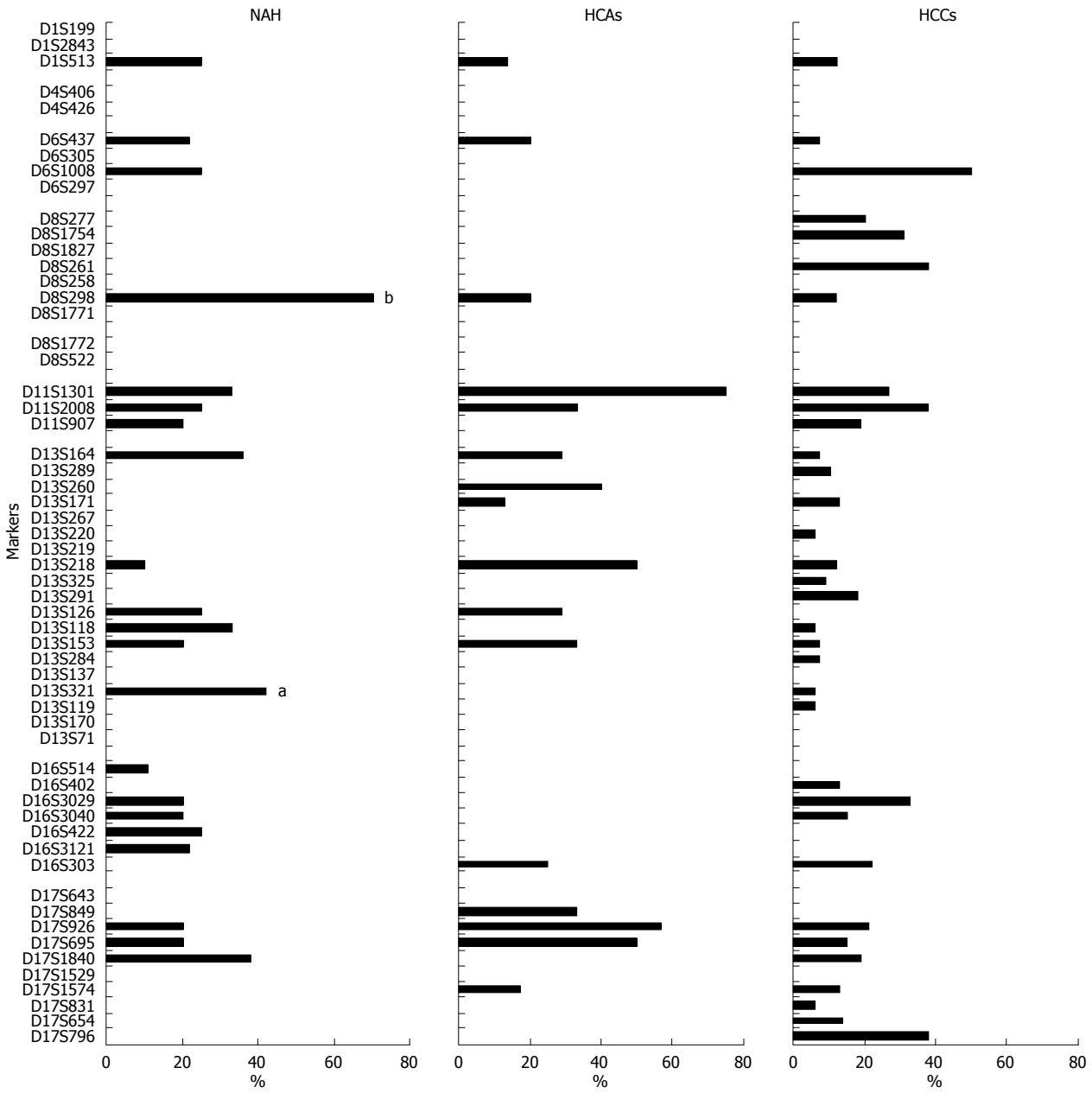

Figure 5 Frequencies of LOH as demonstrated using 57 microsatellite markers in NAH (*n* = 13), HCAs (*n* = 9) and well-differentiated HCCs (*n* = 18). [a]*P* < 0.05, [b]*P* < 0.01 *vs* HCCs.

proliferative response to an arterial malformation[3]. Analysis of angiopoietin (ANGPT) 1 and ANGPT2 mRNA expression revealed an increase in the ANGPT1/ANGPT2 ratio in classical FNH, but not in HCA and telangiectatic FNH[8,9,46]. This may provide a possible mechanism for alterations in proliferation and remodeling of vascular elements within these lesions. However, the nodular hyperplasia of the involved parenchyma needs to be explained. An observation by Paradis *et al*[5], using the XCI analysis, revealed polyclonality in all of the 12 FNH lesions examined. However, data from other laboratories, obtained by similar assays, demonstrated monoclonality in FNH lesions at frequencies ranging from 39%[9] to 75%[7]. The reason for this discrepancy is currently unknown. Insufficient sampling may result in the occurrence of skewed XCI patterns during a test with normal tissues[28,47], which may lead to misinterpretation of the data. The phenomenon was associated with clonal cell clusters (patching) as described in other tissues[28,47-49]. It may also be true that clonal proliferation occurs focally within some of the classical lesions.

In this study, the neoplastic nature of HCC and HCA was confirmed, however, a random XCI inactivation pattern was demonstrated in all of the eight FNH lesions examined. In order to avoid interference from clonal patches, large samples were analyzed for all the lesions. Our results demonstrate that classical FNH, when examined as a whole, is polyclonal in cell composition. The assay is useful for the differential diagnosis between FNH and neoplastic hepatocellular lesions including HCA and HCC.

Human hepatocarcinogenesis is a multi-step process,

including the occurrence of FAH and NAH, neoplastic formation and malignant transformation through high-grade SCC to a fully developed HCC[20,22]. The sequential changes may involve inactivation of multiple tumor suppressors[38]. LOH is one of the main mechanisms for inactivation of tumor suppressor genes. Its occurrence has been localized frequently at 1p, 6q, 8p, 11p and 16q in early-stage, small HCC, while the losses at 4q, 13q and 17p were linked to progression of the lesion[30-38]. As some well-differentiated HCCs and HCAs with high-grade SCC share many morphologic features, it may be difficult to make the differential diagnosis between these disorders with full certainty. This is particularly true when the lesion occurs in a liver without chronic viral hepatitis and cirrhosis. Activation of β-catenin and Wnt-1 signaling pathway has been linked to HCA lesions showing various degrees of phenotypes indicative of malignant transformation[11,14], however, these changes were also found in some HCCs[50,51]. In this study, we examined the allelic integrity of these chromosomal arms using 57 microsatellite markers in nine HCAs and 18 well-differentiated HCCs. Frequent LOH was observed in these well-differentiated HCCs at loci on 6q, 8p, 11p, 16q and 17p. In HCAs, the alterations were observed on similar chromosomal arms, but at different loci (Figure 5). Among the eight loci selected for 8p, four showed LOH, located at D8S261, D8S1754, D8S277 and D8S298, in HCC, and only one in HCA, at D8S298. Among the seven loci for 16q, four showed LOH in HCC, located at D16S3029, D16S303, D16S3040 and D16S402, and only one, at D16S303, in HCA. It seems that the demonstration of 8p losses at D8S261, D8S1754 and D8S277, 16q losses at D16S3029 and D16S3040, and 17p loss at 17S796 is helpful for the identification of HCC from HCA.

In contrast to HCA, FNH did not show TCF1/HNF-1α or CTNNB1 gene mutations and rarely showed DNA losses[14,52,53]. Allelic imbalances are also present in the majority of HCAs, as shown in this study, but results of LOH detection in FNH are confusing. A genome-wide assay by Bioulac-Sage and colleagues demonstrated LOH in 5 (26%) of the 19 classical FNH samples[9], but another group, using a similar approach, did not find any LOH among the 212 informative loci[54]. In the present study, we did not find LOH at any of the 57 loci in the six FNH lesions, as tested using samples as large as 1 cm × 1 cm. This is consistent to the polyclonal nature of FNH as revealed in most of the lesions.

Results from our previous studies on human liver has enabled us to identify FAH and NAH in both explanted and resected liver specimens, and these lesions were associated with SCC and a markedly increased risk of HCC development[20,22]. In a recent study, various kinds of nodules from livers with HBV-associated cirrhosis were microdissected. XCI assay revealed monoclonality in all of the NAH with SCC, 35% of the NAH without SCC, but not in any of the ordinary regenerative nodules examined[27]. These NAH with clonal expansion were considered neoplastic, designated microadenomas and representing hepatic intraepithelial neoplasia (HIN). In this study, multiple FAH and NAH were also identified in FNH lesions, all of these lesions were composed mainly of clear hepatocytes with none showing SCC. A total of 56 NAH were isolated from four FNH lesions, and monoclonality was revealed in 21 (40%) of the 52 informative NAH by XCI analysis, demonstrating multifocal microadenoma formation within FNH. In contrast, all of the five ordinary nodules were proven to be polyclonal.

The occurrence of NAH with different inactivation patterns, as shown in FNH09 (Figure 3B and C), proves that microadenomas develop independently, as described for multiple leiomyomas of the uterus[28]. It is conceivable that FNH is composed of numerous NAH and NAH-forming lesions. The synchronous relationship between different NAH may provide an explanation for the stable behavior of most FNH lesions, in contrast to HCA. In addition, the presence of multiple microadenomas (HIN) with similar XCI patterns in an FNH lesion, as shown in FNH11 (Figure 3D), may result in misinterpretation of the data if the sample examined is small. For this reason, we proposed that sample areas for clonality assessment should be as large as 1 cm × 1 cm for larger FNH or cover the largest cross section for smaller lesions.

While some NAH were shown to be monoclonal, their pathogenesis is unknown. Our assay demonstrated allelic imbalance in all of the 13 lesions, providing additional evidence for their neoplastic nature. The alterations were highly frequent in six loci, involving similar chromosomal arms, as in HCA, including 8p, 11p, 13q and 17p. However, chromosomal arms 8p and 16q were affected in different frequencies in these two types of lesions. D8S298 was affected frequently in both NAH and HCAs, but LOH at this locus was revealed in only a minority of the HCC lesions. Allelic imbalance at D8S298 has been observed in oral and laryngeal squamous cell carcinoma and its precursor lesions. The change was proposed to be an early event in development of the cancer[55]. This change was also observed in HCC specimens, with its frequencies ranging from 15%[56] to 32%[57]. It remains to be determined whether there is a tumor suppressor gene near D8S298 preferentially responsible for HCC development in HBsAg-negative patients.

In summary, classical FNH lesions were shown to be polyclonal by XCI and LOH assays and distinguishable from HCA and HCC. Secondly, multifocal HIN formation, in the form of NAH, was demonstrated within the FNH lesions. In addition, allelic imbalances were also identified in the microdissected NAH, with similar chromosomal arms affected as in HCAs. Therefore, classical FNH is considered a cluster of NAH. Elucidation of the process will be helpful for further understanding of early human hepatocarcinogenesis.

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COMMENTS

Background

Focal nodular hyperplasia (FNH) is a lesion found in an otherwise normal liver, and is considered parenchyma overgrowth responsive to increased blood flow secondary to vascular malformations. While its clinical outcomes are believed to be different from hepatocellular adenoma (HCA) and carcinoma, its pathogenesis is largely unclear and its distinction from HCA is sometimes difficult.

Research frontiers

While FNH was proposed to be a hyperplastic lesion, its clonality status has not been elucidated and the development of hepatocyte nodules within the involved parenchyma needs to be explained. In this study, the authors demonstrate that classical FNH, when examined as a whole, is polyclonal in cell composition. However, the formation of multiple nodules of altered hepatocytes (NAH), showing monoclonality and genetic alterations, was found within all the FNH lesions.

Innovations and breakthroughs

Clonality status of FNH has not been clarified and the mechanism for development of multiple hepatocyte nodules within FNH is unknown. In this study, classical FNH lesions were shown to be polyclonal and distinguishable from HCA and carcinoma. Secondly, the multifocal formation of NAH, representing early-stage hepatic intraepithelial neoplasia (HIN), was demonstrated within the FNH lesions. In addition, allelic imbalances were also identified in microdissected NAH. Classical FNH is considered a cluster of NAH.

Applications

The results of clonality analyses demonstrated polyclonality in all the classical FNH lesions, the approach being useful for the differential diagnosis of FNH from HCA and well-differentiated carcinoma. In addition, elucidation of the pathogenesis of the NAH lesions, representing hepatocytic microadenoma and early-stage HIN, may lead to further understanding of early human hepatocarcinogenesis.

Terminology

Most, if not all, of HCAs and carcinomas develop from a single focus of altered hepatocytes through nodular transformation (formation of NAH) and appearance of cellular and architectural atypia (small-cell change). Monoclonality and nonrandom genetic alterations similar to HCA, as demonstrated in NAH lesions in this study, enable us to consider these lesions hepatocytic microadenoma that represent early-stage HIN.

Peer review

This is an interesting report of clonality and genetic alternations in FNH. The article is well written and deserves publishing.

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BRIEF ARTICLES

Analysis of risk factors for central venous port failure in cancer patients

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Abstract

AIM: To analyze the risk factors for central port failure in cancer patients administered chemotherapy, using univariate and multivariate analyses.

METHODS: A total of 1348 totally implantable venous access devices (TIVADs) were implanted into 1280 cancer patients in this cohort study. A Cox proportional hazard model was applied to analyze risk factors for failure of TIVADs. Log-rank test was used to compare actuarial survival rates. Infection, thrombosis, and surgical complication rates (χ^2 test or Fisher's exact test) were compared in relation to the risk factors.

RESULTS: Increasing age, male gender and open-ended catheter use were significant risk factors reducing survival of TIVADs as determined by univariate and multivariate analyses. Hematogenous malignancy decreased the survival time of TIVADs; this reduction was not statistically significant by univariate analysis [hazard ratio (HR) = 1.336, 95% CI: 0.966-1.849, P = 0.080)]. However, it became a significant risk factor by multivariate analysis (HR = 1.499, 95% CI: 1.079-2.083, P = 0.016) when correlated with variables of age, sex and catheter type. Close-ended (Groshong) catheters had a lower thrombosis rate than open-ended catheters (2.5% *vs* 5%, P = 0.015). Hematogenous malignancy had higher infection rates than solid malignancy (10.5% *vs* 2.5%, P < 0.001).

CONCLUSION: Increasing age, male gender, open-ended catheters and hematogenous malignancy were risk factors for TIVAD failure. Close-ended catheters had lower thrombosis rates and hematogenous malignancy had higher infection rates.

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Key words: Central venous port; Chemotherapy; Risk factor; Cancer patient; Multivariate analysis

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INTRODUCTION

Central venous access is necessary for patients who require long-term intravenous chemotherapy, parenteral nutrition, transfusion of blood components and repetitive blood sampling. Techniques for external cannulation of the subclavian and internal jugular veins were described by Broviac *et al*[1] and Hickman *et al*[2] in the 1970s. In 1982, Niederhuber *et al*[3] introduced the

totally implantable access port. The principal advantages of these access ports are; no external dressing, lower infection rates than non-totally implantable devices and allowance of patients to perform normal physical activities. It is common practice to insert totally implantable venous access devices (TIVADs) in cancer patients beginning a course of chemotherapy to eliminate potential peripheral venous access problems[4,5]; however, risk factors impacting the survival of TIVADs remain unclear. Accordingly, this retrospective cohort study analyzed the risk factors for failure of the TIVADs and compared adverse events among risk factors.

MATERIALS AND METHODS

Patient population

Between January 1, 2003 and December 31, 2006, this retrospective study enrolled 1280 cancer patients who underwent an operation for placement of TIVAD at Chang Gung Memorial Hospital, Chiayi, Taiwan. All devices were indicated for administering prolonged antineoplastic chemotherapy. Medical records for 1280 cancer patients provided data for age, sex, disease, catheter type, surgical procedure, origin of patients, reason of catheter failure, surgical complications and length of implantation. All patients were followed through December 31, 2006, until death or catheter removal due to complications.

Surgical procedure and device care

All procedures were conducted in an operating room by surgeons under fluoroscopic control for correct positioning of the catheter tip in the superior vena cava. The implantation of TIVAD required only local anesthesia (usually 10 mL of 2% mepivacaine hydrochloride) in almost all patients; general anesthesia was used in combination with major surgical procedures for 10 devices. Perioperative antibiotic prophylaxis consisted of a single dose of cephalosporin during the surgical procedure.

Surgical approaches were cephalic cut-down or subclavian vein puncture on either side depending on the surgeon's preference. The right side was usually selected for the TIVAD implantation due to shorter access route to superior vena cava than left side. Implantation from the jugular vein or femoral vein system was considered when there was failure of the cephalic vein and subclavian vein system. The TIVAD system was fixed to the underlying pectoral muscle fascia with one non-absorbable suture. Filling the port system with diluted heparin saline was performed routinely at the end of each procedure.

The TIVAD system was routinely flushed with diluted heparin saline by trained oncologic nurses, following each administration of chemotherapy agents. The device was maintained by flushing with a heparinized solution every 4 wk. Non-coring Huber needles were utilized for all injections into the TIVAD system.

Two catheter tips for port systems are used at our institution: the Groshong valved close-ended catheter (Bard, Salk Lake City, UT, USA), and the open-ended catheter. Port systems were assigned to patients depending on surgeon's preference during the review period.

Study outcomes

The primary endpoint was failure of the TIVAD and the analysis of risk factors affecting device failure among the variables of age, gender, origin of patients, type of catheter, insertion site and type of malignancy using univariate and multivariate methods. The secondary endpoint was to compare the adverse events in relation to the risk factors.

The number of catheter-indwelling-days of TIVAD was calculated from day of insertion to one of the following observation end points: December 31, 2006; date of death; date of catheter removal due to adverse events.

Adverse events of the TIVAD system were divided into three categories: infection events (local erythematous skin change or catheter-related systemic febrile); thrombosis events (intraluminal thrombosis); and surgical complications (pneumothorax, hemothorax, distortion of port system, catheter fracture or malposition). The incidence rate of adverse event was defined as number of events per 1000 catheter-days.

Statistical analysis

The numeric variables were presented as mean ± SD. The number of catheter-indwelling-days was presented as median with inner quartile range (IQR). Log-rank test was conducted for time of death, device removal due to adverse events. For other analyses, independent proportions were compared by using the chi-square test or Fisher's exact test. Using catheter-indwelling-days of TIVAD as a dependent variable and age, sex, catheter type, tumor type, origin of patients and insertion site as independent variables, Cox proportional hazards modeling with forward selection was performed using a two-step technique. First, univariate analysis was performed and included any potential prognostic factor. Thereafter, only variables with a value of $P < 0.10$ by univariate analysis were introduced in the Cox model. $P < 0.05$ indicated a significant statistical difference. All statistical analyses were performed using Stata Statistical Software version 9.2. (StataCorp. 2005. Stata Statistical Software: Release 9.2. College Station, TX, USA).

RESULTS

Distribution of device and device life

From January 1, 2003 and December 31, 2006, 1348 TIVADs were implanted into 1280 consecutive patients. Of the study population, 796 (62%) (842 TIVADs) were male and 484 (38%) (506 TIVADs) were female. The mean age of the subjects was 60.13 ± 13.06 years (range, 13-91 years). Patient origins for insertion of TIVADs were 967 (72%) inpatients and 381 (28%) outpatients. The devices were inserted into 1272 (94%) patients for treatment of solid tumors and 76 (6%) patients for

Table 1 Distribution of 1348 TIVADs and average catheter-indwelling-days

	n (%)	Median (range)
Sex		
Male	842 (62)	151 (60337)
Female	506 (38)	228 (88473)
Origin of patient		
Inpatients	967 (72)	157 (60358)
Outpatients	381 (28)	217 (93455)
Type of malignancy		
Hematogenous	76 (6)	148 (61303)
Solid	1272 (94)	180 (70403)
Type of catheter		
Groshong	830 (61)	218 (81478)
Open-ended	518 (39)	143 (55280)
Total	1348 (100)	178 (70399)

Table 2 Primary malignancy in 1280 patients with 1348 TIVADs for long-term intravenous chemotherapy *n* (%)

Malignancy	Patients	TIVADs
Colorectal	354 (27.7)	359 (26.6)
Lung	348 (27.2)	367 (27.2)
Head and Neck	139 (10.8)	150 (11.1)
Breast	103 (8.0)	109 (8.1)
Gastric	78 (6.1)	91 (6.8)
Hematogenous	70 (5.5)	76 (5.6)
H-B-P	69 (5.5)	72 (5.4)
Esophageal	41 (3.2)	43 (3.2)
Urologic	40 (3.1)	40 (3.0)
Gynecologic	12 (0.9)	14 (1.0)
Others[1]	26 (2.0)	27 (2.0)
Total	1280 (100)	1348 (100)

[1]Others include skin, brain, bone, sarcoma and unknown primary origin. H-B-P: Hepato-biliary-pancreatic.

hematogenous tumors. The catheters used were 830 (61%) Groshong catheters and 518 (39%) open-ended catheters (Table 1). Table 2 lists the distribution of primary malignancies and TIVADs.

Table 3 lists the insertion sites, surgical procedures and catheter type used. Of the 1280 consecutive patients who required 1348 TIVADs, 1100 (81.6%) were suited to a cephalic vein cut-down approach and 196 (14.6%) to a subclavian vein puncture procedure. Of the remaining 52 devices, 23 (1.7%) were placed *via* the jugular vein system due to difficulty approaching the subclavian vein system. The final 29 (2.1%) devices utilized femoral vein placement with or without a saphenous vein approach due to previous neck/thorax radiotherapy or superior vena cava syndrome.

The median (IQR) number of catheter-indwelling-days was 178 (70399) d and total number of catheter-indwelling-days was 368373 d. There were 563 device expires in this study, including 461 deaths (331 males and 130 females) and catheters removed due to 102 adverse events.

Comparisons of risk factors and adverse events

Univariate analysis demonstrated that the following were

Table 3 Insertion site, surgical procedure and catheter type *n* (%)

Site	Surgical procedure	Open-ended catheter	Groshong catheter	Total
Cephalic vein	Cutdown			
Right		287	620	907 (67.3)
Left		111	82	193 (14.3)
Subclavian vein	Puncture			
Right		54	87	141 (10.5)
Left		38	17	55 (4.1)
Internal jugular vein				
Right		8	1	9 (0.7)
Left		0	1	1 (0.1)
External jugular vein				
Right		4	7	11 (0.8)
Left		0	2	2 (0.1)
Femoral vein				
Right		14	9	23 (1.7)
Left		2	4	6 (0.4)
Total		518	830	1348

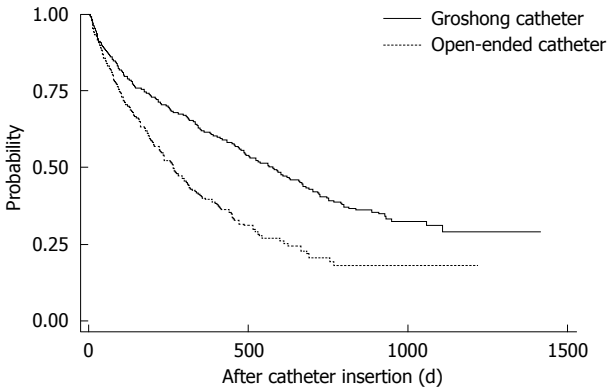

Figure 1 Kaplan-Meier survival curve showing that the Groshong catheter of the TIVAD had better survival time than open-ended catheters by log-rank test (*P* < 0.001).

significant risk factors for TIVAD failure: increasing age; male gender; and use of an open-ended catheter (Table 4). The remaining variables, such as patient origin, insertion site and malignancy type were not statistically significant. Increasing age, male gender, open-ended catheter and hematogenous malignancy [hazard ratio (HR) = 1.499, 95% CI: 1.079-2.083, $P = 0.016$] were significant risk factors by multivariate analysis for reduced TIVAD survival, although hematogenous malignancy (HR = 1.336, 95% CI: 0.966-1.849, $P = 0.080$) was not statistically significant by univariate analysis. The median numbers of catheter-indwelling-days for patients inserted with a Groshong or open-ended tube were 218 and 143 d, respectively. The log-rank test showed highly significant statistical differences between these survival curves ($P < 0.0001$) (Figure 1). Clearly, the patients inserted with open-ended catheter type had much lower survival rates than those with Groshong catheters.

The overall complications were 102 events (7.5%): 40 infection events; 47 thrombosis events; and 15 surgical complications. The overall infection rate was 0.108 events per 1000 catheter-days (40 cases, 2.96%), the thrombosis rate was 0.127 events per 1000 catheter-days

Table 4 Univariate and multivariate analyses of risk factors for TIVAD failure

	Univariate hazard ratio	95% CI	*P* value	Multivariate hazard ratio	95% CI	*P* value
Age (yr)	1.009	1.002-1.015	0.006	1.009	1.003-1.016	0.003
Sex						
Male	1.63	1.373-1.936	< 0.001	1.566	1.318-1.861	< 0.001
Female	1					
Origin of patients						
Inpatient	1					
Outpatient	0.926	0.779-1.100	0.385			
Type of catheter						
Groshong	1					
Open-ended	1.719	1.461-2.023	< 0.001	1.689	1.435-1.989	< 0.001
Insertion site						
RCV	0.891	0.754-1.053	0.179			
Others[1]	1					
Type of malignancy						
Solid	1					
Hematogenous	1.336	0.966-1.849	0.080	1.499	1.079-2.083	0.016

[1]Others include left cephalic vein, right subclavian vein, left subclavian vein, femoral vein and jugular vein. RCV: Right cephalic vein.

Table 5 Comparisons of adverse events for open-ended *vs* Groshong catheter and solid *vs* hematogenous malignancy

	Open-ended catheter (*n* = 518)	Groshong catheter (*n* = 830)	*P* value[1]	Solid malignancy (*n* = 1272)	Hematogenous malignancy (*n* = 76)	*P* value[1]
Infection	14 , 2.7% (0.130)	26, 3.1% (0.099)	0.651	32, 2.5% (0.091)	8, 10.5% (0.456)	< 0.001
Thrombosis	26, 5% (0.242)	21, 2.5% (0.080)	0.015	46, 3.6% (0.131)	1, 1.3% (0.057)	0.515
Surgical complication	9, 1.7% (0.083)	6, 0.7% (0.022)	0.084	12, 0.9% (0.034)	3, 3.9% (0.171)	0.048

[1]χ^2 test or Fisher's exact test. Parenthesis indicated events per 1000 catheter-days.

(47 cases, 3.48%), and the surgical complication rate was 0.04 events per 1000 catheter-days (15 cases, 1.1%).

Table 5 presents comparisons of adverse events for open-ended *vs* Groshong catheters and solid *vs* hematogenous malignancies. Open-ended catheter devices had a higher thrombosis rate than Groshong catheter devices (5% *vs* 2.5%, $P = 0.015$). Hematogenous malignancies had a higher infection rate (10.5% *vs* 2.5%, $P < 0.001$) and surgical complication rate (3.9% *vs* 0.9%, $P = 0.048$) than solid malignancies.

DISCUSSION

Notably, the TIVAD is designed to be a reliable, safe and dependable means of long-term venous access for administering chemotherapy or hyperalimentation nutrition. Several studies have documented the relative superiority of TIVADs over non-totally implanted devices[3-6]. Nevertheless, factors affecting device survival remain a major concern. This study analyzed several risk factors including age, sex, patient origin, catheter type, insertion site and malignancy type to assess whether these factors influence the failure of TIVADs.

According to the literature, the average indwelling duration of the implanted devices varies from 61 to 512 d[7-11]. In this series, the total number of catheter-indwelling-days was 368 373 d and the median duration was 178 d.

The variable of age was a significant risk factor for TIVAD failure by univariate and multivariate analyses (HR = 1.009, 95% CI: 1.003-1.016, $P = 0.003$), signifying that risk of TIVAD failure will increase 1.009 times for each additional year of age. Puig-la Calle *et al*[11] reported an average of 512 catheter-indwelling-days for 123 patients with a mean age of 37 years. Hou *et al*[10] identified an average of 395 catheter-indwelling-days for 298 patients with a mean age of 55 years. In this series, the median of catheter-indwelling-days was 178 d and mean age of 1280 patient was 60 years. Taken together, these three studies indicate that age affects TIVAD survival.

Male gender was another risk factor for TIVAD failure by univariate and multivariate analyses (HR = 1.566, 95% CI: 1.318-1.861, $P < 0.001$). Average number of catheter-indwelling-day for females (228 d) was longer than that for males (151 d). This finding may be due to a higher TIVAD failure rate for males (69%, 417/607 catheter) than females (31%, 190/607) in this series. The causes of TIVAD failure for males were 331 deaths and catheter removal due to 68 adverse events. Advanced stage of cancer with reduced life expectancy for male patients is the major reason for the decreased survival of the devices (data not shown).

The third significant risk factor was catheter type. Open-ended catheters (HR = 1.689, 95% CI: 1.435-1.989, $P < 0.001$) had shorter TIVAD survival time than close-ended catheters (Groshong catheter). We utilized the log-rank test to compare the survival distributions of open-ended and closed-ended catheters. The results, depicted in Figure 1, show that the survival time for close-

ended catheters (Groshong catheter) is significantly better than open-ended catheters using log-rank test ($P < 0.001$). Hou *et al*[10] reported these two catheter types had similar actuarial survival rates in their study. The Groshong catheter[12] has a unique three-way, pressure-sensitive valve that allows infusion and blood aspiration while reducing risk of an air embolism, blood reflux, and clotting. This special design is supposed to increase TIVAD survival, which is confirmed in our study.

Hematogenous malignancy was the last risk factor analysed here. By univariate analysis, hematogenous malignancy decreased TIVAD survival, but was not statistically significant (HR = 1.336, 95% CI: 0.966-1.849, $P = 0.080$). Hematogenous malignancy became a significant risk factor by multivariate analysis (HR = 1.499, 95% CI: 1.079-2.083, $P = 0.016$) when correlated with age, sex and catheter type variables. Hematogenous malignancy has a hypercoagulable status when compared to solid tumors that is considered to be an etiology for shorter device life.

The reported total adverse event rate was 5.1% (13/296 catheters) in the study by Dillon *et al*[13], 11% (33/298 catheters) in the study by Hou *et al*[10], 12.8% (192/1500 catheters) in the study by Kock *et al*[14] and 21% (14/66 catheters) in the study by Grannan *et al*[15]. There was a 7.5% (102/1348 catheters) total adverse event rate in the present series. Groshong catheters (6.3%, 53/830 catheters) had a lower total adverse rate than the open-ended group (9.4%, 49/518 catheters), especially for thrombotic events (2.5% *vs* 5%, $P = 0.015$). This result supports the proposition that the valved tip of Groshong catheters prevents thrombotic events and prolongs catheter survival. Hematogenous malignancies had higher infection and surgical complication rates than solid malignancies ($P < 0.001$, $P = 0.048$, respectively). Vescia *et al*[16] mentioned that sterile precautions are essential when implanting and accessing port systems; infections must be treated with adequate antimicrobial therapy and catheter-related thromboembolic complications constitute a major problem during the device life, but routine anticoagulation cannot be recommended based on the results of four clinical trials[17-20].

Patients from an outpatient background for insertion of TIVAD had a lower risk for device failure than inpatients; however, the difference between the two groups was not statistically significant (HR = 0.926, 95% CI: 0.779-1.100, $P = 0.385$). The insertion site, i.e. right cephalic vein site or other sites, was not a significant risk factor.

In conclusion, increasing age, male gender, open-ended catheter use and hematogenous malignancy were all risk factors for reduced actuarial device survival time in this study by univariate and multivariate analyses. Close-ended (Groshong) catheters had a lower thrombotic rate than open-ended catheters. Hematogenous malignancies had higher infection and surgical complication rates than solid malignancies.

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COMMENTS

Background

Central venous access is crucial for patients who have need of long-term intravenous chemotherapy, parenteral nutrition, transfusion of blood components and repetitive blood sampling. The main advantages of the totally implantable venous access device (TIVAD) are no external dressing, lower infection rates than non-totally implantable devices and allowance of patients to achieve normal physical activities.

Research frontiers

It is common practice to insert the TIVAD in cancer patients beginning a course of chemotherapy to eliminate potential peripheral venous access problems; however, risk factors impacting the survival of TIVADs remain unclear. Accordingly, the research hotspot is to investigate the risk factors for failure of the TIVAD and correlate adverse events with risk factors.

Innovations and breakthroughs

Recent reports have highlighted the relative superiority of TIVADs over non-totally implantable devices. Nevertheless, factors influencing device survival are a major concern. In this study, the authors found that increasing age, male gender, open-ended catheter use and hematogenous malignancy were significant risk factors reducing survival of TIVAD by multivariate analysis. Close-ended catheters (Groshong) had a lower thrombosis rate than open-ended catheters; hematogenous malignancies had higher infection rates than solid malignancies.

Applications

By understanding the risk factors that affect the survival of TIVADs and adverse events relating to the risk factors, this study may represent a future strategy for prolonging the survival time of TIVADs and help prevent the occurrence of adverse events when cancer patients need central venous ports for long-term chemotherapy.

Terminology

TIVAD is a port with a central venous line that does not have an external connector, instead, it has a small reservoir that is covered with silicone rubber and is implanted under the skin. Medication is administered intermittently by placing a small needle through the skin, piercing the silicone and into the reservoir.

Peer review

This is a large-scale analytic study in which authors investigated risk factors and their impact on failure of TIVADs in cancer patients receiving long-term chemotherapy. The findings suggest that increasing age, male gender, open-ended catheter and hematogenous malignancy are the factors that reduce the survival of devices. Additionally, the authors depict that close-ended catheters had lower thrombosis rates and hematogenous malignancies had higher infection rates. The results are interesting and informative, adding to existing literature.

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A systematic review of treating *Helicobacter pylori* infection with Traditional Chinese Medicine

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Abstract

AIM: To evaluate the efficacy and safety of Traditional Chinese Medicine (TCM) in the treatment of *Helicobacter pylori* (*H pylori*) infection.

METHODS: We electronically and manually searched electronic databases, references lists and conferences compilations, and included all randomized clinical trials comparing the treatment of *H pylori* using TCM with proton pump inhibitor or colloidal bismuth subcitrate-based triple therapy as controls. The Jadad score was used to assess trial quality, *H pylori* eradication rate and the incidence of side effects were taken as outcome measurements, and heterogeneity analysis, meta-analysis and funnel plot analysis were conducted.

RESULTS: Sixteen trials were included. The Jadad scores of all the trials were not more than 2. Clinical heterogeneity and substantial statistical heterogeneity existed among the trials (P = 0.001, I^2 = 59%) and meta-analysis was not conducted. The average eradication rates following TCM and triple therapy were 72% and 78% and the incidence of side effects were 2% and 29%, respectively. The funnel plot was obviously asymmetric.

CONCLUSION: Available evidence is not convincing enough to show that TCM has the same efficacy as triple therapy in *H pylori* treatment. TCM may be safer than triple therapy. TCM should not be recommended as monotherapy in *H pylori* infection.

INTRODUCTION

Helicobacter pylori (*H pylori*) plays a crucial role in the pathogenesis of chronic gastritis, peptic ulcer, gastric mucosa associated lymphoid tissue (MALT) lymphoma and gastric carcinoma. Clinical trials have shown that *H pylori* eradication can relieve mucosa inflammation, accelerate ulcer healing, reduce complications and recurrence, alleviate MALT and decrease post-operative relapse of early stage gastric carcinoma. The eradication rates of *H pylori* with proton pump inhibitor (PPI) or colloidal bismuth subcitrate (CBS)-based triple therapy, recommended in the Maastricht consensus report, are around 85%-90%. However, antibiotic resistance in *H pylori* has increased over the past few years. The incidence of primary resistance to metronidazole is over 50%[1] and to clarithromycin is about 18%[2]. Antibiotic resistance decreases the eradication rates of triple therapies significantly. The eradication rate dropped by 38% for *H pylori* resistance to metronidazole and by 55% for the strain resistant to clarithromycin[3]. Additionally, the high dosages of antibiotics applied in triple therapies readily induce a high incidence of adverse effects in about 16%-29% of patients, and poor patient compliance decreases the efficacy indirectly[4]. Researchers are exploring more effective and safer treatment regimens.

Since Warren and Marshall isolated *H pylori* in 1982, many *in vitro* and *in vivo* studies on the treatment of *H pylori* with Traditional Chinese Medicine (TCM) have been carried out. The *in vitro* studies showed that some

herbs such as *Radix scutellariae*, *Lonicera*, *Radix Isatidis Seu Baphicacanthi*, *Coptis chinensis Franch* and *Fructus Aurantii Immaturus* have obvious inhibitory effects on *H pylori*[5,6]. The results of clinical studies are, however, variable. There have been no systematic reviews on the efficacy and safety of TCM in the treatment of *H pylori* infection. Therefore, we reviewed the clinical trials treating *H pylori* with TCM published between 1982 and 2008 which compared the efficacy and safety of TCM with those of triple therapies, in order to define the status of TCM in *H pylori* treatment.

MATERIALS AND METHODS

Inclusion criteria

Inclusion criteria were as follows: (1) Randomized, positive-controlled and parallel trials, irrespective of whether blinding was adopted; (2) Subjects with chronic gastritis, peptic ulcers, remnant gastritis or gastro-esophageal reflux disease who were *H pylori* positive. Subjects with bleeding ulcer or gastric cancer were excluded. (3) Determination of *H pylori* positive status was in accordance with the criteria of the 2007 3rd National Consensus Report on *H pylori* Infection (Consensus Report)[7]; (4) The treatment groups received TCM including single herb, formulae or Chinese medicine products; (5) The control groups received the first line therapy regimens recommended in the Consensus Report; (6) Outcome measurements included *H pylori* eradication rate and the incidence of adverse effects. The definition of *H pylori* eradication must meet the requirements of the Consensus Report.

Literature searching strategy

The electronic databases of MEDLINE (1982-2008), Cochrane Controlled Trials Register (1982-2008), Wei-Pu database (1989-2008) and Wan-Fang database (1998-2008) were searched by using a combination of MESH subject headings of "*H pylori*", "Medicine", "Traditional Chinese" and "clinical trial" without language limitation. Reference lists from trials selected by electronic searching and conference compilations were hand searched.

Data collection and assessment

Two independent reviewers screened all retrieved trials according to the inclusion criteria. If the two reviewers disagreed, the difference was discussed. If a consensus could not be reached, a third reviewer was consulted. The quality of all the eligible trials was scored using the Jadad criteria[8]. Allocation concealment was assessed. Central randomization, pharmacy controlled randomization or random numbers sealed in envelopes were regarded as appropriate concealment. If the method of allocation concealment was not mentioned or the subjects were allocated by the sequence produced by an open random number table, it was regarded as inappropriate concealment[9].

Statistical analysis

H pylori eradication rate and the incidence of adverse effects were used to evaluate the efficacy and safety of the regimens. The relative risk of *H pylori* eradication rate and the incidence of adverse effects were calculated using the original data and presented with 95% confidence interval. If there was no heterogeneity among the trials, a fixed effects model was applied in the meta-analysis. If there was heterogeneity among the trials and $I^2 < 50\%$, a random effects model was used instead in the meta-analysis. If $I^2 > 50\%$, the meta-analysis was aborted. Potential publication bias was analyzed by the funnel plot. All statistics were conducted using RevMan4.2 software.

RESULTS

Trials characteristics and quality

Three hundred and nine papers were obtained after initial searching. 293 papers were excluded after reading titles, abstracts or texts due to the non-clinical nature of the studies, repetitious publication or failure to meet the inclusion criteria. Sixteen papers[10-25] were finally included with a total number of 1983 subjects (124/per trial). All the clinical trials were conducted in China and published in Chinese language. The characteristics of the included trials are presented in Table 1.

The Jadad scores of all the included trials were not more than two. One trial presented details of the randomization methods (random number table)[22] while the rest did not present any such detail. All the trials were single-centered and non-blinded without reporting allocation concealment and sample size calculation. Three trials[10,17,23] reported dropout cases and one of these trials[23] conducted intention-to-treatment analysis.

Clinical efficacy assessment

Due to the wide variety of TCM treatment applied in each trial and the statistical heterogeneity of the trials ($\chi^2 = 36.97$, $P = 0.001$, $I^2 = 59.4\%$), the data could not been pooled to conduct a meta-analysis. The *H pylori* eradication rates of the TCM treatment in each trial are presented in Table 2. The average *H pylori* eradication rates following TCM treatment and triple therapy were 72% (36%-90%) and 78% (44%-93%), respectively. The eradication rates following *Chang Wei Qing* oral liquid and *Jian Pi Qing Hua* formula were lower than those of CBS-based triple therapies. The eradication rates following *Jian Wei Yu Yang* granule and *Wei Kang* formula were lower than those of PPI-based triple therapies. There were no differences in the eradication rates between the remaining TCM treatments and triple therapy. Worst-case scenario was performed in two trials[10,17] which reported dropout cases and did not conduct intention-to-treat analysis. There was no significant difference between the new result and the original result.

Clinical safety assessment

Eleven trials[10,11,14,16-20,23-25] reported adverse effects without presenting the methods of acquiring the information on adverse effects. Eight of 11 reported that TCM treatment had adverse effects with an average incidence of 2%

Table 1 Characteristics of randomized and controlled clinical trials treating *Helicobacter pylori* infection with TCM

Trials	No. of cases	Age (yr)	Gender (male) (%)	Jadad score	Regimen of TCM group	Regimen of triple therapies group
Chen *et al*[10] (2001)	419	23-68	62.1	2	Fixed formula × 7 d	(PPI + A + F) × 7 d
Hua *et al*[11] (2003)	155	19-65	54.2	2	*Changweiqing* oral liquid × 14 d	(CBS + A + F) × 14 d
Fan *et al*[12] (2006)	50	NA	NA	1	*Anzhong Yin* × 28 d	(CBS + A + F) × 14 d
Hua *et al*[13] (2006)	150	23-85	61.3	1	*Jianwei Mieyou* Inspissant × 60 d	(CBS + A + C) × 7 d + CBS × 49 d
Ma *et al*[14] (2006)	106	44.2/43.8	79.3	2	*Weikang* Capsule × 60 d	(RBC + A + M) × 14 d
Wang *et al*[15] (2006)	77	NA	47.7	1	*Jianpi Qinghua* formula × 30 d	(CBS + A + T) × 14 d
Wu[16] (2006)	71	19-65	67.6	2	*Jiawei Liumo* Decoction × 56 d	(PPI + A + M) × 10 d
Yang *et al*[17] (2006)	80	22-65	70	2	*Weitongning* Tab × 28 d	(CBS + A + M) × 14 d
Zhou *et al*[18] (2006)	56	23-70	47.2	2	*Qingwei* Decoction × 14 d	(PPI + A + M) × 14 d
Huang[19] (2007)	320	18-77	55.6	2	*Maimendong* Granule × 28 d	(PPI + A + M) × 14 d
Jin *et al*[20] (2007)	98	18-72	67.4	2	*Maimendong* Granule × 28 d	(PPI + A + M) × 7 d
Wang *et al*[21] (2008)	60	20-64	61.7	1	*Mieyou* Decoction × 14 d	(CBS + A + T) × 14 d
Ling *et al*[22] (2008)	46	33.2/35.1	71.7	2	*Jianwei Yuyang* Granule × 6 wk	(PPI + A + M) × 1 wk + PPI × 1 wk
Wang[23] (2008)	149	16-66	61.7	2	Formulae × 2 wk	(CBS + A + M) × 2 wk
Xiao *et al*[24] (2008)	80	19-77	62.5	2	*Weiyan* Decoction × 20 d	(PPI + A + C) × 7 d
Xin *et al*[25] (2008)	70	48.6/44.6	60	2	*Weikang* formula × 1 mo	(PPI + C + T) × 7 d

TCM: Traditional Chinese Medicine; PPI: Proton pump inhibitor; CBS: Colloidal bismuth subcitrate; RBC: Ranitidine bismuth citrate; A: Amoxillin; M: Metronidazole; T: Tinidazole; C: Clarithromycin; F: Furazolidone. NA: No available.

Table 2 *H pylori* eradication rates comparison between TCM and triple therapies

Trials	TCM (*n*/N)	Triple therapies (*n*/N)	RR (95% CI)	*P*	Ref.
Fixed formula *vs* PPI + A + F	161/211	160/204	1.02 (0.91, 1.13)	0.60	[10]
Anzhong Yin vs CBS + A + F	19/30	14/20	0.90 (0.61, 1.34)	0.63	[12]
Jianwei Mieyou Inspissant *vs* CBS + A + C	60/100	22/50	1.43 (1.01, 2.03)	0.06	[13]
Weikang Capsule *vs* RBC + A + M	46/56	42/50	0.98 (0.82, 1.16)	0.80	[14]
Jiawei Liumo Decoction *vs* PPI + A + M	37/41	28/30	0.97 (0.84, 1.11)	0.64	[16]
Weitongning Tab *vs* CBS + A + M	32/40	31/40	1.03 (0.82, 1.30)	0.78	[17]
Qingwei Decoction *vs* PPI + A + M	13/29	19/27	0.64 (0.40, 1.02)	0.05	[18]
Maimendong Granule *vs* PPI + A + M	166/200	98/120	1.02 (0.91, 1.13)	0.76	[19]
Maimendong Granule *vs* PPI + A + M	43/50	40/48	1.03 (0.87, 1.22)	0.71	[20]
Mieyou Decoction *vs* CBS + A + T	22/32	19/28	1.01 (0.73, 1.43)	0.88	[21]
Formulae *vs* CBS + A + M	81/93	49/56	1.00 (0.88, 1.13)	0.94	[23]
Weiyan Decoction *vs* PPI + A + C	28/40	29/40	0.97 (0.73, 1.28)	0.80	[24]
Changweiqing oral liquie *vs* CBS + A + F	53/103	36/52	0.74 (0.57, 0.96)	0.03	[11]
Jianpi Qinghua formula *vs* CBS + A + T	15/42	29/35	0.43 (0.28, 0.66)	< 0.01	[15]
Jianwei Yuyang Granule *vs* PPI + A + M	11/24	18/22	0.56 (0.35, 0.90)	0.01	[22]
Weikang formula *vs* PPI + C + T	26/40	26/30	0.75 (0.57, 0.98)	0.04	[25]

RR: Relative risk; CI: Confidence interval.

(21/903), while 10 of 11 reported that triple therapy resulted in adverse effects with an average incidence of 29% (204/697). The adverse effects associated with TCM included dizziness, loss of taste, nausea/vomiting, poor appetite, belching and loose stool/diarrhea. There were no abnormalities in routine blood tests, liver or kidney function tests in the TCM groups. Twenty subjects withdrew from the trials due to adverse effects and all were in the triple therapy groups[10,11,17,23].

Funnel plot analysis

Funnel plot analysis was conducted using the eradication rate as the index. The figure was obviously asymmetrical (Figure 1).

DISCUSSION

Although the statistical data in this review showed that there were no differences in eradication rate between TCM treatment and triple therapy in the majority of the trials (12/16), there is no powerful evidence to conclude that TCM treatment has the same efficacy as triple therapy in the treatment of *H pylori* infection due to the following reasons:

Poor quality of the clinical trials

All 16 trials were of poor quality and did not meet the requirements of the CONSORT statement[26]. Although these trials claimed to adopt randomization, only one trial presented details of the randomization method and the remaining trials did not give any details of the randomization method. None of the trials described the methods of allocation concealment. Thus, whether the randomization was truly or effectively conducted in these trials is doubtful. Inappropriate randomization or allocation concealment can lead to selection bias. All trials were single-centered and non-blinded, and the treatment courses of the study groups were longer

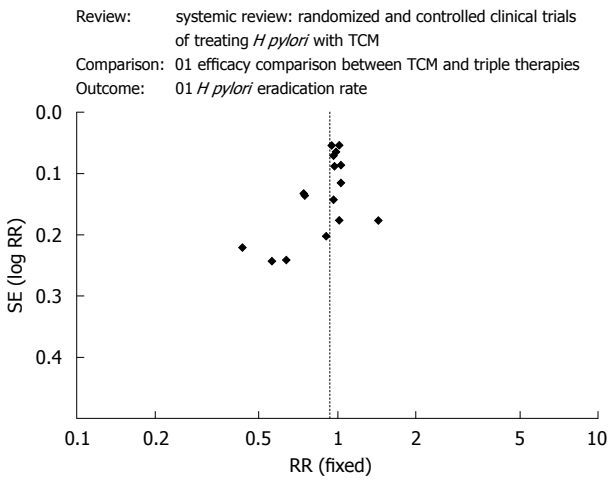

Figure 1 Funnel plot analysis of clinical trials treating *H pylori* with TCM. RR: Relative risk.

than those of the control groups in some trials, which may have resulted in performance bias. Dropout cases were reported in only three of 16 trials and were not mentioned in the other trials. It is not clear whether these trials had any dropout cases or whether they were simply not reported. Having dropout cases without reporting can result in attrition bias. Selection bias, performance bias and attrition bias may overestimate the efficacy of treatment groups. Additionally, none of the trials showed the calculation of sample size, thus, whether the sample size met the statistical requirement or the reliability of the statistical results cannot be judged. Therefore, confidence in the results obtained from these poor quality trials is questionable.

Asymmetrical funnel plot

The asymmetry of the funnel plot may have been caused by the poor quality of the trials and publication bias. All 16 trials were conducted in China and published in Chinese language. Fourteen trials were published in TCM journals, one in a journal of integrated Chinese and Western medicine and one in a college journal. TCM journals are inclined to publish only positive results showing efficacy of TCM treatment. Therefore, when we searched the literature, most of the papers we found reported positive results, with only a few presenting negative results.

Heterogeneity

The TCM formulae used in the 16 trials were composed of various herbs which can clear heat, strengthen the spleen, regulate *Qi* or promote blood circulation. The composition of the formula and the dosage of each herb were different in each trial. The treatment courses also varied in each trial, from 1 wk to 2 mo. This resulted in clinical heterogeneity. Heterogeneity analysis also showed that there was substantial statistical heterogeneity. Due to the limited number of trials and the variety of composition and treatment courses, these trials could not be classified into subgroups for meta-analysis. An overall comparison between TCM and triple therapy could not be conducted.

Eight of 11 trials showed that the incidence of adverse effects following TCM treatment was lower than those following triple therapy, while three trials showed that there was no difference between them. Although non-blinded and lacking in detail on how the information on adverse effects was obtained, due to the obvious difference in the incidence of adverse effects between the two groups (2% *vs* 29%), we speculate that TCM treatment might be safer than triple therapy.

On the basis of the above, we cannot conclude that TCM treatment has the same efficacy as triple therapy in the treatment of *H pylori* infection. TCM should not be recommended as monotherapy in the treatment of *H pylori* infection until stronger positive evidence is obtained. However, neither can we completely ignore the efficacy of TCM in the treatment of *H pylori* infection because of the poor quality of the trials. We suggest that a scientific proposal should be designed and clinical trials performed strictly in accordance with the requirements of evidence-based medicine, in order to truly show and objectively assess the efficacy of TCM in *H pylori* treatment. Additionally, based on the tenet that medical study originates from clinical practice and serves clinical practice, studies on TCM in the treatment of *H pylori* infection should focus on the problems faced in western medicine, such as antibiotic resistance, the high incidence of adverse effects and high cost of the treatment, in addition to comparing the efficacy between TCM and Western medical treatment.

COMMENTS

Background

As antibiotic resistance to *Helicobacter pylori* (*H pylori*) has increased over the past few years, the eradication rates following triple therapies have decreased. New approaches to eradicate *H pylori* are under development.

Research frontiers

Some herbs show obvious inhibitory effects on *H pylori in vitro*. Traditional Chinese Medicine (TCM) alone or integrated with western medicine have been used to treat *H pylori* infection in many clinical trials. However, the results of these trials are controversial and the efficacy of TCM in *H pylori* treatment is indefinite.

Innovations and breakthroughs

This is the first systematic review to assess the efficacy of TCM in the treatment of *H pylori* infection. It truly presents the quality of the clinical trials using TCM in *H pylori* treatment. The authors objectively concluded that the validity of TCM in the treatment of *H pylori* infection could not be determined due to the poor quality of the trials.

Applications

This study suggested that TCM should not be recommended as monotherapy to treat *H pylori* infection and the quality of TCM clinical trials need to be improved in the future.

Terminology

A systematic review is a summary of research that uses explicit methods to perform a thorough literature search and critical appraisal of individual studies to identify valid and applicable evidence.

Peer review

This is a good paper.

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BRIEF ARTICLES

Correlation between anti-fibrotic effect of baicalin and serum cytokines in rat hepatic fibrosis

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Abstract

AIM: To investigate the correlation between the antifibrotic effect of baicalin and serum cytokine production in rat hepatic fibrosis.

METHODS: Forty male Sprague-Dawley rats were divided randomly into four groups: normal control group, model group, baicalin-treated group, and colchicine-treated group. Except for the normal control group, all rats in the other groups were administered with carbon tetrachloride to induce hepatic fibrosis. At the same time, the last two groups were also treated with baicalin or colchicine. At the end of the 8 wk, all animals were sacrificed. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 were measured. Liver index, hepatic hydroxyproline content and the degree of liver fibrosis were also evaluated.

RESULTS: The levels of ALT, AST and liver index in the baicalin-treated group were markedly lower than those in the model group (ALT: 143.88 ± 14.55 U/L *vs* 193.58 ± 24.35 U/L; AST: 263.66 ± 44.23 U/L *vs* 404.37 ± 68.29 U/L; liver index: 0.033 ± 0.005 *vs* 0.049 ± 0.009, $P < 0.01$). Baicalin therapy also significantly attenuated the degree of hepatic fibrosis, collagen area and collagen area percentage in liver tissue ($P < 0.01$). Furthermore, the levels of serum TGFβ1, TNF-α and IL-6 were strikingly reduced in the baicalin-treated group compared with the model group, while the production of IL-10 was up-regulated: (TGF-β1: 260.21 ± 31.01 pg/mL *vs* 375.49 ± 57.47 pg/mL; TNF-α: 193.40 ± 15.18 pg/mL *vs* 260.04 ± 37.70 pg/mL; IL-6: 339.87 ± 72.95 pg/mL *vs* 606.47 ± 130.73 pg/mL; IL-10: 506.22 ± 112.07 pg/mL *vs* 316.95 ± 62.74 pg/mL, $P < 0.01$).

CONCLUSION: Baicalin shows certain therapeutic effects on hepatic fibrosis, probably by immunoregulating the imbalance between profibrotic and antifibrotic cytokines.

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Key words: Baicalin; Hepatic fibrosis; Hepatic stellate cell; Cytokines

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INTRODUCTION

Hepatic fibrosis is a common pathological process of chronic liver injury, regardless of etiology, and its progression leads to cirrhosis and liver cancer[1]. Despite extensive efforts, its etiology and pathogenesis remain unclear, and effective therapy with limited side effects is still lacking[2]. Baicalin is a major bioactive flavonoid contained in dried roots of Scutellaria baicalensis

Georgi (common name: *Huangqin* in China, a traditional Chinese herbal medicine) and it possesses a multitude of pharmacological activities. For instance, Baicalin exerts the inhibitory effects against several virus including influenza virus, human T cell leukemia virus and acquired human immunodeficiency virus type I [3-5]. It can act as potent anti-inflammatory, anti-allergic and anti-bacterial agent in a variety of inflammatory diseases[6-8]. It may also be potentially useful in the treatment of prostate and bladder cancers as well as hepatoma *via* multiple cellular mechanisms[9-11]. Most importantly, previous studies show that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injury induced by iron overload and CCl_4, suggesting that it is a potent free radical scavenger and hepatoprotective drug[12,13]. However, the exact mechanisms of anti-fibrotic effect of baicalin remain unclear. Therefore, it is necessary to be further elucidated.

CCl_4-induced hepatic fibrosis is a well-established animal model to study the pathogenesis and therapy of chronic liver injury. Zhang *et al*[14] have reported that several profibrotic cytokines, including transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α and interleukin (IL)-6, play an important role in the initiation and perpetuation of CCl_4-induced liver fibrosis, whereas IL-10 plays an antifibrogenic role by counterbalancing the former effects .This study aimed to further investigate the effect of baicalin on hepatic fibrosis induced by CCl_4 and its relationship with the expression of TGF-β1, TNF-α, IL-6 and IL-10.

MATERIALS AND METHODS

Reagents and rats

Baicalin (7-glucuronic acid, 5,6-dihydroxyflavone, CAS No.: 21967-41-9) was provided by Sichuan Guanghan Bencao Plant Chemical Co., Ltd (Sichuan, China), and purity was assessed by HPLC (> 98%). Colchicine was purchased from Xiamen Sanland Chemical Co., Ltd (Fujian, China). CCl_4 was obtained from Chongqing Chemical Reagent Co., Ltd (Chongqing, China). Male Sprague-Dawley (SD) rats weighing 150-180 g were purchased from the Experimental Animal Center of Third Military Medical University. All studies involving rats were approved by the Institutional Animal Care and Use Committee.

Induction of liver fibrosis and baicalin treatment

Forty male SD rats were divided randomly into four groups: normal (n = 9); model (n = 11); baicalin-treated (n = 10); and colchicine-treated (n = 10). Except for the normal control group, all rats in the other groups were treated with subcutaneous injection of 40% CCl_4 (initial dose of 0.5 mL/kg, followed by 0.3 mL/kg), mixed with vegetable oil, twice weekly for 8 wk. The latter two groups were also treated with baicalin (70 mg/kg, dissolved in sterile saline water, intraperitoneal injection, once daily), or colchicine (50 μg/kg, dissolved in sterile saline water, intraperitoneal injection, once daily) on the same day as CCl_4 administration and continued for the 8-wk experimental period. The two drug doses were selected based on a previous study[13]. Simultaneously, normal control and model groups were intraperitoneally administered with the same volume of vehicle (sterile saline water) once daily. At the end of the 8-wk experimental period, all animals were anesthetized with 3% chloral hydrate and dissected. Blood and liver were obtained for further analysis.

Table 1 Level of liver index and serum AST, ALT in different treatment groups (mean ± SD)

Group	n	Liver index	ALT (U/L)	AST (U/L)
Normal	9	0.026 ± 0.004	114.50 ± 8.16	183.09 ± 26.70
Model	7	0.049 ± 0.009^{b}	193.58 ± 24.35^{b}	404.37 ± 68.29^{b}
Baicalin	9	0.033 ± 0.005^{b,d}	143.88 ± 14.55^{b,d}	263.66 ± 44.23^{b,d}
Colchicine	9	0.031 ± 0.004^{a,d}	167.60 ± 21.66^{b,c}	325.61 ± 52.83^{b,c}

$^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Measurement of serum aspartate aminotransferase (AST)

Serum AST and alanine aminotransferase (ALT) levels were measured using on an automated analyzer of biochemistry (Hitachi 7170, Tokyo, Japan) according to the manufacturer's instructions.

Liver index calculation

Liver index was measured according to the formula: (rat liver weight/rat weight) × 100%[15].

Histopathology

Samples were obtained from the same liver lobe in all animals and fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) or van gieson (VG) stain.

The degree of liver fibrosis was evaluated on HE-stained sections as described previously[16,17]. The collagen content of the sections was also determined on VG-stained sections by a computer image analysis system (CM2000B, Beijing University of Aeronautics & Astronautics, China). Five random fields were chosen in each section and the amount of total collagen was detected in the area stained by VG, and expressed as percentage relative to the total area[18].

ELISA for serum TGF-β1, TNF-α, IL-6 and IL-10

Cytokine levels in the serum samples were measured by a commercially available ELISA kit (Biosources, San Jose, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS for Windows, version 13.0 (Chicago, IL, USA). Parametric data were analyzed statistically by one-way ANOVA followed by post-hoc tests when appropriate. Degree of hepatic fibrosis was analyzed by Kruskal-Wallis nonparametric test. Data were expressed as the means ± SD. A significant difference was defined as $P < 0.05$.

Table 2 Degree of liver fibrosis in different treatment groups

Group	*n*	Degree of hepatic fibrosis					Average
		0	I	II	III	IV	
Normal	9	9	0	0	0	0	0
Model	7	0	0	0	4	3	3.43[b]
Baicalin	9	0	3	4	2	0	1.89[b,d]
Colchicine	9	0	3	5	1	0	1.78[b,d]

[b]$P < 0.01$ *vs* normal control group; [d]$P < 0.01$ *vs* model group.

Table 3 Comparison of collagen area and collagen area percentage in liver tissue from rats of different treatment groups (mean ± SD)

Group	*n*	Collagen area (μm^2)	Collagen area percentage
Normal	9	993.54 ± 145.31	1.97 ± 0.30
Model	7	2599.99 ± 488.32[b]	10.15 ± 2.87[b]
Baicalin	9	1407.74 ± 284.49[b,d]	4.54 ± 2.08[b,d]
Colchicine	9	1396.00 ± 276.07[b,d]	4.65 ± 2.11[b,d]

[b]$P < 0.01$ *vs* normal control group; [d]$P < 0.01$ *vs* model group.

Table 4 Serum levels of TGF-β1, TNF-α, IL-6 and IL-10 in rats in the different treatment groups (mean ± SD) (pg/mL)

Group	*n*	TGF-β1	TNF-α	IL-6	IL-10
Normal	7	199.78 ± 18.92	157.62 ± 11.77	187.98 ± 51.97	700.52 ± 138.63
Model	7	375.49 ± 57.47[b]	260.04 ± 37.70[b]	606.47 ± 130.73[b]	316.95 ± 62.74[b]
Baicalin	7	260.21 ± 31.01[b,d]	193.40 ± 15.18[b,d]	339.87 ± 72.95[b,d]	506.22 ± 112.07[a,d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal control group; [d]$P < 0.01$ *vs* model group.

RESULTS

Animals

Irritability, aggression and weight loss were present predominantly in the model group. At the end of the 8-wk experimental period, no death was found in the normal control group. There were four deaths in the model group, one in the colchicine-treated group, and one in the baicalin-treated group.

Liver index and serum aminotransferases

Liver index in the normal control group was 0.026 ± 0.004. However, 8 wk after CCl_4 injection, the liver index increased markedly. The increase was significantly attenuated by baicalin or colchicine treatment ($P < 0.01$, Table 1).

We then measured serum aminotransferase activity in different experimental groups. The levels of serum AST and ALT were significantly increased in the model group compared with those in the normal control group. In contrast, baicalin or colchicine treatment significantly suppressed upregulation of these parameters induced by CCl_4 ($P < 0.05$ or $P < 0.01$, Table 1).

Histopathology

Using HE staining, we observed that the liver tissue in normal control rats showed normal lobular architecture with central veins and radiating hepatic cords. However, liver sections taken from rats in the model group exhibited more inflammatory infiltration, steatosis, hepatocyte coagulative necrosis and fibrous septa compared with the normal control rats after 8 wk of CCl_4 treatment. In contrast, baicalin or colchicine treatment markedly ameliorated these histopathological changes (Figure 1A-D). The results were further supported by a significantly decreased staging score of hepatic fibrosis after baicalin or colchicine therapy ($P < 0.01$, Table 2).

We evaluated by VG staining the collagen level in the liver tissue from different treatment groups. Compared with the normal control group, both collagen area and collagen area percentage were significantly increased in the model group. The increases were reduced by baicalin or colchicine treatment, similar to the changes in hepatic histological examination ($P < 0.01$; Figure 1E-H, Table 3). Therefore, the above findings show that baicalin can prevent CCl_4-induced hepatic fibrosis in rats.

Effect of baicalin on serum TGF-β1, TNF-α, IL-6, IL-10 production

As shown in Table 4, the levels of serum TGF-β1, TNF-α and IL-6 in the model group were significantly higher than those in the normal control group ($P < 0.01$). Upregulation was markedly inhibited by treatment with 70 mg/kg baicalin ($P < 0.01$). On the other hand, IL-10 production in the model group was sharply decreased compared with that in the normal control group (55% reduction, $P < 0.01$). However, baicalin therapy significantly recovered the decrease induced by CCl_4 ($P < 0.01$).

DISCUSSION

In the present study, baicalin significantly lowered the levels of serum ALT, AST and liver index, reduced histological changes of liver fibrosis, suppressed the expression of cytokines, including TGF-β1, TNF-α and IL-6, and improved significantly the serum level of IL-10. Furthermore, our previous study showed that the increases of several fibrosis indices including serum hyaluronic acid, type Ⅳ collagen and hepatic hydroxyproline content after the CCl_4 injection can be notablely inhibited by baicalin treatment[13]. The above findings demonstrated that baicalin can effectively prevent CCl_4-induced hepatic fibrosis in rats and regulate the production of cytokines correlated with fibrosis.

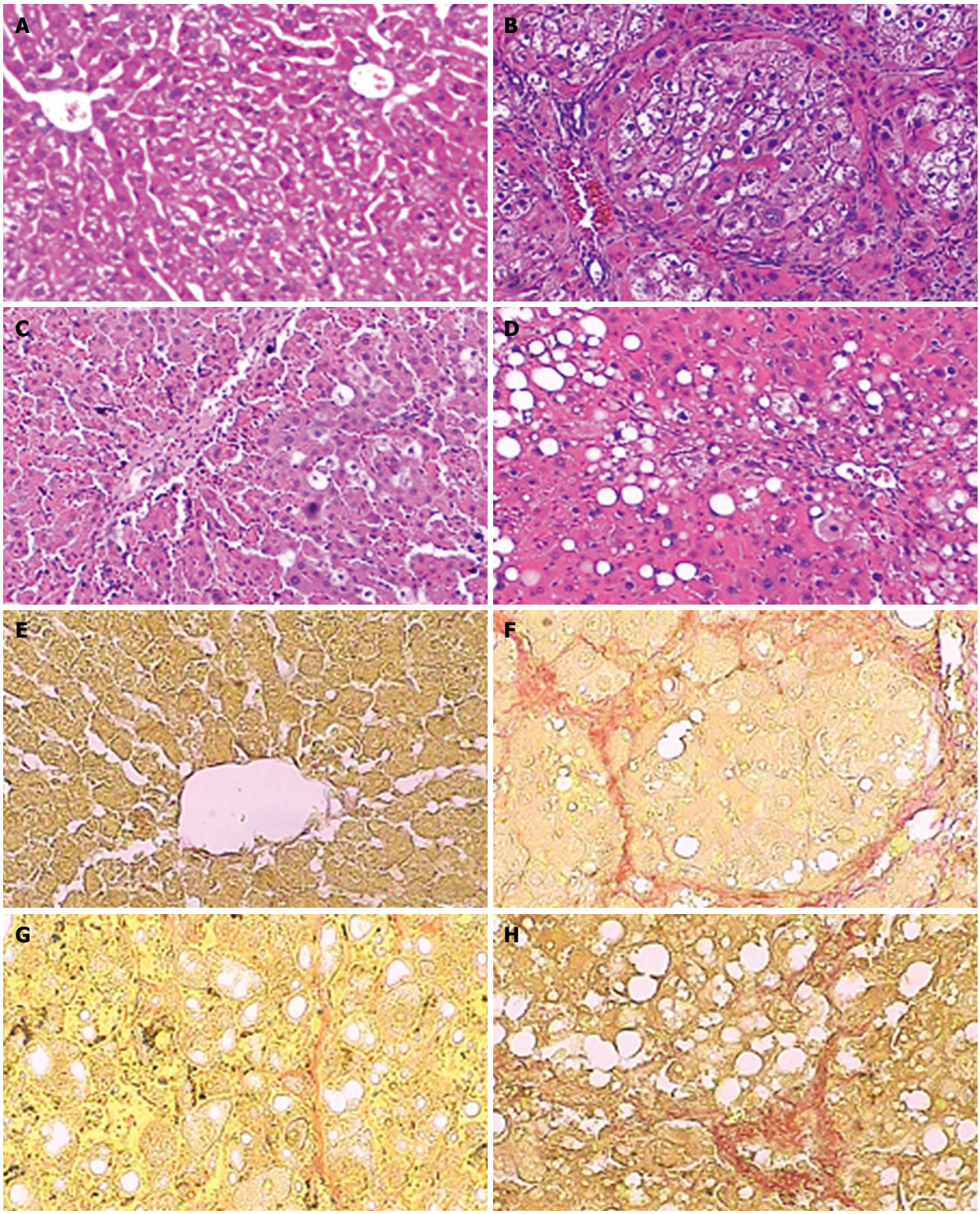

Figure 1 Representative pathological changes in liver sections taken from four experimental groups (A-D: HE, × 100; E-H: VG, × 200)**.** A, E: Normal; B, F: Model; C, G: Baicalin; D, H: Colchicine.

Carbon tetrachloride (CCl_4), is a known hepatotoxin that can cause liver necrosis, fibrosis and cirrhosis when administered repeatedly. Hepatotoxicity is thought to involve two phases[19,20]. The initial phase involves bioactivation by a microsomal cytochrome-P450-dependent monooxygenase system, which results in the formation of free radicals and oxidative stress/lipid peroxidation which exhibits the increase of malondialdehyde (MDA) amounts and decrease of superoxide dismutase (SOD) levels[19,21]. The second step involves the activation of Kupffer cells, which is accompanied by the production of profibrotic mediators such as TGF-β1, TNF-α and IL-6[22]. Hepatic stellate cells (HSC), activated by pro-fibrotic factors, lose

vitamin A and transform into myofibroblasts (MFB), expressing α-smooth muscle actin (α-SMA) and thus gaining the function of contractibility, proliferation and fibrogenesis[23,24]. In this study, we observed that baicalin significantly reduced the increase in profibrotic cytokines such as TGF-β1, TNF-α and IL-6 induced by CCl_4. The reduction in profibrotic cytokines may be correlated closely with previous results that baicalin has good radical scavenging action (lessening the MDA level and activating the SOD activity) and can thus reduce the production of activated Kupffer cells[12,13]. The down-regulation of pro-fibrotic cytokines induced by baicalin treatment then significantly inhibits the activation and proliferation of HSC and enhances HSC apoptosis *in vitro* or *vivo* studies[13], which results in the extenuation of hepatic fibrosis. Thus, the reduction of pro-fibrotic cytokines such as TGF-β1, TNF-α and IL-6 levels is one important mechanism associated with anti-fibrotic effect of baicalin.

IL-10 is a pluripotent cytokine produced by many activated immune cell types, including T-helper cells, B cells, macrophages, monocytes and keratinocytes[25]. Recent studies have indicated that IL-10 might play an important role in antifibrogenesis during CCl_4-induced hepatic fibrogenesis[26-28]. Our study showed that the level of circulating IL-10 in the model group was lower than that in the normal control group, which was consistent with a previous study[14]. In contrast, baicalin significantly restored the decrease in IL-10 content induced by CCl_4, probably contributing to the antifibrotic effect of baicalin.

In conclusion, baicalin has significant antifibrogenic effects on CCl_4-induced liver fibrosis in rats. In addition to the inhibition of HSC activation and lipid peroxidation, as previously reported, immunoregulation of the imbalance between profibrotic and antifibrotic cytokines is one of the most important factors involved in the preventive effect of baicalin on CCl_4-induced liver fibrosis. The exact molecular mechanisms remain to be explored.

COMMENTS

Background

Hepatic fibrosis is a common pathological process of chronic liver injuries, regardless of etiology, and its progression leads to cirrhosis and liver cancer. Despite extensive efforts, its etiology and pathogenesis remain unclear and effective therapies with limited side effects are still deficient. Baicalin is a flavonoid purified from the medicinal plant Scutellaria baicalensis Georgi, a well known Traditional Chinese Medicine. The previous studies show that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injuries induced by iron overload and CCl_4, however, its exact mechanisms remain unclear.

Research frontiers

Baicalin is a flavonoid purified from the medicinal plant Scutellaria baicalensis Georgi, a well known Traditional Chinese Medicine. In the area of prevention of hepatic fibrosis with baicalin, one of research hotspots is that how is the actual mechanism of anti-fibrotic effect of baicalin.

Innovations and breakthroughs

The previous studies have shown that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injuries induced by CCl_4. In this study, the effect of baicalin on hepatic fibrosis induced by CCl_4 and its relationship with the expression of pro-fibrotic and anti-fibrotic cytokines were first investigated. The levels of liver index and serum aminotransferases in baicalin-treated group were markedly lower than those in model group. Baicalin therapy also significantly attenuated the degree of hepatic fibrosis, collagen area and collagen area percent in liver tissue. Furthermore, the levels of serum transforming growth factor-β1, tumor necrosis factor-α and interleukin (IL)-6 were strikingly reduced in baicalin-treated group compared with model group while the production of IL-10 was up-regulated .The above results show that baicalin has certain therapeutic effects on hepatic fibrosis probably by immunoregulating the imbalance between pro-fibrotic and anti-fibrotic cytokines.

Applications

The study demonstrates that baicalin is a good hepatoprotective drug for preventing and treating human liver fibrosis probably by immunoregulating the imbalance between pro-fibrotic and anti-fibrotic cytokines.

Terminology

Hepatic fibrosis is characterized by elevated deposition and altered composition of extracellular matrix, which is a common stage in most chronic liver injuries. Baicalin is a bioactive anti-inflammatory flavone purified from the medicinal plant Scutellaria baicalensis Georgi, a well known Traditional Chinese Medicine.

Peer review

The authors analyzed the preventative effects and mechanism of baicalin in the treatment of liver fibrosis in rats. The results are interesting and suggest that baicalin may be a clinical useful agent for preventing and treating human liver fibrosis.

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CASE REPORT

Tumor lysis syndrome after transarterial chemoembolization of hepatocellular carcinoma: Case reports and literature review

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Abstract

Tumor lysis syndrome (TLS) is a potentially lethal complication in cancer therapy. It may occur in highly sensitive tumors, especially in childhood cancer and leukemia, whereas, it is rare in the treatment of solid tumors in adults. TLS results from a sudden and rapid release of nuclear and cytoplasmic degradation products of malignant cells. The release of these can lead to severe alterations in the metabolic profile. Here, we present two cases of large hepatocellular carcinoma (HCC) treated by transarterial chemoembolization (TACE) that resulted in TLS. Although TLS rarely happens in the treatment of adult hepatic tumor, only a few cases have been reported. We should keep in mind that all patients with HCC, particularly those with large and rapidly growing tumors, must be closely watched for evidence of TLS after TACE.

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Key words: Hepatocellular carcinoma; Therapeutic chemoembolization; Tumor lysis syndrome

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm worldwide, and the third most common cause of cancer-related death. Despite the development of several methods for unresectable HCC, transarterial embolization (TAE) is the most widely used[1]. Although TAE is an effective modality, there are several potential side effects such as hepatic failure, internal bleeding, liver abscess, biliary tract injury, and renal failure[2]. Among these varied complications, tumor lysis syndrome (TLS) is easily neglected and may be fatal.

TLS was first reported in 1929, and is defined by the release of intracellular components into the bloodstream as a result of massive lysis of malignant cells after effective therapy. The release of these components can cause severe metabolic alterations, characterized by hyperuricemia, hyperkalemia, hyperphosphatemia, and hypocalcemia[3-5]. Hyperuricemia is caused by purine degradation and can lead to precipitation of uric acid crystals in the kidney collecting tubules, which results in obstructive nephropathy[6]. Hyperkalemia, following potassium effusion from the cytoplasm, may lead to cardiac arrhythmia and arrest. Therefore, TLS can lead to acute renal failure and can be life-threatening, if it is not recognized early and treated. It is most commonly observed following chemotherapy for high-grade lymphoproliferative malignancy, such as acute lymphocytic leukemia and Burkitt lymphoma[7,8]. Although TLS also has been reported in many different types of solid tumors, including lung and breast carcinoma[9-11], it is still very rare in the treatment of HCC with transarterial chemoembolization (TACE). As far as we know, only a few cases have been reported in the English-language literature[12,13].

Here, we report two cases of acute TLS in patients with HCC following TACE, and review the literature.

CASE REPORT

Case 1

A previously well 76-year-old woman suffered from epigastric fullness and dull pain for about 1 mo. Abdominal sonography revealed a large liver tumor that occupied the majority of the right lobe (Figure 1A), and biopsy of the tumor showed HCC. TACE with 20 mg adriamycin (Figure 1B) was performed during admission.

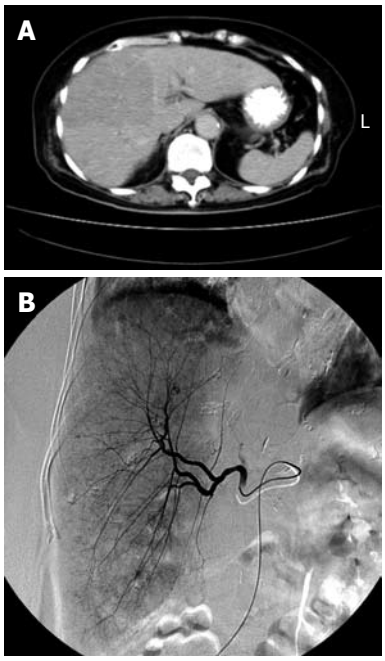

Figure 1 Computed tomography scan obtained before TACE showed a large HCC over the right lobe (A), and TACE revealed hypervascularization of the tumor (B).

The patient developed acute renal insufficiency on the next day. Contrast-induced acute renal failure was suspected in the beginning, but the symptom did not improved after adequate resuscitation. On the third day post-TACE, the acute renal failure worsened. In addition, hyperuricemia (16.6 mg/dL), hyperkalemia (5.7 mEq/L) and hypocalcemia (6.8 mg/dL) were noted later. Under suspicion of TLS, oral allopurinol and urine alkalization, combined with hemodialysis, were given immediately. The patient's renal function stabilized and the urine output increased progressively after treatment. Unfortunately, severe aspiration pneumonia developed during this period, and the patient expired because of sepsis on day 17 after TACE.

Case 2

A 56-year-old male patient was a hepatitis B virus carrier. Regular surveillance revealed a large hepatic tumor over segment 6 and a high α-fetoprotein level (247 ng/dL). Under suspicion of HCC, he was admitted for further treatment. After discussion with the hepatobiliary team, TACE (10 mg lipiodol + 20 mg adriamycin) was suggested as the treatment of choice. Oliguria occurred on the same night. The previous experience reminded us about the possibility of TLS in such a situation. We checked the laboratory profile for TLS immediately. Hyperuricemia (15.1 mg/dL), hyperphosphatemia (4.7 mg/dL) and hypocalcemia (6.6 mg/dL) were noted, and TLS was diagnosed. After standard treatment including oral allopurinol and urine alkalization, this patient's symptoms improved dramatically. Finally, he was discharged on day 6 post-TACE without any other complication.

DISCUSSION

HCC is the fifth most common neoplasm in worldwide, and the third most common cause of cancer-related death. Only a minority of HCCs are diagnosed at an early stage for potentially curative therapy such as surgical resection or local ablation[1]. Several methods have been developed for unresectable HCC, including radiotherapy, chemotherapy and TACE. Among the various treatments, TACE has shown evidence of a beneficial survival effect compared with other methods[1,2]. As the most widely used treatment for advanced HCC, TACE still has some complications. Common adverse effects of TACE include liver dysfunction, post-embolization syndrome, hepatic infarction, and liver abscess. Besides these, TLS is a very rare and easily neglected complication.

The first two cases of TACE-induced TLS were reported by Burney in 1998, and both had large HCCs (> 5 cm in size)[12]. As in our situation, the first case was diagnosed too late and the patient died. The second patient's syndrome was detected early and treated appropriately. Another case report was published by Sakamoto *et al*[13]. This patient had a large HCC > 20 cm in size. After treatment with TACE, the patient also died of acute TLS. Both authors concluded that TLS is a possible and lethal complication of TACE for large HCC.

TLS is a known consequence of treatment of hematological malignancies such as leukemia and lymphoma[7,8]. In general, TLS is less likely to occur in adult patients with solid tumors. However, some solid malignancies in adults, such as breast cancer, small cell lung cancer, seminoma, metastatic Meckel's cell tumor, medulloblastoma, and even hepatoma have been reported to be the cause of TLS[9-11]. TLS may occur after various treatments of HCC, including radiofrequency ablation[14], oral thalidomide[15] and TACE[12,13].

TLS arises from spontaneous cell death or within the first few days after cytotoxic therapy, and results from rapid tumor destruction. It leads to hyperuricemia, hyperkalemia, hyperphosphatemia, hypocalcemia, or often, acute renal failure[3-6]. Early identification of patients at risk and prevention of TLS development is critical. Acute renal failure may occur from intravascular volume depletion or from deposition of uric acid and calcium phosphate crystals in the renal tubules. Without prompt and proper management, derangements of TLS can result in death, as in case 1. Early recognition and treatment will reduce the risk of this fatal complication. For early recognition, it is essential to monitor patients at high risk, especially during the early course of treatment. Traditionally, patients with large tumor burdens and/or rapidly dividing tumors are at greatest risk for TLS. Other risk factors include chemosensitive tumors, large area of tumor necrosis, and pretreatment renal dysfunction. Serum potassium, uric acid, calcium, phosphate and lactate dehydrogenase level, and renal function should be monitored intensively in these high-risk patients after cancer therapy[3-5].

According to the Cairo-Bishop classification, TLS is defined as a 25% change or a level above or below normal for any two or more serum values of uric acid, potassium, phosphate, and calcium within 7 d after cancer therapy[5] (Table 1). The principles of treatment of TLS

Table 1 Cairo-Bishop definition of TLS

Uric acid	⩾ 8.0 mg/dL or 25% increase from baseline
Potassium	⩾ 6.0 mEq/L or 25% increase from baseline
Phosphorus	⩾ 4.5 mg/dL or 25% increase from baseline
Calcium	⩾ 7.0 mg/dL or 25% decrease from baseline

should address three critical areas: hydration, correction of metabolic abnormalities, and aggressive treatment of renal failure. Aggressive fluid administration has been recommended in all high-risk patients. Volume expansion with isotonic saline can reduce serum concentrations of uric acid, phosphate and potassium. As renal blood flow, glomerular filtration rate, and urine output are all increased, the concentration of solutes in the renal tubules is decreased, which makes precipitation less likely[16]. Prevention and treatment of abnormal metabolites are the next step, especially hyperuricemia. Uric acid crystals can precipitate in renal tubules and cause obstructive nephropathy and renal failure. Oral or intravenous allopurinol (100 mg/m^2 every 8 h) is recommended for the treatment of hyperuricemia. Recently, another urate oxidase (rasburicase; 0.05-0.2 mg/kg) was demonstrated to be more effective than allopurinol for the treatment of hyperuricemia[3-6]. If obstructive nephropathy persists and progresses to acute renal failure, aggressive hemodialysis should not be delayed[16].

Oliguria is not a rare occurrence post-TACE. The most common cause is radiocontrast-induced renal insufficiency. However, oliguria also can be one of the early clinical signs of TLS[5]. The incidence of acute renal insufficiency caused by TACE *per se* has been shown to be 3%-8% for each treatment session[17]. It is much more common than that induced by TLS. Adequate hydration is sufficient to overcome this complication. Therefore, many physicians may misdiagnose oliguria as a sign of contrast-induced renal insufficiency and delay proper treatment. Hydration alone is not sufficient for the treatment of TLS. Correction of abnormal metabolites, such as reduction of uric acid level, is another essential feature. If we overlook the potential risk of TLS, the results can be tragic.

In conclusion, although rare, TLS can occur after TACE of HCC in adults. Pretreatment evaluation of risk factors, including large tumor size, rapid tumor growth, and renal insufficiency should be completed. Close monitoring after TACE in these high-risk patients is warranted. When TLS develops, effective treatment, including adequate hydration, oral medication (allopurinol and/or urate oxidase) or hemodialysis, should be undertaken immediately.

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CASE REPORT

A rare case of periampullary carcinoma with ectopic ending of Vater's ampulla

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Abstract

A 71-year-old woman was referred to our department complaining of painless progressive jaundice for the last 3 mo. Magnetic resonance imaging and magnetic resonance cholangiopancreatography (MRCP) showed the ectopic hepatopancreatic ampulla draining into the fourth part of the duodenum adjacent to the duodenojejunal flexure; the irregular morphology of the duodenojejunal flexure likely due to a soft tissue mass. Laparotomy confirmed the presence of the abnormal ampulla of Vater located at the fourth part of the duodenum and a soft tissue tumor about 6 cm × 5 cm × 5 cm with a peduncle adjoining the ampulla. Resection of the tumor, including some peripheral tissue, and a Roux-Y loop anastomosis choledochojejunostomy were performed. Pathological examination indicated an intestinal villous adenoma accompanied by severe dysplasia and focal canceration. Periampullary carcinoma with ectopic ending of the Vater's ampulla into the fourth part of the duodenum is rather rare. The embryonic genetic background of this anomaly has not yet been fully explained. It is worth mentioning that MRCP is useful for demonstrating anomalies and anatomic variants of the biliary tract system and pancreatic duct.

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Key words: Ectopia; Ampulla of Vater; Periampullary carcinoma; Magnetic resonance cholangiopancreatography; Treatment

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INTRODUCTION

The usual location of the major papilla of Vater is in the mid-portion of the descending duodenum, closer to the posterior wall than the anterior wall. On rare occasions it may be found in other sites, along a line extending from the stomach to the fourth part of the duodenum[1-5]. Here we present a case of periampullary carcinoma combined with ectopic opening of the Vater's ampulla into the fourth part of the duodenum and a low confluence of the cystic duct into the common bile duct. To our knowledge, such a case has never been described previously, so the pathogenesis, diagnosis and treatment will be discussed in this report.

CASE REPORT

A 71-year-old woman was referred to our department complaining of painless progressive jaundice for the last 3 mo. Hypertension and diabetes mellitus were diagnosed 10 years previously, and a cholecystectomy was performed 18 years before admission. Physical examination did not show any abnormalities except jaundice. Laboratory data (Table 1) showed the levels of the serum total bilirubin, direct bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and glutamyl transpeptidase were 138.5 μmol/L, 120.0 μmol/L, 78 IU/L, 184 IU/L, 1031 IU/L, and 944 IU/L, respectively (significantly higher than normal); tumor markers including serum α-fetoprotein, carcinoembryonic antigen, carbohydrate antigen 199 (CA199), and carbohydrate antigen 125 (CA125) were 1.44 ng/mL, 3.27 ng/mL, 34.54 U/mL, and 89.87 U/mL, respectively (the latter two were a little higher than normal); the hepatitis B & C markers were negative; blood routine test (BRT) was normal. Sonography of the upper abdomen revealed only hepatic biliary and main pancreatic duct dilation. Subsequently magnetic resonance imaging and magnetic resonance cholangiopancreatography

Table 1 Some lab tests of patient

Lab items	Values		Normal values
TBIL (μmol/L)	138.5	↑	1.7-17
DBIL (μmol/L)	120.0	↑	0-6.8
ALT (IU/L)	78	↑	10-40
AST (IU/L)	184	↑	10-40
ALP (IU/L)	1031	↑	40-110
GT (IU/L)	944	↑	< 50
AFP (ng/mL)	1.44		< 20
CEA (ng/mL)	3.27		< 15
CA199 (U/mL)	34.54	↑	< 33
CA125 (U/mL)	89.87	↑	0-35

TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GT: Glutamyl transpeptidase; AFP: α-fetoprotein; CEA: Carcinoembryonic antigen.

Figure 1 Magnetic resonance imaging showed the expansion of hepatic biliary duct and the ectopic hepatopancreatic ampulla (arrow) draining into the fourth part of the duodenum.

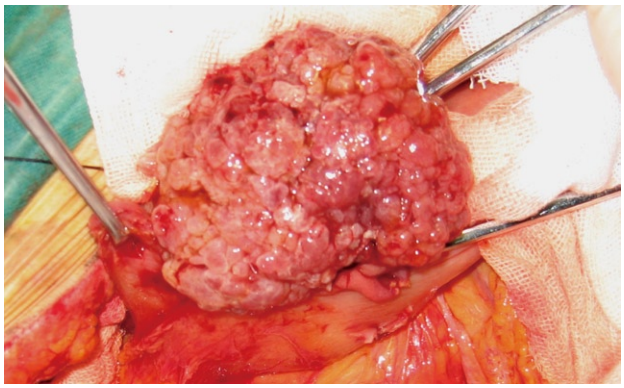

Figure 2 A soft texture tumor about 6 cm × 5 cm × 5 cm with a peduncle conjunct to the ampulla.

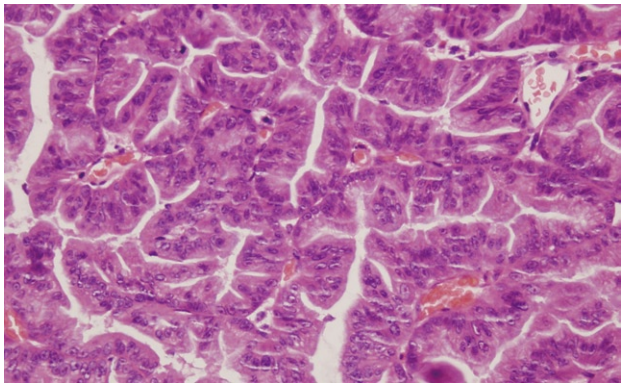

Figure 3 Microscopic examination (HE, × 400) showed severe dysplasia and focal carcinomatous change.

(MRCP) were performed (Figure 1) which showed the expansion of the extrahepatic and intrahepatic biliary ducts and the main pancreatic duct, the ectopic hepatopancreatic ampulla draining into the fourth part of the duodenum adjacent to the duodenojejunal flexure, a low confluence of the cystic duct into the common bile duct with a distance of approx 4 cm to the Vater's ampulla, and the irregular morphology of the duodenojejunal flexure; likely due to a soft tissue mass, which did not rule out the possibility of periampullary tumor resulting in the common bile duct stricture. Gastroscopy revealed chronic superficial antral inflammation without orifice or mucosal ulceration in the stomach and without any duodenal papilla in the descending duodenum.

Laparotomy confirmed the presence of an abnormal ampulla of Vater located at the fourth part of the duodenum and a soft tissue tumor about 6 cm × 5 cm × 5 cm with a peduncle joined to the ampulla (Figure 2). Intraoperative choledochoscopic examination displayed no mass in the common bile duct. Consequently, complete resection of the tumor including some peripheral tissue was performed from an incision of the jejunum, close to the duodenojejunal flexure. Frozen pathological examination indicated an intestinal villous adenoma accompanied by severe dysplasia and highly suspect focal canceration, however, no incisal margin tumor cells were seen. A Roux-Y loop anastomosis at side-to-side choledochojejunostomy and end-to-side jejunojejunostomy was carried out in order to prevent stricture at the end of the common bile duct. Pancreatoduodenectomy was not done. Paraffin block histopathologic examination confirmed a diagnosis of intestinal villous adenoma accompanied by severe dysplasia and focal carcinomatous change without incisal margin infiltration (Figure 3). After operation, the patient recovered without incident and liver function tests gradually reverted to normal levels.

DISCUSSION

The ectopic drainage of the biliary tract is a rare congenital anomaly, which consists of abnormal communication of the common bile duct, the cystic duct, or an intrahepatic duct with the gastrointestinal tract. As noted by previous authors, ectopic drainage of the common bile duct may be located along a line extending from the stomach to the fourth part of the duodenum[1-5]. The frequency of this anomaly fluctuates between 0% and 13%[1,6]. However, there only four cases with ectopic ampulla draining into the fourth part of duodenum were reported[5,7]. Periampullary carcinoma combined with this anomaly has never described previously.

The embryogenetic background of this anomaly has not yet been fully explained. As is known, the extrahepatic biliary duct and the ventral pancreas arise

from the hepatic diverticulum of the end of foregut during the 4th to 6th week of embryo life[8,9]. The dorsal pancreatic bud appears opposite the hepatic diverticulum. During development, as the duodenum rotates to the right and becomes C-shaped, the ventral pancreatic bud is carried dorsally with the bile duct. The ventral pancreatic duct joins the distal portion of the dorsal pancreatic duct to form the main pancreatic duct, which merges with the common bile duct, inserting into the duodenum *via* the papilla of Vater[10]. Then, the proximal portion of the dorsal pancreatic duct gradually regresses and becomes obliterated at the junction with the ventral pancreatic duct. By the end of the 7th week, the proximal portion of the dorsal pancreatic duct may disappear or persist as the accessory pancreatic duct[11,12]. Thus, we can make the hypothesis that the ampulla of Vater may be found in the sites along a line extending from the stomach to the fourth part of the duodenum, or in another words, along the end of the foregut. However, other factors may play a role in this process.

It is worth mentioning that MRCP is useful for demonstrating anomalies and anatomic variants of the biliary tract system and pancreatic duct[13,14]. In our case, MRCP was of great value not only for depicting the ectopic common bile duct and pancreatic duct draining into the fourth part of duodenum but also for depicting the abnormal junction of the cystic duct and intraluminal small bowel lesion. According to Ruge's[15] categorization, this variation of lower confluence of the cystic duct belongs to type Ⅱ - the double-barreled type in which the cystic duct follows the hepatic duct for some distance before entering it. In addition to MRCP, gastroscopy should be used to assess the gastric mucosa and other malformations which could possibly exist.

Periampullary carcinoma includes cancer of the terminal end of the common bile duct, the ampulla of Vater and its adjacent duodenum. These lesions are similar to each other in many aspects, such as their clinical manifestations, changes noted by laboratory determinations and approach and technique of surgical excision. The prognosis is much better than for carcinoma of the head of pancreas. Endoscopic ampullectomy may be considered as a viable procedure in patients with small ampullary tumors who are unfit for surgery or who prefer a nonsurgical approach[16-18]. But in our case, on the accout of the ectopic hepatopancreatic ampulla and a likely major periampullary tumor located in the duodenojejunal flexure, endoscopic ampullectomy was unfit. So local excision, combined with frozen section, is a low morbidity and a valid alternative to pancreaticoduodenectomy[19]. However, a choledochojejunostomy was carried out in order to prevent stricture of the lower end of the common bile duct after operation.

In conclusion, periampullary carcinoma with ectopic ending of the Vater's ampulla in the fourth part of the duodenum is rather rare. Although pathogenesis is not quite clear, accurate diagnosis and appropriate investigations are necessary for surgeons before operation. Use of MRCP is recommended for the diagnosis and exclusion of associated anomalies of the biliary and pancreatic ducts.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
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Acknowledgments

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.

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