MARIA DA GRAÇA BEÇA GONÇALVES PORTO

HEMOCROMATOSE IDIOPÁTICA:
ASPECTOS EPIDEMIOLÓGICOS, HEMATOLÓGICOS E IMUNOLÓGICOS

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HEMOCROMATOSE IDIOPÁTICA
ASPECTOS EPIDEMIOLÓGICOS, HEMATOLÓGICOS E IMUNOLÓGICOS

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ORIENTADOR: Prof. Doutora Maria de Sousa
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Prof.Doutora Corália Vicente

PORTO, 1993

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Vicente C, **Porto G** & De Sousa M. Method for establishing serum ferritin reference values depending on sex and age. *J Lab Clin Med*, 1990; 116: 779-784

Porto G, Vicente C, Fraga J, Martins da Silva B & De Sousa M. The Importance of Establishing Appropriate Local Reference Values for the Screening of Hemochromatosis: a study of 3 different control populations and 136 hemochromatosis family members. *J Lab Clin Med*, 1992; 119: 295-305

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Porto G, Reimão R, Gonçalves C, Vicente C, Justiça B & De Sousa M. Hemochromatosis as a window into the study of the immunological system: novel correlation between CD4/CD8 populations and iron absorption in man. (submetido a publicação)

Porto G, Vicente C, Gonçalves C, Reimão R, Martins da Silva B, Fraga J, Macedo G, Justiça B & De Sousa M. Relative impact of HLA phenotype and T cell subset ratios in predicting iron overload. (submetido a publicação)

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LISTA DE ABREVIATURAS:

Na Introdução e Discussão:

ADN = ácido deoxiribonucleico
ARNm = ácido ribonucleico mensageiro
HLA = antigénios leucocitários humanos
MHC = complexo maior de histocompatibilidade
SRE = sistema retículo-endotelial
TNF = factor de necrose tumoral

Nos outros capítulos (artigos):

APAAP = alkaline phosphatase anti-alkaline phosphatase
FACS = fluorescent activated cell sorter
γGT = gamma glutamyl transferase
MCV = mean corpuscular volume
MRI = magnetic ressonance imaging
PBMC = peripheral blood mononuclear cells
TIBC = total iron binding capacity
TCR = T cell receptor

CAPÍTULO 1

INTRODUÇÃO GERAL

HEMOCROMATOSE IDIOPÁTICA: UM DIAGNÓSTICO QUE NÃO PODE SER ESQUECIDO

A Hemocromatose Idiopática (HI) é uma doença autossómica recessiva, ligada ao HLA, na qual um defeito na regulação da absorção do ferro leva à acumulação progressiva deste metal no organismo. A acumulação de ferro é clinicamente silenciosa nos estadios iniciais da doença, mas, com o decorrer dos anos, a deposição de ferro vai atingir níveis tóxicos com consequente injúria tecidular e insuficiência funcional dos órgãos atingidos. Os órgãos mais frequentemente afectados são o fígado (que é o principal local de armazenamento de ferro), o pâncreas, o coração, os orgãos endócrinos, a pele e as articulações. Vários mecanismos têm sido propostos para explicar a destruição tecidular induzida pelo ferro incluindo a peroxidação lipídica mediada por radicais livres, o aumento da produção de colagénio e mesmo um efeito mutagénico por interacção directa entre o ferro e o ADN (*Nichols & Bacon, 1989; Bacon & Britton, 1990*).

A HI manifesta-se geralmente a partir da quarta década de vida por sintomas não específicos acompanhando quadros clínicos comuns a outras doenças tais como cirrose, diabetes, artropatia, impotência, insuficiência cardíaca congestiva, etc. Por isso muitos doentes são diagnosticados em fases tardias da doença, ou não chegam mesmo a ser diagnosticados, e em muitos casos documentados o diagnóstico foi feito apenas na autópsia (*Sheldon, 1935; Edwards et al, 1989*). No entando, se a doença for diagnosticada precocemente pode ser facilmente tratada e os doentes poderão ter uma esperança de vida normal (*Niederau et al, 1985; Adams et al, 1991; Fargion et al, 1991*). O diagnóstico precoce dependerá em parte do estado de alerta dos clínicos ou mesmo da população em geral para esta entidade, mas poderá também ser conseguido por meio de programas bem conduzidos de despiste da doença, especialmente em áreas onde se sabe que a hemocromatose tem uma alta prevalência. Assim a HI deixa de ser a doença rara e geralmente fatal, conforme

caracterizada há mais de 50 anos (*Sheldon, 1935*), mas passa a ser considerada uma doença comum de fácil diagnóstico e fácil tratamento. O diagnóstico é baseado em testes bioquímicos simples para os parâmetros do ferro no sangue periférico (saturação da transferrina e ferritina sérica) e confirmado pela demonstração de uma sobrecarga importante de ferro no fígado. Uma vez diagnosticado um caso, é mandatório o despiste da doença em todos os membros familiares de primeiro grau. O tratamento consiste na remoção do ferro em excesso por meio de um programa de flebotomias intensivas (geralmente semanais) seguido de um tratamento regular de manutenção.

Apesar do crescente interesse e avanços na investigação desta patologia, sobretudo nos últimos anos, existem certos aspectos importantes da doença que são ainda desconhecidos: o gene da HI ainda não foi identificado, embora tenha sido localizado no cromossoma 6, perto do locus A do MHC (*Simon et al, 1980, 1987; Edwards et al, 1980, 1986; Jouanolle et al, 1990; Yaouanq et al, 1992*); a variabilidade individual na expressão da doença permanece por explicar e não é ainda claro se todos os indivíduos com a doença genética desenvolverão obrigatoriamente uma fase clínica; finalmente, não se sabe como é regulada a absorção do ferro nem qual o defeito responsável pela patogénese da HI.

HI: UMA DOENÇA COMUM COM EXPRESSÃO HETEROGÉNEA

A descoberta da associação da HI com os antigénios HLA (*Simon et al, 1977; Cartwright et al, 1979*), que se veio a demonstrar ser o reflexo de uma ligação genética entre a região do HLA no cromossoma 6 e o locus da HI (*Simon et al, 1980, 1987; Edwards et al, 1980, 1986; Jouanolle et al, 1990; Yaouanq et al, 1992*), tem permitido a identificação precoce dos chamados homozigóticos, ou seja, os membros familiares idênticos, em ambos os haplótipos HLA, ao probando. O uso do genótipo HLA como marcador do gene da HI, embora sem valor no despiste da doença na população em geral, tem também permitido, por meio dos estudos familiares, estimar a prevalência do gene (*Beaumont et al, 1979; Dadone et al, 1982; Basset et al, 1982; Borwein et al, 1983*). A frequência de homozigóticos e heterozigóticos numa população pode ser calculada a partir da frequência genotípica observada assumindo um equilíbrio genético e empregando o método de cálculo de Hardy-Weinberg (*Vogel*

& Motulsky, 1979). Contudo, as estimativas feitas por estudos da expressão fenotípica da doença na população em geral nem sempre têm confirmado as previsões baseadas nos genótipos HLA (*Edwards et al, 1988; Olsson et al, 1983, 1984; Lindmark et al, 1985; Hallberg et al, 1989; Meyer et al, 1990; Wiggers et al, 1991; Leggett et al, 1990; Karlsson et al, 1988; Velati et al, 1990*) (Tabela 1). As diferenças de frequência encontradas nos diversos estudos podem ser reais e traduzir as variações na prevalência da HI em regiões geográficas distintas, como se infere a partir dos vários estudos realizados na Suécia (*Olsson et al, 1983, 1984; Lindmark et al, 1985*), ou podem ser falsas, reflectindo apenas as diferenças nos métodos utilizados na selecção das populações estudadas (Tabela 1). A prevalência das doenças transmitidas de forma recessiva sabe-se não ser geograficamente uniforme, dependendo da densidade e diversidade populacionais e de factores (sociais, religiosos, de comunicação, etc) que se sabe poderem influenciar a escolha de pares no sentido de uma selecção não ao acaso (*Lindmark et al, 1985*). As estimativas de prevalência baseadas em estudos familiares são naturalmente feitas em populações onde vários doentes são observados, e este facto pode ser causa de desvio nos cálculos da prevalência na população como um todo. Por outro lado, a doença pode não se exprimir clinicamente em todos os homozigóticos para o gene putativo ligado ao HLA, o que poderá originar erros de cálculo quando a estimativa é feita em estudos de expressão fenotípica na população em geral. Sabe-se que a expressão clínica da HI é influenciada pelo sexo e pela idade. Embora a frequência genotípica teórica seja semelhante em ambos os sexos, os homens manifestam clinicamente a doença mais frequentemente que as mulheres, facto que tem sido atribuído à perda regular de ferro que ocorre nas mulheres durante a idade reprodutiva através da menstruação e gravidez. A idade de início dos sintomas, geralmente a partir da quarta década de vida, está relacionada com o tempo necessário para que a acumulação de ferro nos tecidos se torne tóxica. Estão contudo descritas formas de hemocromatose juvenil e neonatal (*Cazzola et al, 1983; Haddy et al, 1988; Knisely, 1992; Barnard & Manci, 1991*), ainda que a sua relação com a HI não esteja estabelecida. Embora o sexo e a idade sejam os principais factores que se sabe influenciarem a sobrecarga de ferro e, consequentemente, a expressão clínica da doença, o facto de grupos de doentes do mesmo sexo e do mesmo escalão etário apresentarem uma grande heterogeneidade, quer nos sintomas quer nos depósitos de ferro, obriga a pensar na existência de outros factores que possam influenciar a absorção e acumulação de ferro.

Tabela 1. *Prevalência da HI estimada em diversos estudos familiares e populacionais*

País (região)	Referência	Tipo de estudo	(n)	Populações seleccionadas	Prevalência (%)
França (Bretanha)	Beaumont, 1979	**Familiar**	174		**0.25**
Estados Unidos (Utah)	Dadone, 1982	**Familiar**	421		**0.50**
	Edwards, 1988	Populacional	5840	homens dadores de sangue	0.45*
Austrália (Queensland)	Basset, 1982	**Familiar**	24		**0.77**
	Legget, 1990	Populacional	1968	homens e mulheres empregados de 2 grandes empresas	0.36
Suécia (Ostersund-Central)	Olsson, 1983	Populacional	623	homens empregados	0.48
	Olsson, 1984	Populacional	4700	homens e mulheres doentes e dadores	0.24
(Goteborg)	Hallberg, 1989	Populacional	1660	homens 50-51 anos normais random.	0.00
(Stockholm)	Hallberg, 1989	Populacional	11920	homens doentes random.	0.027
Canadá (Ontario)	Borwein, 1983	**Familiar**	132		**0.30**
Finlândia (Central e do Sul)	Karlsson, 1988	Populacional	22070	homens e mulheres exame de rotina	0.05
Dinamarca (Ilha Fyn)	Wiggers, 1991	Populacional	4502	homens e mulheres dadores de sangue	0.37-0.46
África do Sul (Cabo-Sudoeste)	Meyer, 1990	Populacional	1783	homens >40 anos (freq.abuso de álcool)	0.95*
Itália	Velati, 1990	Populacional	1301	homens e mulheres dadores de sangue	0.20
Portugal (Norte)	Porto, 1992	**Familiar**	136		1.9

(n) = número de indivíduos na amostra
* diagnóstico de homozigótico para HI não confirmado por biópsia hepática

HI: UMA DOENÇA DE PATOGÉNESE DESCONHECIDA

A HI é caracterizada por uma absorção de ferro anormalmente elevada (*Crosby et al, 1963; Whittaker et al, 1989; Lynch et al, 1989; McLaren et al, 1991*) mas o defeito metabólico responsável por essa condição é desconhecido, tal como continua sem se saber o mecanismo ou mecanismos que regulam a absorção de ferro nos indivíduos normais. Aceita-se clàssicamente que a modulação da quantidade de ferro absorvido é feita por um processo de "inteligência da mucosa" que absorve mais quando há deficiência e menos quando há excesso (*Crosby, 1963; Conrad & Crosby, 1963; Wheby & Crosby, 1963*), mas o "sinal" ou "sinais" que são transmitidos ao intestino para absorver mais ou menos ferro constituem ainda uma incógnita. Possíveis anomalias têm sido procuradas: (1) ao nível das proteínas de ligação do ferro (*Jacobs et al, 1981; Ward et al, 1984; Sciot et al, 1987; Lombard et al, 1989; Pietrangelo et al, 1991*); (2) ao nível do sistema retículo-endotelial (SRE), que é o local principal de armazenamento do ferro em circunstâncias normais, mas não na HI (*Basset et al, 1982; Van Asbeck et al, 1984; Bjorn-Rasmussen et al, 1985; Saab et al, 1986; Flanagan et al, 1989; Fillet et al, 1989; McLaren, 1989; Dullmann et al, 1991; Worrall & Worwood, 1991; Gordeuk et al, 1992*); (3) e ainda ao nível da mucosa duodenal, onde se processa a absorção de ferro (*Whittaker et al, 1989; Dullmann et al, 1991; Fracanzani et al, 1989; Flanagan, 1989; Lombard et al, 1990; Pietrangelo, 1992*).

Até à data não foi encontrada qualquer anomalia na expressão das proteínas de ligação do ferro que possa ser considerada como um defeito primário na HI. Estudos da expressão do receptor da transferrina em biópsias de fígado por métodos imunohistoquímicos têm demonstrado uma expressão adequadamente baixa nos doentes com HI (*Sciot et al, 1987; Lombard et al, 1989*) e que é restaurada após a terapêutica de depleção de ferro (*Lombard et al, 1989*). Estes achados constituem a evidência de que há uma regulação fisiológica adequada do receptor da transferrina a nível hepático. Um estudo mais recente dos níveis basais de ARNm para a transferrina, para o receptor da transferrina e para a ferritina no tecido hepático demonstrou que a regulação concertada da expressão dos genes destas proteínas está mantida na HI (*Pietrangelo et al, 1991*).

Na HI não existe sobrecarga de ferro nas células do SRE, em contraste com a sobrecarga parenquimatosa (*Flanagan et al, 1989; Fillet et al, 1989; McLaren, 1989; Dullmann et al, 1991*). Este facto constitui um paradoxo que tem motivado a

procura de um defeito primário nessas células. Embora o teor em ferritina nos monócitos de doentes com HI pareça ser "inapropriadamente" baixo (*Saab et al, 1986; Worrall & Worwood, 1991*), está demonstrado que a síntese de ferritina nestas células é normal (*Jacobs et al, 1981; Basset et al, 1982*). Está descrito um aumento da secreção de ferritina, não relacionado com o nível de depósitos de ferro, nos monócitos do sangue periférico de doentes com HI (*Flanagan et al, 1989*), estando esse achado em conformidade com os resultados obtidos a partir de estudos da cinética do ferro "in vivo", nos quais se demonstrou um aumento da passagem do ferro, resultante do catabolismo da hemoglobina, dos macrófagos para o plasma (*Fillet et al, 1989*). Contudo, no estudo de Flanagan havia uma grande variabilidade nos resultados de secreção da ferritina, quer nos doentes com HI quer nos controlos normais, sugerindo que factores ainda não definidos estão envolvidos nesse processo (*McLaren, 1989*). Recentemente foi descrito que a concentração de TNF-α em sobrenadantes de células mononucleares do sangue periférico de doentes com HI está diminuida (*Gordeuk et al, 1992*). Esta achado pode constituir uma pista interessante para a compreensão da patogénese da HI já que o TNF-α, cujo gene está localizado no cromossoma 6, entre muitos dos seus efeitos metabólicos como mediador da resposta inflamatória, é capaz de diminuir o nível de ferro circulante (*Michie et al, 1988*).

Vários estudos em doentes com HI mostram que o teor em ferro das células da mucosa duodenal encontra-se paradoxalmente baixo (*Banerjee et al, 1986; Fracanzani et al, 1989; Whittaker et al, 1989; Lombard et al, 1990*): estudos em homogeneisados de células mucosas duodenais demonstraram que o seu nível de ferritina não varia em paralelo com a ferritina sérica, tal como ocorre em indivíduos normais (*Whittaker et al, 1989*); por meio de técnicas imunohistoquímicas foi demonstrada uma diminuição da expressão de ferritina naquelas células (*Fracanzani et al, 1989*); o aumento da expressão de receptores da transferrina na área basolateral do epitélio duodenal (*Banerjee et al, 1986; Lombard et al, 1990*) constitui evidência adicional de que existe uma "relativa deficiência de ferro" nas células duodenais dos doentes. Todos estes dados parecem indicar a existência de um defeito de sequestração do ferro na célula mucosa duodenal na HI, o que poderia aumentar a libertação do metal para o plasma. Esta interpretação está de acordo com os resultados de estudos ferrocinéticos (*Marx, 1979; McLaren et al, 1991*) nos quais se verificou que, nos doentes com HI, o aumento da transferência de ferro da mucosa para o plasma constitui o principal factor determinante do aumento de absorção de

ferro, sendo a baixa de ferro intracelular que estimularia o seu maior "uptake". Esta explicação é consistente com a diminuição da ferritina na mucosa duodenal já que, na síntese de ferritina, o ARNm é controlado, a nível translaccional, pelo teor intracelular em ferro (*Arosio et al, 1989*). Também está de acordo com as observações de Whittaker que demonstrou que a ferritina da mucosa, embora inadequadamente baixa para o nível de ferritina sérica, é adequada para o nível de absorção (*Whittaker et al,1989*).

Com base nos conhecimentos atrás expostos propõe-se que a patogénese da HI envolve um aumento da libertação de ferro quer das células da mucosa intestinal quer das células do SRE (*McLaren et al, 1991*). Para explicar este aumento de transferência de ferro Marx postulou que deverá existir um aumento de actividade de uma proteína capaz de transportar o ferro para fora do epitélio duodenal e das células do SRE. Está em curso um projecto para a caracterização dessa proteína (*Marx, trabalho em curso*) mas até à data ela ainda não foi identificada. Em resumo, embora haja já evidência suficiente para admitir que o aumento de absorção de ferro na HI resulta de uma transferência anormal de ferro da mucosa para o plasma, é ainda desconhecido qual o mecanismo que regula essa transferência.

O METABOLISMO DO FERRO E AS CÉLULAS DO SISTEMA IMUNOLÓGICO: A HI COMO UM MODELO DE ESTUDO

O postulado de que as células do sistema imunológico podem participar activamente na protecção da toxicidade potencial do ferro foi inicialmente formulado por De Sousa com base na evidência que a migração dos linfócitos T ocorre frequentemente para os locais, fisiológicos ou patológicos, de depósito de ferro (*De Sousa, 1978, 1981*) e que os macrófagos e linfócitos T sintetizam proteinas de ligação do ferro (*De Sousa, 1981; Nishiya et al, 1980*). Desde então tem-se assistido a um acumular de dados que evidenciam a importância do ferro como imuno-regulador (*De Sousa et al, 1988, 1991; De Sousa, 1989a, 1989b; Brock, 1989*). Estudos imunológicos em doentes com sobrecarga de ferro mostraram desproporções nas subpopulações de células T circulantes e deficiência de algumas funções mediadas por células T (*Guglielmo et al, 1984; Kaplan et al, 1984; Grady et al, 1985; Dwyer et al, 1987*). Esses estudos, contudo, realizaram-se em doentes com β-talassemia major e sobrecarga transfusional, tornando difícil distinguir entre os efeitos atribuíveis à

sobrecarga de ferro, à esplenectomia ou à transfusão. A Hemocromatose Idiopática constitui um modelo alternativo ideal para o estudo "in vivo" da interacção entre o ferro e as células do sistema imunológico por três razões essenciais: em primeiro lugar, porque é uma forma primária de sobrecarga de ferro, não complicada por esplenectomia ou transfusões de sangue; em segundo lugar porque é uma doença ligada ao MHC, constituindo uma oportunidade de estudar o envolvimento do MHC no metabolismo do ferro; em terceiro lugar porque é uma doença tratável, i.e., a sobrecarga de ferro pode ser corrigida por meio de flebotomias, facto que permite distinguir entre uma anomalia secundária à sobrecarga de ferro (que poderá ser corrigida com o tratamento) de uma anomalia associada primàriamente à doença (neste caso não susceptível de correcção pelo tratamento). Além disso, a eventual identificação de uma anomalia imunológica nos doentes com HI constituirá certamente uma importante contribuição para a compreensão da patogénese da doença e dos mecanismos que regulam a absorção do ferro em geral.

RESUMO DO PRESENTE TRABALHO

Em 1986 iniciamos um programa de despiste da HI em Portugal, que inclui não só estudos em membros familiares dos doentes mas também o despiste na população em geral. Os estudos familiares foram feitos sistematicamente com tipagem HLA tendo permitido demonstrar a associação da doença com o haplótipo HLA A3B7 nesta região (*Capítulo 2*). A todos os doentes e membros familiares diagnosticados foi oferecido apoio no tratamento e vigilância clínica. No decurso do programa de despiste da doença tornou-se aparente a necessidade de estabelecer valores de referência locais para os parâmetros do metabolismo do ferro. Não existem limites normais universalmente aplicáveis a esses parâmetros, facto que é particularmente relevante para a ferritina sérica cujos valores de referência descritos em vários estudos são notavelmente diferentes. Foi por isso desenvolvido um método original para o estabelecimento de valores de referência locais para a ferritina, dependendo do sexo e da idade. Esse método está descrito no *Capítulo 3,* onde são também comparados os valores encontrados nos vários estudos descritos na literatura, bem como os métodos utilizados nos respectivos estudos. A importância da definição de valores de referência locais para o metabolismo do ferro é também abordada no *Capítulo 4* onde são analisados os factores que podem fazer variar esses parâmetros, nomeadamente: o sexo, a idade, a ingestão regular de álcool e mesmo a própria região de origem dos

indivíduos. Com base em estudos familiares com tipagem HLA foi calculada uma prevalência da HI no norte de Portugal muito elevada, mesmo em relação a outros países onde a doença é mais frequente (também descrito no *Capítulo 4*). Essa estimativa de uma alta prevalência da doença no norte do país foi posteriormente confirmada num estudo da população de Refoios-Ponte de Lima (*Capítulo 5*).

Motivados pelo interesse no estudo da interacção entre o ferro e as células do sistema imunológico, fizemos uma análise seriada das populações circulantes no sangue periférico, incluindo as proporções relativas das subpopulações linfocitárias, em doentes submetidos ao tratamento por meio de flebotomias regulares intensivas (*Capítulo 6*). Esse estudo constituiu uma oportunidade única de estudar a homeostasia das populações celulares numa situação de constante renovação do pool periférico já que, durante o tratamento, são removidas grandes quantidades de sangue (em muitos casos correspondendo a mais de 10 vezes o volume sanguíneo original) com progressiva deplecção dos depósitos de ferro. Foi assim demonstrado, pela primeira vez no homem, que existe uma notável estabilidade do número e proporções relativas das subpopulações de linfócitos T à periferia. No decurso do mesmo estudo foram feitas mais observações que constituem dados novos na caracterização da HI: num número significativo de doentes foram encontradas leucopenias constitucionais; a macrocitose eritrocitária foi um achado constante nos doentes, independentemente do tratamento e da disfunção hepática; e ainda, em oposição ao que está descrito na sobrecarga de ferro transfusional, foram encontradas razões CD4/CD8 anormalmente elevadas num subgrupo de doentes. Esta anomalia correlaciona-se com o comportamento clínico dos doentes, nomeadamente a resposta à remoção de ferro e a recuperação da saturação da transferrina após o tratamento. Para melhor caracterizar subgrupos de doentes que diferem entre si nas razões CD4/CD8 e na quantidade de ferro mobilizado pelas flebotomias, foi feita uma análise estatística exaustiva incluindo o modo de apresentação da doença, as manifestações clínicas, o fenótipo HLA e as razões CD4/CD8 (*Capítulo 7*), tendo sido demonstrado que o fenótipo HLA e a razão CD4/CD8 influenciam, em conjunto, a sobrecarga de ferro e, consequentemente, as manifestações clínicas da doença. Finalmente são discutidas as implicações de uma possível anomalia da expansão das células CD8+ (no contexto do MHC) na patogénese e heterogeneidade clínica da Hemocromatose Idiopática (*Capítulo 8*).

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PARTE I

ASPECTOS EPIDEMIOLÓGICOS:

Trabalho de campo para a definição de métodos e identificação de doentes

CAPÍTULO 2

IDIOPATHIC HAEMOCHROMATOSIS IN THE NORTH OF PORTUGAL: ASSOCIATION WITH THE HAPLOTYPE A3B7

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(Letters to the Editor)

IDIOPATHIC HAEMOCHROMATOSIS IN THE NORTH OF PORTUGAL: ASSOCIATION WITH HAPLOTYPE A3B7

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The association of certain HLA antigens and haplotypes with idiopathic haemochromatosis is well documented, and their pattern of distribution has been defined in several countries. The antigen most strongly associated with idiopathic haemochromatosis is HLA-A3 and the haplotypes more frequently linked to the disease are A3B7 and A3B14 [1-3].

In 1986 we started a systematic search for cases of idiopathic haemochromatosis in the north of Portugal that has led to the identification of 30 patients to date. Preliminary studies of the first unrelated patients and families indicated an association of antigen A3 and haplotype A3B7 with the disease in this region [4].

Seventeen unrelated, HLA typed, patients with idiopathic haemochromatosis (15 men and two women), aged between 21 and 69 years were included in this study. Haemochromatosis was diagnosed according to previously established clinical, biochemical and histopathological criteria [5].

One hundred and eighteen family members (55 men and 63 women) of 10 unrelated subjects were studied. All subjects were HLA typed; evaluation of their iron status was carried out by routine measurements of serum iron, total iron binding capacity, transferrin saturation and serum ferritin. In the course of the family screening seven additional patients were identified in five of the families. To calculate the haplotype prevalence we followed the criteria defined by Simon *et al* [1].

The phenotype prevalence of the HLA antigens in the control population was determined in 203 healthy, unrelated blood donors from the blood bank of the Hospital Geral de Santo António-Porto.

HLA haplotype prevalence in the control population was calculated in a control group of unrelated subjects from the north of Portugal, including the spouses of some family members in whom the disease was excluded, heterozygous family members (only the non-idiopathic haemochromatosis associated haplotype was considered), and normal subjects previously genotyped.

The significance of the differences observed between the prevalence of antigens and haplotypes in the patient and control groups was determined by the χ^2 test. The relative risk was calculated by the method of Woolf.

Table. *Prevalence of phenotypes and haplotypes in patients and controls*

	Patients *(n=17)*	***Controls*** *(n=203)*	*Relative risk*
HLA antigens*			
A2	0.471	0.419	ns
A3	0.529	0.217	4.065 a
A9	0.294	0.222	ns
B7	0.353	0.089	5.606 b
B12	0.353	0.315	ns
B35	0.235	0.108	ns
HLA haplotype*			
	(16 haplotypes)	(110 haplotypes)	
A3B7	0.176	0.018	12.46 c
A9B5	0.118	0.027	ns
A2B12	0.118	0.118	ns

a $\chi^2 = 7.419$ $p < 0.01$
b $\chi^2 = 9.329$ $p < 0.01$
c $\chi^2 = 6.920$ $p < 0.01$
* only the most common antigens and haplotypes are represented

The comparison of the phenotype prevalences of HLA antigens showed a significantly higher prevalence of the A3 and B7 ($p<0.01$) in the patient group. The antigen A3 was represented in 52.9% of the subjects in the patient group and in 21.7% of the controls. The antigen B7 was represented in 35.3% of the cases in the

patient group and in 8.9% of the controls. These figures correspond to relative risks of 4.065 and 5.606 for A3 and B7, respectively. Among the other antigens tested, high prevalences of A2 and B12 were also found, but they were seen as often in the control group.

Sixteen haplotypes were associated with the disease in the 10 families studied. In two subjects it was not possible to identify the haplotypes. A3B7 was the haplotype found most frequently in the patient group (17.6%). This was significantly higher than that observed in the control population (1.8%), representing a relative risk of 12.46. The haplotype A3B14, which has also been shown in association with idiopathic haemochromatosis in certain regions [1], was not found to be linked to the disease in this patient population; this haplotype, however, was not represented in the control population studied.

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CAPÍTULO 3

METHOD FOR ESTABLISHING SERUM FERRITIN REFERENCE VALUES DEPENDING ON SEX AND AGE

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Method for establishing serum ferritin reference values depending on sex and age

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A method is described for the establishment of reference values for serum ferritin depending on sex and age. The results obtained in a stratified random sample of 353 healthy individuals from the Portuguese population revealed that 23% of the variation observed on serum ferritin values was related to sex differences and 6% of the variation was explained by age differences. Furthermore, the effect of age was different in both sexes: serum ferritin values increase with age in females over 40 years and in males under 40. The minimum and maximum reference values were established for the different subgroups of subjects as follows: for females under 40 years and males over 40, reference values were considered as the 2.5 and 97.5 percentiles from the adjusted lognormal distribution; for females over 40 years and males under 40, reference values were considered as the exponential transformation of the upper and lower limits of the 95% prediction intervals for the log of serum ferritin for each particular age point. (J LAB CLIN MED 1990;116:779-84)

Abbreviation: IH = idiopathic hemochromatosis

Serum ferritin level is a valuable parameter for the assessment of iron stores.[1-3] Besides being a sensitive method in the detection of iron deficiency,[4] serum ferritin level has been used, in addition to transferrin saturation, as a reliable phenotypic marker in the screening of idiopathic hemochromatosis.[5-7] No clear "universal" cutoff values have yet been established because a great variability of this parameter seems to occur from study to study described in the literature (Table I).[1,2,5,6,8-13] In the course of a screening program for idiopathic hemochromatosis in Portugal,[14,15] we were faced with the necessity of establishing appropriate upper limits for serum ferritin values to help define *iron overload.* In the present study a method is described that enabled us to establish reference values for serum ferritin in a Portuguese population aged from 12 to 82 years, eliminating, as much as possible, the variation caused by age and sex.

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5/1/24264

METHODS

Serum ferritin level was determined in a random sample stratified for age and gender from three Portuguese communities. A total of 353 subjects were included in the study: 152 (78 males and 74 females) from Cabeceiras de Basto, a village in the north with 1259 inhabitants; 91 (27 males and 64 females) from S. João de Lobrigos, another northern village with 1790 inhabitants; and 110 (53 males and 57 females) from Castelo de Vide, a town in the southern region of the country with 3383 inhabitants. The ages of the individuals in all three populations ranged from 12 to 82 years, with similar representatives in all decade groups. Individuals were recruited into the study with the help of local community leaders, namely the priest and the village doctors. All subjects were apparently healthy, with no chronic diseases or biochemical abnormalities referred to in their clinical

Table I. Published values for serum ferritin in normal subjects

Reference	Sample origin	No.	Sex	Age group (yr)	Statistical method	Serum ferritin (ng/ml) Males	Serum ferritin (ng/ml) Females
Addison et al. (1972)	Healthy controls (not specified)	33	M	–	Mean (range)	52 (12-128)	
		18	F	–			28.8 (10-56)
Jacobs et al. (1972)	Healthy male workers in a factory	75	M	18-65	Mean (range)	69.2 (6-186)	
	Female members of hospital staff	44	F	–			34.8 (3-162)
Walters et al. (1973)	Healthy subjects (not specified) including blood donors	12	M	19-46	Mean (range)	103 (36-224)	
		10	F	22-40			35.6 (2-83)
Halliday et al. (1975)	Blood donors and laboratory staff	40	M	–	Geometric mean (95% limits)	56.9 (18-180)	
		40	F	–			34 (10-143)
Cook et al. (1976)	Subjects from a Washington State nutritional survey	323	N.S.	5-11	Median (10/90 percentiles)		21 (10-45)
		126	M	12-18		23 (10-63)	
		125	F				21 (6-48)
		240	M	18-45		94 (34-196)	
		370	F				25 (7-14)
		165	M	>45		124 (29-455)	
		215	F				89 (12-170)
Beaumont et al. (1979)	Healthy persons attending a social security medical examination	66	M		Median (range)	67 (14-360)	
		75	F				48 (4-182)
Cartwright et al. (1979)	Relatives of patients with idiopathic hemochromatosis with no HLA haplotypes of probands (from Mormon pedigrees)	41	M	0-77	Geometric mean (95% limits)	93 (16-542)	
		50	F	0-80			48 (7-330)
Milman and Sondergaard (1984)	First-time male blood donors	21	M	–	Geometric mean (range)	52 (15-114)	
	Multiple male blood donors	1327	M	–		36 (4-247)	
Milman et al. (1986)	Healthy subjects (not specified)	125	M	18-45	Geometric mean (95% limits)	67 (20-227)	
		115	F				23 (5-104)
	Geriatric patients (excluding disease associated with abnormal serum ferritin values)	67	M	60-79		73 (18-432)	
		75	F				62 (7-410)
		61	M	80-93		77 (17-348)	
		64	F				57 (9-383)
Present study	Healthy subjects from three restricted areas from different regions	See Table II					

N.S., Not specified.

records and with no signs or symptoms related to iron overload or deficiency. No blood donors were included.

Serum ferritin determinations were performed by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, Calif.).

The statistical analysis included linear regression analysis and distribution fitting. Because the serum ferritin values follow a lognormal distribution, the regression analysis performed considered as the dependent variable the logarithmic transformation (log) of the ferritin values. Data were analyzed within each gender group separately and also according to age.

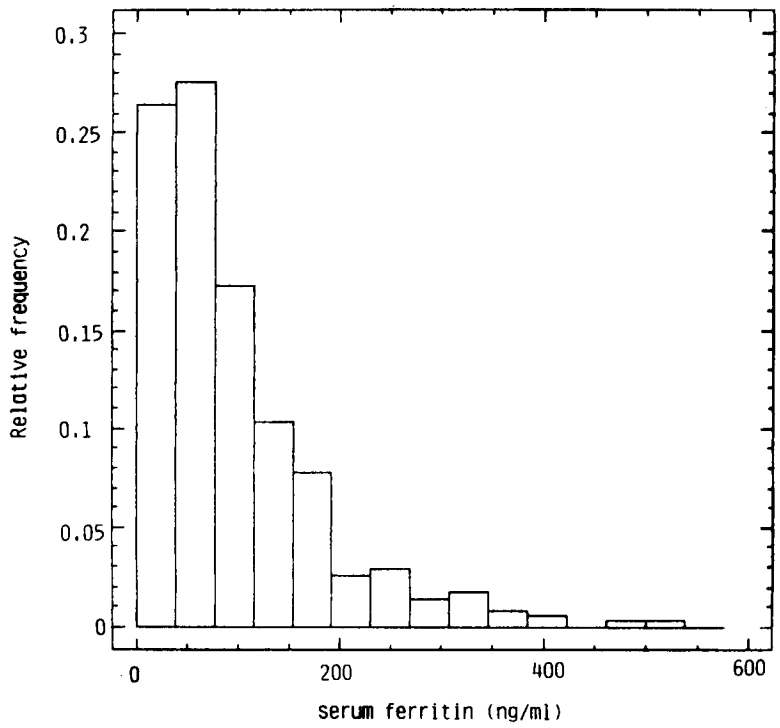

Fig. 1. Sample distribution of serum ferritin values (relative frequency histogram).

RESULTS

Three of the 353 serum ferritin values obtained were above 1000 ng/ml. They were discarded from the sample to be studied and were later repeated and confirmed as abnormal. The relative frequency histogram of the remaining 350 values is given in Fig. 1, showing the lognormal distribution for this iron-related parameter.

The stepwise linear regression for the log of serum ferritin values disclosed the influence of sex and age. On the whole sample, sex and age variation explain 29% of the total variation, 23% being explained by sex and 6% by age. Because of the considerable variation of serum ferritin value and the importance of gender, reference values were established within each gender group separately.

The dependence on age was first looked into by plotting serum ferritin value and age for each sex (Fig. 2). As is clearly seen in Fig. 2, the variation pattern is different for each gender group: females showed a clearly wider variation of values after the age of 40 years; in the male group a break was seen in the increasing trend of values at around 40 years. This led us to a further division of the group of subjects analyzed into those under 40 years of age and those over 40.

Females over 40 years and males under 40 years of age. For these two subsamples a further age dependence was found. A linear regression model relating the log of serum ferritin and age was determined. The equations obtained for females over 40 years of age (Fig. 3) are:

$$\log(\text{serum ferritin}) = 2.71 + 0.0230 \times \text{age}$$
$$<=>$$
$$\text{serum ferritin} = 15.03 \times 1.023^{\text{age}}$$

For this model the standard error for the slope was equal to 7.49×10^{-3}, giving a p value of 2.611×10^{-3}; the coefficient of determination (R^2) was equal to 0.074, and the correlation coefficient (r) was 0.272. The equations obtained for males under 40 years of age (Fig. 4) are:

$$\log(\text{serum ferritin}) = 2.85 + 0.0612 \times \text{age}$$
$$<=>$$
$$\text{serum ferritin} = 17.29 \times 1.063^{\text{age}}$$

For this model the standard error for the slope was equal to 0.011, giving a p value of 2.292×10^{-7}; the coefficient of determination (R^2) was equal to 0.327, and the correlation coefficient (r) was 0.572.

Clearly a greater variation exists in the females than in the males. The coefficient of determination found in males being higher than that found in females signifies that a greater variation in females exists that cannot be attributed to age.

With these models it is possible to estimate the mean, the 95% confidence interval for the mean,

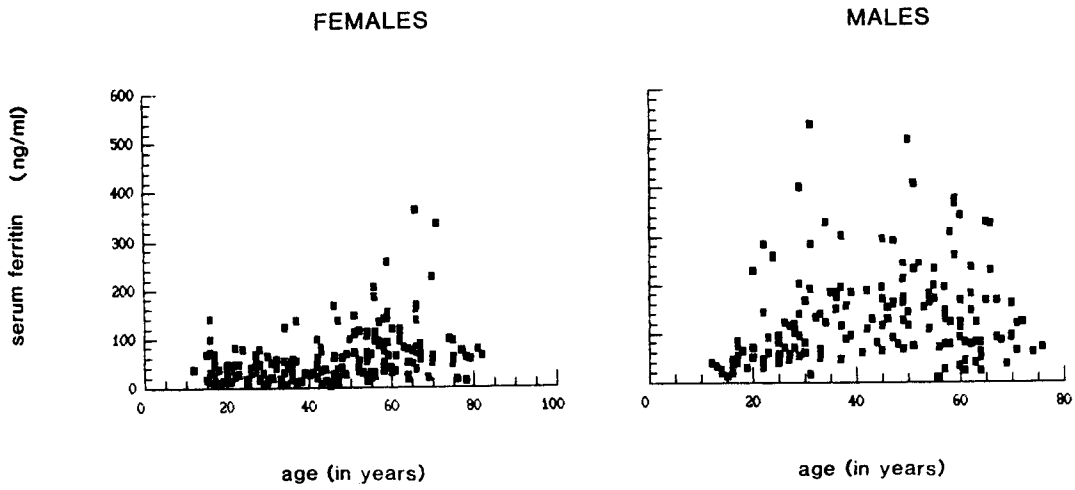

Fig. 2. Plot of age and log of serum ferritin for males and females.

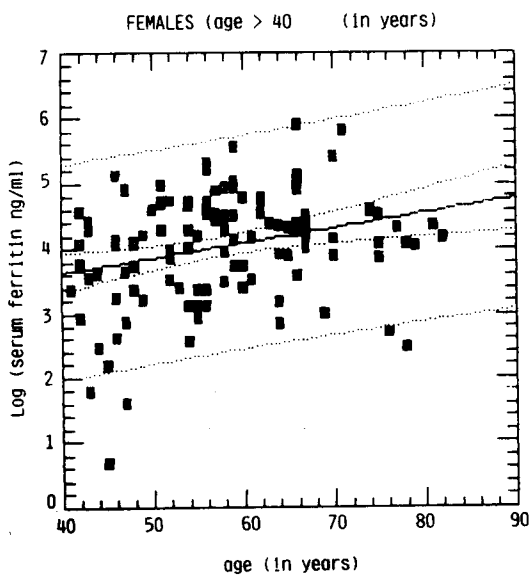

Fig. 3. Linear regression of log of serum ferritin and age for females over 40 years. Plot of the least square straight line, the 95% confidence bands, and 95% prediction bands.

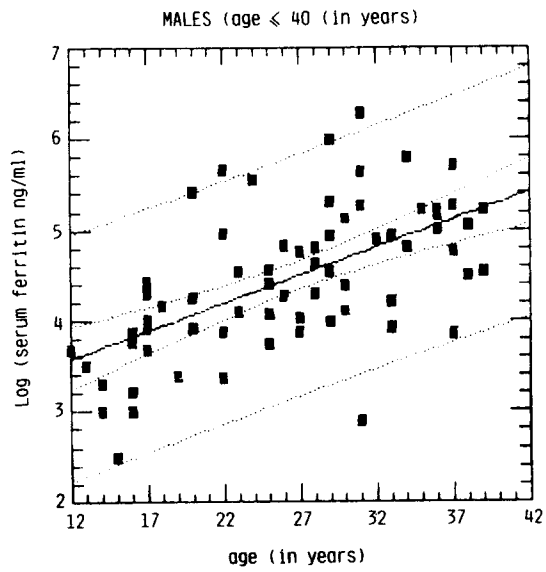

Fig. 4. Linear regression of log of serum ferritin and age for males under 40 years. Plot of the least square straight line, the 95% confidence bands, and the 95% prediction bands.

and the 95% prediction interval for log of serum ferritin for a particular age within the age range of each according to standard formulas.[16]

The minimum and maximum reference values for serum ferritin for different ages are the exponential transformation of the upper and lower limits of the 95% prediction intervals for the log of serum ferritin for that particular age point. Examples of values obtained for different ages in the population studied by applying this method are shown in Table II.

Females under 40 years and males over 40 years of age. In these two groups no further age dependence was found, therefore, a lognormal distribution was adjusted for each subsample. The goodness of fit was tested by the Kolmogorov Smirnov one-sample test, giving in both cases p values close to 1.

The reference values in these cases were considered as the 2.5 and the 97.5 percentiles from the adjusted distribution. The results are also included in Table II; in females under 40 years the minimum and maximum predicted values for serum ferritin

are 7 and 134, respectively (geometric mean = 40), in males over 40 years the minimum and maximum predicted values are 30 and 483, respectively (geometric mean = 155).

DISCUSSION

Serum ferritin level is a parameter of iron metabolism that shows a great variability in normal subjects. The main purpose of this study was to curtail this variability in a parameter that may be decisive to the diagnosis of iron overload. The results obtained with the present population showed that sex and age explain 29% of the total variation. The effect of age on serum ferritin values was different in the two genders: an increase with age is seen in females over 40 years and in males under 40. A possible reason for this increase in the case of the older females could be the cessation of regular menstrual blood loss; in the case of the younger males, the increase could be associated with the expansion of iron stores through absorption of dietary iron after the period of maximal growth until a normal storage iron level is reached. The influence of other factors on serum ferritin values, namely, the level of alcohol intake and the region of origin of subjects, as well as the relation with other independent markers of iron status—namely, serum iron, total iron binding capacity, and transferrin saturation—is discussed elsewhere (Porto et al., submitted for publication). The present results reinforce the importance of considering age dependence for each sex in the determination of serum ferritin reference values within one population. Although in females the age factor does not influence serum ferritin values as markedly as in younger males, with this model of age dependency it was still possible to eliminate 7.4% of the variation in those values. The need to define as accurately as possible upper limits for serum ferritin values was motivated by the screening of hemochromatosis in this region.

In the case of a screening program for iron deficiency, a greater emphasis should be placed in the establishment of reference lower limits for serum ferritin. Moreover, different risk groups should be targeted that should include infants, children, pregnant women, and fast-growing young males.

The conclusion of this study leads to the recommendation that a regression equation should be determined for each population under study and applied to the determination of the respective reference values. Having done that for a Portuguese population, we obtained values for the females and for younger males that are higher than those reported in other studies (Table I).[1,2,5,6,8-13] We do not know whether this difference represents a real difference in values or simply results from the application of a different method. It is our conviction that the present method is a more exact representation of the changes in ferritin values with sex and age. The data illustrate well the need to test a regression equation within each sex and within each age group separately.

Table II. Reference values for serum ferritin (ng/ml)

Age (yr)	Males (n = 157) [min, max]	Males (n = 157) Mean	Females (n = 193)	
10	[8,123]	32	[7,134]	32
20	[16,218]	59		
30	[29,401]	108		
40	[30,483]	155	[7,196]	38
50			[9,242]	47
60			[12,304]	60
70			[15,387]	75

Reference values are indicated as minimum and maximum intervals followed by the geometric mean. Minimum and maximum intervals are given by the 2.5 and 97.5 percentiles from the adjusted lognormal distribution for females under 40 years and males over 40, and by the exponential transformation of the 95% prediction limits of the log of serum ferritin for each particular age for the other groups.

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CAPÍTULO 4

IMPORTANCE OF ESTABLISHING APPROPRIATE REFERENCE VALUES FOR THE SCREENING OF HEMOCHROMATOSIS: A STUDY OF THREE DIFFERENT CONTROL POPULATIONS AND 136 HEMOCHROMATOSIS FAMILY MEMBERS

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Importance of establishing appropriate local reference values for the screening of hemochromatosis: A study of three different control populations and 136 hemochromatosis family members

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Hemochromatosis is a human leukocyte antigen–linked (HLA-linked), potentially lethal disorder of iron metabolism with a high prevalence in white populations albeit an autosomal recessive mode of transmission. The diagnosis and treatment at early stages of the disease are critical to the prevention of the morbidity and mortality caused by the iron overload. After the identification of the first cases of hemochromatosis in Portugal, a screening program was started with a systematic search for the disease among family members of the patients, as well as in subjects from the normal population. In this study we analyze the results obtained with a total of 136 family members from 15 different families and 353 control subjects from three different villages, two in the north and one in the south of Portugal. We establish reference values for the biochemical tests used in the screening for iron overload and analyze the factors that affect those results. Besides sex-related differences, factors that were found to influence biochemical parameters most significantly included age and levels of daily alcohol intake. In addition, differences in iron status were identified between the populations from the regions in the north and the south of the country. We estimate, by HLA typing and family studies, a gene frequency for hemochromatosis of 0.14 that corresponds to a frequency of homozygotes and heterozygotes of 0.019 and 0.24, respectively. (J LAB CLIN MED 1992;119:295-305)

Abbreviation: DN = maximum absolute deviation between the two cumulative distribution functions; HLA = human leukocyte antigen

Hemochromatosis is a disorder of iron metabolism. Failure of the regulation of iron absorption leads to the progressive accumulation of iron in some organs, with eventual tissue damage. Hemochromatosis is inherited as an autosomal recessive disorder whose putative gene is linked to the HLA locus on the short arm of chromosome 6.[1-4] This association with the HLA locus has been used to identify family cases before clinical manifestations occur. If the disease is diagnosed and treated early, a fatal outcome can be prevented and a normal life expectancy anticipated for the patient.[5,6] Therefore it is of major interest to carry out a program of systematic screening for this disorder. In our study we report results of a screening program for hemochromatosis started in Portugal in 1986.[7,8]

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5/1/34389

METHODS

Clinical study. This study involved populations from two regions, (1) people from the north, in areas known to have previously diagnosed cases of hemochromatosis, and people from the south, in areas where no cases had been reported. Two different approaches have been adopted: fam-

Table I. Region of origin and age distribution of subjects from the control populations

Region Village/town	Sex	Total number	Age range group (yr) 10-20	20-30	30-40	40-50	50-60	60-70	70-80	>80
North										
Cabeceiras de Basto	M	78	9 (11.5%)	21 (27%)	7 (9%)	8 (10%)	13 (17%)	16 (20.5%)	4 (5%)	— —
	F	74	7 (9.5%)	12 (16%)	4 (5%)	10 (13.5%)	18 (24%)	17 (23%)	5 (7%)	1 (1%)
S. João de Lobrigos	M	27	4 (15%)	5 (18.5%)	3 (11%)	5 (18.5%)	6 (22%)	3 (11%)	1 (4%)	— —
	F	64	9 (14%)	11 (17%)	9 (14%)	11 (17%)	12 (23%)	8 (12.5%)	3 (5%)	1 (1.5%)
South										
Castelo de Vide	M	53	6 (11%)	3 (6%)	12 (23%)	13 (24.5%)	12 (23%)	7 (13%)	— —	— —
	F	57	7 (12%)	3 (5%)	10 (17.5%)	12 (21%)	16 (28%)	6 (10.5%)	3 (5%)	— —

ily studies, and screening of populations from restricted areas in those two regions.

Family studies. Patients with hemochromatosis were refered to the Hemochromatosis Clinical and Research Group (Hemocare) study by the clinicians in the area. The disease was diagnosed according to established criteria that included clinical, biochemical, and histopathologic parameters.[9] The screening was then carried out in all first-degree and some second-degree relatives aged over 10 years. Each individual was interviewed, and a standard questionaire was filled out to collect data on general health, with particular reference to clinical manifestations of iron overload and other relevant information such as alcohol intake and dietary habits. Venous blood samples were obtained for serum iron, transferrin saturation, and serum ferritin determinations. If an abnormality was found in these biochemical tests, they were repeated, and if they were consistently abnormal, a liver biopsy was done and the disease confirmed. HLA typing was done systematically in all members of the family. Subjects with HLA type identical to their proband were considered homozygous for the hemochromatosis gene. A total of 136 subjects (67 males and 69 females) aged between 10 and 78 years, who were family members of 15 probands (13 males and 2 females), were included in the study.

Population studies. Individuals were recruited into the study with the help of local community leaders, namely the priest, the family physician, or the old village doctor. The population selected from subjects attending Sunday Mass (with the collaboration of the priest) or from subjects being followed up at public health posts (with the collaboration of the physicians) was considered representative of the whole village population (see Table I). Faced with a very high response of the population to our request for blood samples, limits on the number of subjects had to be imposed based on age and sex group quotas. The screening methods used in the population studies were the same as for the family studies, except that HLA typing was not done.

A total of 353 subjects were studied, as follows: 152 (78 males and 74 females) from Cabeceiras de Basto, a village in the north with 1259 inhabitants; 91 (27 males and 64 females) from S. João de Lobrigos, another northern village, with 1790 inhabitants; and 110 (53 males and 57 females) from Castelo de Vide, a town in the south with 3383 inhabitants. None of the subjects was a regular blood donor. The age of the individuals in all three populations ranged from 12 to 82 years, with representatives in all decade groups (Table I).

All the apparently healthy subjects from the population studies who had no chronic diseases or biochemical abnormalities referred in their clinical records and with no signs or symptoms related with iron overload or deficiency were considered normal subjects.

Biochemical parameters. Serum iron level was determined by the bathophenantroline method with deproteinization (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany). Total iron-binding capacity was measured by the method of Ramsay (Boehringer Mannheim). Transferrin saturation was calculated from the ratio of Fe and total iron-binding capacity. The serum ferritin determinations were done by radioimmunoassay (Diagnostic Products Corp. Los Angeles, Calif.) HLA typing for the A, B, C, and Dr loci was done by the microlymphocytotoxicity test.[10] Hepatic iron was graded on histologic sections obtained at the time of the liver biopsy, which were stained for iron by the Perls method according to previously established criteria.[11]

Statistical methods. Variables included in the statistical analysis are listed in Fig. 1. For purposes of analysis, the square root transformation was used for serum iron and transferrin saturation, and the logarithm transformation was used for serum ferritin level. In each case, the goal was to achieve an empirical distribution compatible with the normal (Gaussian) assumptions. For presentation in tables and in plots, the means, percentiles, and confidence intervals have been transformed back to the original scales. Thus, for serum iron and transferrin saturation, the mean of the square roots has been squared, and for serum ferritin, the geometric means are used.

To establish reference values for the biochemical vari-

For the familial studies

A. Demographic variables
- (1) Status
  - (a) Proband (diagnosed with hemochromatosis)
  - (b) First or second degree relative of proband
    - (i) HLA-identical with Proband
    - (ii) Sharing one haplotype with proband
    - (iii) Differing from proband in both haplotypes
- (2) Sex
- (3) Age at interview
- (4) Regular daily alcohol intake
  - (a) Under 10 gm
  - (b) 10 to 60 gm
  - (c) Over 60 gm

B. Biochemical variables
- (1) Serum iron level
- (2) Transferrin saturation
- (3) Serum ferritin level

For the control population studies

A. Demographic variables
- (1) Region/town
  - (a) North of Portugal (Area with known cases of hemochromatosis)
    - (i) Cabeceiras de Basto
    - (ii) S. João de Lobrigos
  - (b) South of Portugal (area with no known cases of hemochromatosis)
    - (i) Castelo de Vide
- (2) Sex
- (3) Age at interview
- (4) Regular daily alcohol intake
  - (a) Under 10 gm
  - (b) 10 to 60 gm
  - (c) Over 60 gm

B. Biochemical variables
- (1) Serum iron level
- (2) Transferrin saturation
- (3) Serum ferritin level

Fig. 1. Variables included in statistical analysis.

ables, regression analyses were performed to test for an effect of sex, age, and/or the interaction between age and sex (all at the 0.005 level of significance). Once significant factors were identified, 95% prediction intervals were used to estimate the 2.5th and 97.5th percentiles of the normal population, and these were used as the lower and upper limits for the reference values; the influence of other relevant factors on iron parameters was evaluated by analysis of variance and linear regression analyses.[12] All testing was performed at the 0.05 level of significance, and all p values are two-sided. Homogeneity of variances was tested with Bartlett's test. Multiple comparisons were performed by using the Scheffe test. Stepwise multiple linear regression analysis was performed by using each iron parameter (or the appropriate transformation) as the dependent variable and the possible factors influencing those values as independent variables.[13,14] For overall comparison of the distributions of the different iron parameters in the groups of subjects from two different regions, the nonparametric two sample Kolmogorov-Smirnov test was used. In this analysis, data were neither standardized nor transformed. The data were analysed by using the Statgraphics Statistical Graphics System (STSC).

RESULTS

Variation of biochemical parameters in normal subjects. The total normal population from the three regions studied (350 subjects) was analyzed for the effects of sex, age, and alcohol intake on serum iron level, transferrin saturation, and serum ferritin level. In addition, the possibility of regional differences for those three parameters was also tested.

Influence of age and sex. Significant differences were found between males and females for the mean serum iron level ($p = 0.0005$), the transferrin saturation ($p = 0.0004$), and the log of serum ferritin ($p = 0.0004$). Consequently we studied males and females separately. For serum iron and transferrin saturation, we found no significant effect of age (all p values greater than 0.2). A strong effect of age was found, however, on serum ferritin for males under 40 years and females over 40 years, as described in full detail in Vicente et al.[15] The reference values thus established are summarized in Table II, where the mean, minimum, and maximum intervals for each gender are indicated. Serum ferritin values are given according to age. The values for males younger than 40 and females older than 40 years were established for the ages specified (Table II). Reference values for intermediate ages may be estimated by assuming a linear increase between two consecutive values.[15]

Influence of alcohol intake. To evaluate the effect of alcohol intake on the biochemical variables, males and females were analyzed separately. All individuals were classified into three groups, according to the level of regular daily alcohol intake, as follows: under 10 gm, between 10 and 60 gm, and over 60 gm. All subjects come from communities with similar habits in terms of the age at which they begin to drink regularly (between 10 and 15 years). Lifetime alcohol intake therefore was not considered as a parameter for classifying the subjects, because the mean age, both in

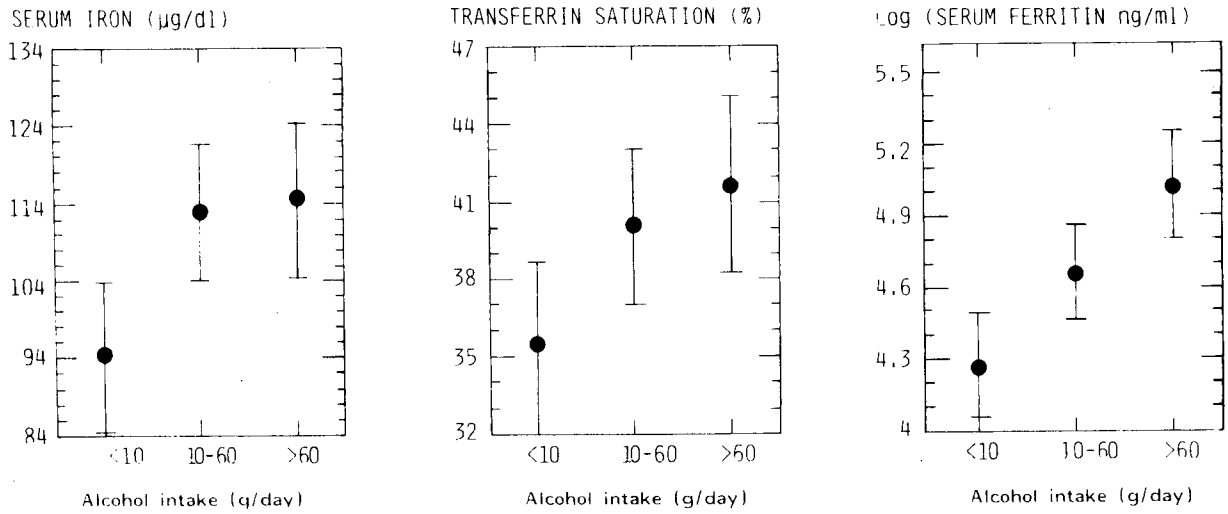

Fig. 2. Influence of the level of alcohol intake on serum iron level, transferrin saturation, and the log of serum ferritin values in males. The arithmetic means and the 95% confidence intervals for the means (two times the pooled standard errors) are indicated.

Table II. Reference values for serum iron level, transferrin saturation, and serum ferritin level

	Males (n = 157)		Females (n = 193)	
	Mean*	(min, max)	Mean*	(min, max)
Serum iron (µg/dl)	107	(50,181)	95	(44,160)
Transferrin saturation (%)	39	(19, 62)	34	(15, 59)
Serum ferritin (ng/ml)†				
Aged 10 yr	32	(8,123)		
Aged 20 yr	59	(16,218)	32	(7,134)
Aged 30 yr	108	(29,401)		
Aged 40 yr	—		38	(7,196)
Aged 50 yr	155	30, 483	47	(9,242)
Aged 60 yr	—		60	(12,304)
Aged 70 yr	—		75	(15,387)

*Arithmetic mean for serum iron and transferrin saturation; geometric mean for serum ferritin.
†Collapsing of male and female age categories for serum ferritin values is based on criteria established previously.[15]

Table III. Mean age and range of males and females belonging to different groups according to the level of daily alcohol intake

Sex	Daily alcohol intake (gm)	(n)	Age (yr) Mean	(Range)
Females	<10	(111)	43	(15-82)
	10-60	(53)	52	(12-81)
	>60	(17)	46	(22-76)
Males	<10	(44)	43	(13-76)
	10-60	(52)	45	(12-70)
	>60	(40)	47	(16-71)

males and females, was not significantly different in the three groups (Table III). As also presented in Table III, the age ranges were also similar in all groups. A one-way analysis of variance was performed to test for differences in the levels of alcohol consumption. As shown in Fig. 2, alcohol intake influenced strongly the serum iron level, transferrin saturation, and serum ferritin level in males; that is, an increase of these values was seen with a corresponding increase of alcohol intake. For serum iron level and transferrin saturation, significant differences were found between males whose daily alcohol intake was under 10 gm and those of the other two groups taken together. For serum ferritin level, significant differences were found between the three groups of males. In the female group the effect of alcohol intake was noted for serum ferritin levels only: a significant difference was found between the group of females who drank less than 10 gm alcohol a day and the other two groups.

Region of origin. To identify differences related to region of origin, two indicator variables (north vs

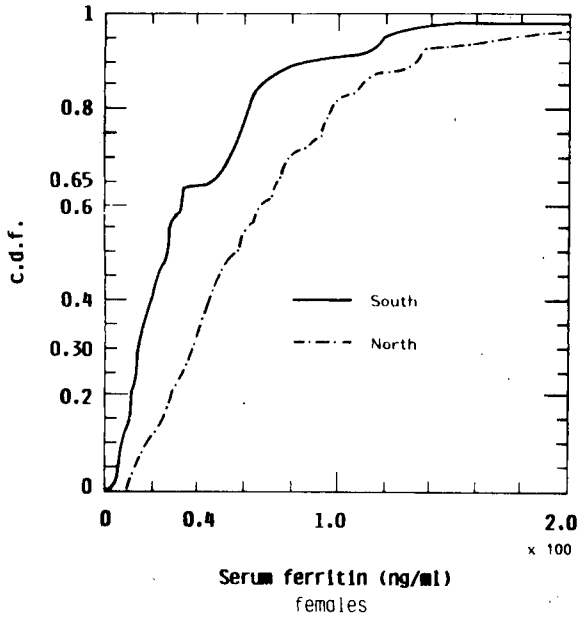

Fig. 3. Sample cumulative distribution frequencies *(c.d.f.)* of serum ferritin values in the female populations from the north and south of Portugal.

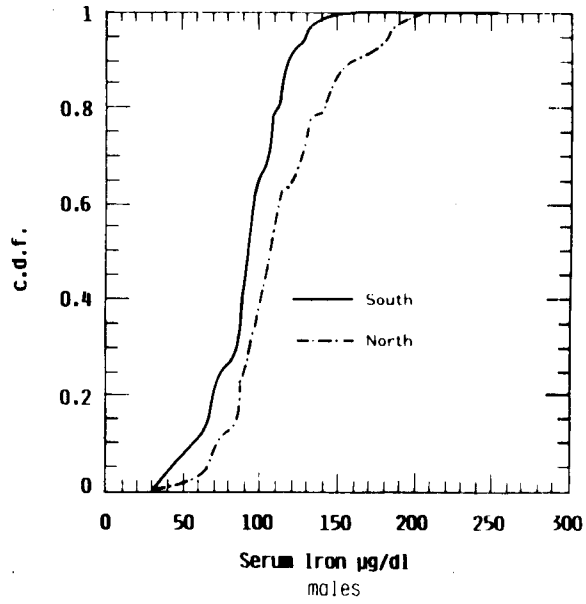

Fig. 4. Sample cumulative distribution frequencies *(c.d.f.)* of serum iron values in the male populations from the north and south of Portugal.

south and Cabeceiras de Basto vs S. João de Lobrigos) were added to the regression analysis, which included the variables previously identified as influencing the biochemical measures (alcohol intake for serum iron and transferrin saturation, and age and alcohol intake for serum ferritin). Because no differences were found between the populations of Cabeceiras de Basto and S. João de Lobrigos, these were grouped and designated as population from the north and were compared with the subjects from Castelo de Vide, designated as population from the south. Using the Kolmogorov-Smirnov two-sample test, we found significant differences between these two populations in the distribution of serum ferritin values in females (DN = 0.421309; $p = 1.24472 \times 10^{-6}$); as can be seen in the sample cumulative distribution functions represented on Fig. 3, about 65% of women from the south had serum ferritin values under 40 ng/ml, in contrast to only 30% of women from the north. This difference was not observed for males. Significant differences were also observed between the two regions when the levels of serum iron in males were compared (DN = 0.351064; $p = 8.84704 \times 10^{-4}$); as shown in the cumulative distribution functions represented on Fig. 4, virtually no men from the south had serum iron values above 130 µg/dl, whereas values above this were observed in about 27% of men from the north. This difference was not observed in females.

Abnormalities in biochemical parameters

Control population. For the definition of iron overload in the control population, we have considered as cutoff values for transferrin saturation 62% for males and 59% for females, according to the reference values calculated. All subjects with transferrin saturation higher than these cutoff values had their biochemical tests repeated. If tests were consistently abnormal, subjects were advised to undergo a liver biopsy. Additionally, individuals who, as the only biochemical abnormality, had serum ferritin values higher than the upper reference values established for their sex and age (for exact values see Table II) were also considered as suspects of iron overload. Following these combined criteria, from a total of 353 subjects, nine (seven from the population from the north and two from the population from the south) were considered to have biochemical evidence of iron overload; six of these had abnormal transferrin saturation values as well as high serum ferritin levels; the other three subjects had high serum ferritin values as the only biochemical abnormality. The biochemical and histologic data in these nine subjects are summarized in Table IV. From these nine subjects, six agreed to have a liver biopsy. In none of them the amount of iron in the liver sections was graded greater than 2; these subjects are being followed up.

In addition, iron deficiency was defined in subjects with serum ferritin values abnormally low according to the reference values established previously for their sex- and age-matched control groups.[15] Iron-deficient subjects were not equally distributed in the popula-

Table IV. Iron parameters in nine subjects with biochemical evidence of iron overload, screened from the control population

Region Village/town	Sex	Age (yr)	Transferrin saturation (%)	Serum ferritin (ng/ml)	Liver biopsy results	
					Histology	Grade
North						
Cabeceiras de Basto	M	22	48	286	Normal	1
	F	16	28	140	Normal	1
	F	37	41	161	np	—
S. João de Lobrigos	M	29	77	403	np	—
	M	31	74	595	Steatosis/fibrosis	1
	M	51	69	410	Steatosis	1
	F	61	86	1860	Inconclusive	—
South						
Castelo de Vide	M	67	67	1180	np	—
	F	53	59	2140	np	—

np, Not performed.

tions from the north and from the south; in the north, 2 out of 243 subjects (0.82%) were found to have iron deficiency; in the south, 9 out of 110 (8.18%) were iron deficient. The 95% confidence intervals for the frequency of iron deficiency were thus estimated to be 0% and 1.96% in the population from the north and 3.06% and 13.3% in the population from the south.

Hemochromatosis families. Family members were grouped according to HLA typing in the following classifications: (1) HLA identical to the probands (HH), (2) those who shared one haplotype in common with their probands (HO), and (3) those who differed from probands in both haplotypes (OO). To examine the biochemical levels in the families with hemochromatosis, a regression was run that included the sex, age, and alcohol intake variables and their (significant) interactions, as well as indicator variables to represent the three types of relatives (HH, HO, and OO) and to represent interactions. To compare the HO relatives with the controls, the regression model was rerun with just those two groups of subjects. As expected, HH family members showed significantly higher values for serum iron level, transferrin saturation, and serum ferritin level than the other two groups (Fig. 5). Significant differences were also found between HO and OO family members for serum iron and transferrin saturation in females and for transferrin saturation in males. No differences were found between HO and OO subjects for serum ferritin level. Because factors exist that were shown to influence iron values—such as age and the level of daily alcohol intake—one could be led to think that the differences observed in family members could be attributed to differences in the distribution of those factors. This was not the case, however, because the three groups did not differ in the distribution of age and alcohol intake.

Family members with biochemical evidence of iron overload or HLA identical to the respective proband were considered as suspected of having hemochromatosis and therefore advised to have a liver biopsy done. This happened in 25 of the 136 subjects in the study (18 males and seven females). From these, 17 agreed to have a liver biopsy. Their clinical, biochemical, and histologic findings at the time of diagnosis are shown in Table V. In three of these subjects, no stainable iron was detected (subjects 3, 11, and 12 in Table V); these subjects were two brothers, aged 29 and 21, and one sister, aged 31, all HLA identical to their respective probands; the hepatic histology showed steatosis in one subject and was normal in the other two subjects. In one additional subject (subject 10 in Table V), a 41-year-old brother with HLA identical to the proband, iron stores in liver were graded as 1 and, therefore, the disease could not be confirmed. The liver histology in this subject showed steatosis. In the remaining 13 subjects (see Table V), stainable iron graded greater than 2 was demonstrated in liver biopsy samples. All were without symptoms except for a 57-year-old woman with severe arthropathy and a 43-year-old man who complained of arthralgia.

From the 13 subjects in whom diagnoses were made, six were siblings identical to their probands on both HLA haplotypes; in one subject we could not define the genotype; the remaining six subjects shared only one haplotype in common with their respective probands. These latter six subjects did not belong to the same sibship of their probands, but they included two half-brothers, two sons, one father, and one mother of each of the respective probands. Assuming that two hemochromatosis alleles, linked to the HLA haplotypes, are required for full expression of the disease,[1,3] these six HLA haploidentical family members were assigned the genotype homozygous for the dis-

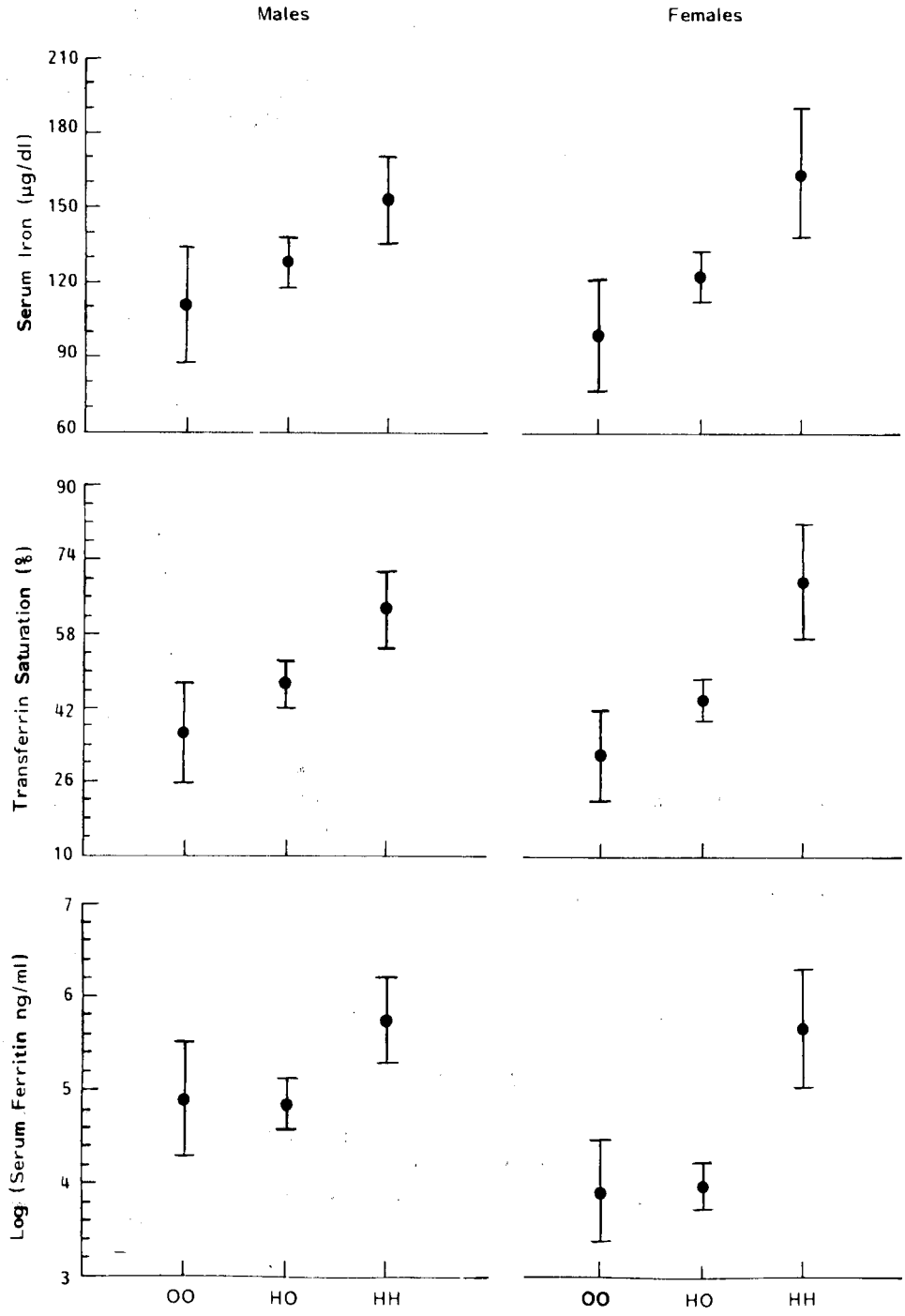

Fig. 5. Variation of serum iron level, transferrin saturation, and the log of serum ferritin values in family members who share in common with their probands the following characteristics: both HLA haplotypes (HH), one HLA haplotype (HO), or no HLA haplotype (OO). Arithmetic means and the 95% confidence intervals for factor means (two times the pooled standard error) are indicated.

ease. This implies that a new entry of the hemochromatosis trait into each pedigree occurred through spouses, that is, through homozygous-heterozygous matings. Therefore, from a total of 70 HLA haplotypes defined in the family studies, 34 were defined as linked to hemochromatosis. Of these 34 haplotypes, 28 were contributed by probands and six were identified in the course of the family studies. Based on these data, a gene frequency of 0.14 (6 of 42) was estimated. This gene frequency corresponds to a het-

Table V. Clinical, biochemical, and histologic data in 17 subjects screened for family studies

Subject number	Relation to the proband	Number of HLA haplotypes identical to proband	Clinical picture	Sex	Age (yr)	Transferrin saturation (%)	Serum ferritin (ng/ml)	Liver biopsy	
								Histology	Grade
1	Half brother	1	Arthralgia	M	43	83	1110	Cirrhosis	3
2	Half brother	1	*	M	34	72	—	Normal	3
3	Brother	2	*	M	21	73	176	Normal	0
4	Brother	2	*	M	39	92	854	Normal	4
5	Son	1	*	M	23	90	432	Normal	4
6	Brother	2	*	M	22	81	427	Normal	3
7	Father	1	*	M	65	38	473	Fibrosis	2
8	Mother	1	Arthrosis	F	57	39	395	Cirrhosis	3
9	Brother	†	*	M	49	54	619	Steatosis	2
10	Brother	2	*	M	41	58	308	Steatosis	1
11	Brother	2	*	M	29	65	476	Normal	0
12	Sister	2	*	F	31	62	219	Steatosis	0
13	Brother	2	*	M	35	68	199	Normal	3
14	Son	1	*	M	16	69	100	Normal	2
15	Brother	2	*	M	37	98	880	Normal	4
16	Brother	2	*	M	39	100	526	Normal	4
17	Sister	2	*	F	34	93	345	Normal	3

*No symptoms.
†Undefined genotype.

erozygote frequency of 0.24 and a frequency of homozygote of 0.019, assuming a Hardy-Weinberg equilibrium.[16]

Phenotypic expression of heterozygotes. To verify the eventual influence of the hemochromatosis allele on iron parameters, we have analyzed the phenotypic expression of family members heterozygous for hemochromatosis. These were, by definition, those who shared one HLA haplotype in common with their probands. In six of those subjects (subjects 1, 2, 5, 7, 8 and 14 in Table V), hemochromatosis was diagnosed by liver biopsy, and therefore these subjects were considered, as stated previously, to be homozygous for the disease.

From a total of 68 heterozygotes (29 males and 39 females) seven showed sporadic abnormalities of some biochemical parameters; one female had abnormally high transferrin saturation as well as serum ferritin values, and one male and five additional females had high transferrin saturation values as the only biochemical alteration. In all these subjects the abnormalities were not consistent, and these subjects are being followed up.

The influence of the hemochromatosis allele on iron values was analyzed by comparing the values between the heterozygotes and the control population. After correction for the effects of age, alcohol intake, and region of origin by stepwise multiple linear-regression analysis, significant differences were found in females for serum iron level ($p < 0.0001$), transferrin saturation ($p = 0.001$), and serum ferritin ($p = 0.005$) values. In the male group no significant differences were found between heterozygotes and controls except for serum iron level ($p = 0.01$). Because the strongest significant regional differences found before were the distribution of serum iron values in males and serum ferritin values in females (see Fig. 3), we compared separately, for the same parameters, the heterozygous population to the control population from the north and the heterozygous population to the control population from the south. Serum iron values in heterozygous males were even more strikingly different ($p = 0.0001$) from controls when analysis of these values was confined to those in the southern populations, whereas no differences were observed when serum iron values in heterozygous males in the north were compared with those in the northern controls. In similar fashion, for serum ferritin values in females, the difference found between the heterozygotes and the controls from the south ($p = 0.01$) was not observed when comparing heterozygotes and controls from the north.

DISCUSSION

This study illustrates well the importance of establishing appropriate reference values for the biochemical tests used for the screening of iron overload, because significant regional variations can occur as a result of both environmental and genetic factors. Previous studies have addressed the question of defining

the best method for the early detection of hemochromatosis and the most appropriate screening programs.[9,2,17,18]

HLA typing in the screening of hemochromatosis. HLA typing has an unquestionable importance in the detection of family members at risk of developing the disease,[2] although it is of no value for screening programs in the population at large. In the present study we confirm an HLA-linked transmission of hemochromatosis, because family members identical to their probands in both HLA haplotypes had significantly higher values for serum iron level, transferrin saturation, and serum ferritin level than did the other family members. In four HLA-identical family members, no excess iron was demonstrated in the liver, although these subjects had biochemical evidence of iron overload. These were considered homozygotes at early stages of development of iron overload and are currently being followed up. These findings reinforce the importance and the need of HLA typing in the early diagnosis of hemochromatosis and prevention of tissue damage.[2]

Biochemical parameters of iron overload. The screening for hemochromatosis implies a choice of biochemical tests that better define iron overload. Transferrin saturation seems to be the single most reliable screening test for iron overload, but there is no definitive agreement on the cutoff values representing the most appropriate control thresholds. Serum ferritin level has been indicated as a second-line test in population studies,[17,18] but as with transferrin saturation, no clear cutoff values have yet been established for this test, perhaps because of the great variability of serum ferritin values referred to in the literature.[12] In this study we used, for the screening of iron overload in the population, the combined criteria of high transferrin saturation and high serum ferritin values, as related to previously established normal values. The importance of establishing our own reference and cutoff values for iron parameters became clear for two reasons: (1) because of eventual minor differences in techniques between different laboratories; (2) as a result of environmental regional differences that could be expected to influence the biochemical tests. Serum iron level and transferrin saturation values found in the Portuguese population did not differ from those defined in populations from other studies.[9,17,19] For the definition of reference values for serum ferritin, we took into account the effects of several factors on those values. We found an increase with age in serum ferritin values in males under 40 years and females over 40 years of age.[15] The level of daily alcohol intake strongly influenced serum ferritin values in the sense of an increase with increasing amounts of regular alcohol consumption. This could explain the tendency for high serum ferritin values that we found, because about half of the females and two thirds of the males drink regularly more than 10 gm of alcohol per day, and 10% of females and 30% of males drink more than 60 gm/day (Table III). Besides those differences, we found a regional effect on serum ferritin level, with a tendency for lower values in the southern populations. This effect could not be attributed to differences in age or alcohol intake. In agreement with this was the finding of a much higher estimate of iron deficiency in the population from the south than that found in the northern controls.

Phenotypic expression of heterozygotes. One possible explanation for the regional variation observed in iron parameters could be the effect, at least in part, of the high prevalence of the hemochromatosis gene found in the population from the north. We tried to answer this question by studying the influence of the hemochromatosis gene on the different biochemical tests through the analysis of the phenotypic expression of individuals heterozygous for the disease. This has been already a matter of some debate. Beaumont et al.[19] and later Simon et al.[20] described as the only biochemical abnormality in heterozygotes high serum ferritin values in males. Basset et al.[16] found abnormal serum ferritin values only in family members who had a history of excessive alcohol intake. Cartwright et al.[9] did not find differences in serum ferritin values in heterozygotes but instead found abnormalities in transferrin saturation, in both males and females; they found abnormalities in serum iron levels only in males. In the Portuguese population in our study we found a clearly different expression of biochemical values between males and females. Heterozygous males did not differ from normal controls except for serum iron values. However, this difference was observed when the heterozygotes were compared with the population from the south and not with the northern controls. Because we found a difference in the distribution of serum iron values between northern and southern populations (see Fig. 3), the different results observed could be attributed to differences between the control populations themselves. On the contrary, the female heterozygous population showed marked differences from the controls for the three parameters (serum iron level, transferrin saturation, and serum ferritin level), although for serum ferritin level the differences were only observed with the controls from the south.

An advantageous gene. The hemochromatosis gene is thought to confer survival advantage to individuals sustaining physiologic loss of blood or to individuals

living in areas of iron-poor diet.[9,21,22] We hoped this study would give us insight into the importance of the prevalence of the hemochromatosis gene in a population. In fact, our findings seem to support the hypothesis that the hemochromatosis allele confers a selective advantage to the numerous heterozygous females[22] who could absorb more iron and so be protected from iron deficiency caused by malnutrition, menstruation, and repeated pregnancies. This fact is particularly relevant in the population from the north of Portugal where a very high rate of pregnancies has been reported: more than 30% of females over 40 years from Cabeceiras de Basto had had more than 10 pregnancies.[23] Instead, in the population studied in the south of Portugal the mean number of pregnancies in women over 40 years was 2.8 (Porto and Transmontano, unpublished data). In view of the above-mentioned possible selective advantage of the hemochromatosis allele, it is not surprising the finding of a very high estimate of the gene frequency for hemochromatosis. The frequency of homozygotes and heterozygotes, based on HLA typing and family studies, was estimated to be 0.019 and 0.24, respectively. This is a very high estimate when compared with the frequency of homozygosity of 0.003 to 0.008 reported before for the white population.[9,18,19,24,25] The estimated high prevalence of the hemochromatosis allele corresponds to a high prevalence of families in which pseudodominant or vertical inheritance was found (5 out of 15 families studied), which is assumed to be the result of homozygous-heterozygous matings.

The migration of populations, having occurred differently in the north and south of Portugal,[26] could be the cause of a different prevalence of the disease in the two regions studied. On the other hand, the concentration of a gene in some regions could be favored by particular demographic characteristics in this country, namely the unipolar mode of migration and the low rate of mobility from other regions.[26] So far we have only studied families from the north of Portugal. Family studies in the south are needed to establish the prevalence of heterozygotes there and to confirm the effect of a possible different prevalence of the hemochromatosis gene on the differences found in iron deficiency prevalence and ferritin values between the populations from the two regions studied.

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CAPÍTULO 5

SCREENING FOR HEMOCHROMATOSIS IN PORTUGAL : A STUDY OF THE POPULATION FROM REFOIOS-PONTE DE LIMA

SCREENING FOR HEMOCHROMATOSIS IN PORTUGAL: A STUDY OF THE POPULATION FROM REFOIOS-PONTE DE LIMA

(submitted for publication)

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ABSTRACT

It is now generally accepted that, in patients with idiopathic hemochromatosis (IH), iron loading and organ damage can be prevented by prophylactic phlebotomy treatment if it is done early in the course of the disease. These findings emphasize the importance of early detection of this disorder, before the occurence of clinical signs, and the need for well conducted screening programs in regions where the disease is known to be prevalent. These programs depend upon reliable reference values of the biochemical parameters of iron overload which were previously shown to be influenced by several factors including sex, age, level of alcohol intake and the region of origin of subjects. In this study we have used transferrin saturation and serum ferritin determinations for the screening of hemochromatosis in a sample of 113 apparently normal subjects from Refoios-Ponte de Lima, a village in the north of Portugal where a high prevalence of the IH gene was suspected. The results confirm the validity of the reference values established for serum ferritin in the portuguese population and support the previous estimation, based on family studies with HLA typing, of a high frequency of the IH gene in this region.

INTRODUCTION

Idiopathic Hemochromatosis (IH) is a genetic disease of iron metabolism characterised by excessive iron absorption and progressive iron overload of parenchymal organs. The underlying metabolic defect is unknown but the gene is known to be linked to the HLA class I region of the MHC complex (2). IH is an autossomic recessive disorder with prevalences of 0.3-0.8%, estimated on the basis of family studies with HLA typing (1,3-5). Therefore it constitutes one of the most common recessive metabolic disorders detected to date in Caucasian populations.

There is now enough evidence that the complications of the disease may be prevented by early phlebotomy treatment. Patients treated before the development of tissue damage have a normal life expectancy in contrast with patients diagnosed after the development of cirrhosis (6). The early diagnosis of hemochromatosis depends in part on awareness of the clinicians for the disease; it can also be achieved by well conducted screening programs which are specially pertinent in regions where IH is known to have a high prevalence. In the absence of a specific marker of the IH gene, two strategies are used for the screening of the disease: 1.family screening with HLA typing, which is the most effective means of identifying homozygotes without manifestations; 2.detection of asymptomatic patients in the population at large based on raised transferrin saturation and serum ferritin levels. This last strategy depends heavily on the establishment of appropriate reference values for those biochemical tests.

In 1986 we have started a systematic screening for IH in this country both by family and population studies (7). In the course of that program, the necessity of establishing appropriate reference values for the biochemical tests of iron metabolism became apparent. This was specially relevant for serum ferritin values since no universal cutoff values were available given the great variability of this parameter from study to study in the literature (reviewed in 8). As a result, a method was developed to establish reference values for serum ferritin in a Portuguese population aged between 12 to 82 years, eliminating, as much as possible, the variation caused by sex and age (8). Other variables were shown to influence serum ferritin values, namely the level of daily alcohol intake and the region of origin of subjects (7). Subjects from populations in the north of Portugal were found to have higher serum ferritin values than subjects from the south, independently of the effects of sex, age and alcohol intake. This finding was interpreted as the possible representation of the high

prevalence values of the IH gene calculated from family studies in the population from the north (7).

In this study we have extended the program of screening of IH to another population from the north of Portugal. The present results confirm the validity of the reference values established for serum ferritin in the portuguese population and support the previous estimation of a high frequency of the IH gene in this region.

MATERIAL AND METHODS

The Study Population

The population selected for this study was a volunteer population from Refoios-Ponte de Lima, a village in the north of Portugal with 2436 inhabitants. Individuals were recruited into the study with the help of the local priest and the clinicians from the public health service. The subjects volunteered to participate after explanatory sessions of the aims of the study; their presence was thus considered as the expression

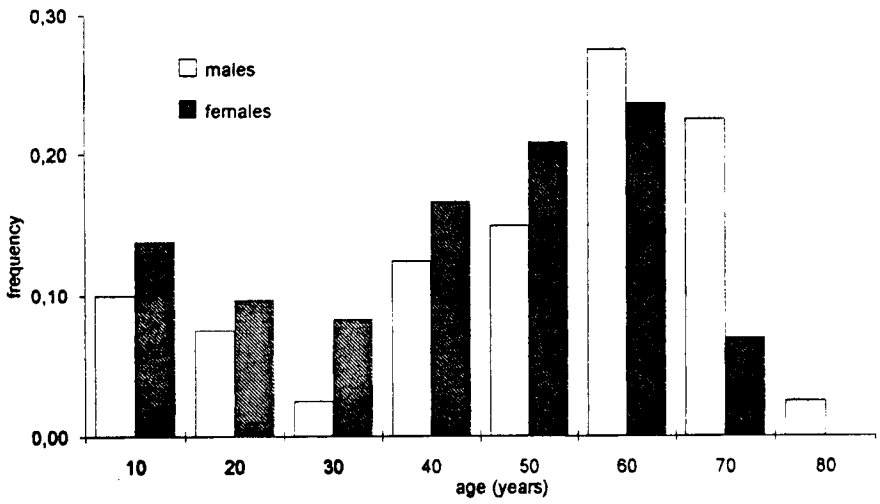

Fig.1 Sample distribution according to age (relative frequency histogram. n=112)

of informed consent. The population was sampled from subjects attending Sunday mass and was considered representative of the whole village population. A total of 112 subjects was studied (40 males and 72 females within an age range between 10 and 83 years). The age distribution, as illustrated in Fig.1, denotes the typical population of a village with a high rate of emigration, i.e., subjects are mainly concentrated in younger and older age groups with few representatives of the middle

decades (20-40 years). All subjects were interviewed and a questionnaire was filled in with relevant information regarding their health status and dietary habits, namely the level of daily alcohol consumption. Subjects were grouped in 4 levels according to their daily alcohol intake: 1=less than 10g/day; 2=10-60 g/day; 3=60-100g/day; 4=more than 100g/day. Lifetime alcohol intake was not the variable chosen to evaluate the influence of alcohol on iron parameters because it was too complex to be measured accurately.

Peripheral venous blood samples were colected during the morning in all subjects for determinations of iron parameters (transferrin saturation and serum ferritin), complete hematological counts and determination of serum gamma-glutamyl transferase (γ-GT).

Controls

Reference values for iron parameters were obtained from a previous study of 353 subjects from 3 different populations: 2 from the north (Cabeceiras de Basto and S. João de Lobrigos) and one from the south of Portugal (Castelo de Vide) (7). The reference values are summarised in Table 1.

Table 1. *Reference values for iron parameters previously published (Ref.7)*

	Males (n=157)		Females (n=193)	
	Mean*	min, max	Mean*	min, max
Transferrin saturation (%)	39	19,62	34	15,59
Serum ferritin (ng/ml)				
Aged 10 yr	32	8,123		
Aged 20 yr	59	16,218	32	7,134
Aged 30 yr	108	29,401		
Aged 40 yr			38	7,196
Aged 50 yr	155	30,483	47	9,242
Aged 60 yr			60	12,304
Aged 70 yr			75	15,387

* Arithmetic mean for transferrin saturation; geometric mean for serum ferritin

Data from that study were reanalysed for comparison with the present data. Control values for the hematological parameters were obtained from a population of 482 (226 males and 256 females) healthy blood donors from Hospital Geral de Santo

António. The confidence values for γ-GT noted by the reagent kit's manufacturer (Boehringer Manheim-GmbH Diagnostica) were used for reference.

Laboratory Parameters

Transferrin saturation was calculated from the ratio of serum iron (Fe) and total iron-binding capacity (TIBC). Serum iron, total iron-binding capacity and γ-GT concentration were determined in an Hitachi automatic analyser. Serum ferritin was determined by microplate immunoenzymatic assay (Abbott Diagnostics, Wiesbaden-Delkenheim). Hematological counts were done in a Coulter automatic cell counter.

Magnetic Ressonance Imaging (MRI)

Liver iron overload was evaluated by MRI according to the method of Gandon (9). Using a MRMAX-0.5T, various breathhold gradient echo sequences were obtained. The liver signal to noise ratio and various tissue ratios were then calculated. The liver iron concentration was estimated from these ratios by comparing to the values previously established by Gandon in a large sample of IH patients in whom the same ratios were correlated with the liver iron concentration calculated on liver biopsy (Gandon, unpublished data).

Statistical Methods

For purposes of the statistical analysis, the square root transformation was used for transferrin saturation and the logarithm transformation was used for serum ferritin and γ-GT. In each case, the goal was to achieve an empirical distribution compatible with the normal (Gaussian) assumptions. Correlation between variables was analysed by linear regression. Regression analyses were performed to test the effect of age on serum ferritin and γ-GT values. Because of the considerable variations in these parameters and the importance of gender, regressions were run in each gender group separately. The influence of daily alcohol intake on serum ferritin and γ-GT was evaluated by analysis of variance. For overall comparison of the distribution of serum ferritin values in the groups of subjects from different regions, i.e., from the north and from the south, the nonparametric two sample Kolmogorov-Smirnov test was used. For the correction of the effects of sex, age and alcohol intake on the distribution of serum ferritin values we calculated, in males and females separately, the residuals of the multiple regression of log serum ferritinin on age and level of alcohol intake in the groups of subjects from each region. The distribution of the residuals in both groups

was then compared using the Kolmogorov-Smirnov two-sample test. All testing was performed at the 0.05 level of significance, and all p values are two sided. The data were analysed by using the Statgraphics Statistical Graphics System (STSC).

RESULTS

Confirmation of the factors affecting serum ferritin values

Sex and age

Confirming our previous results (8) there was an age dependence for serum ferritin

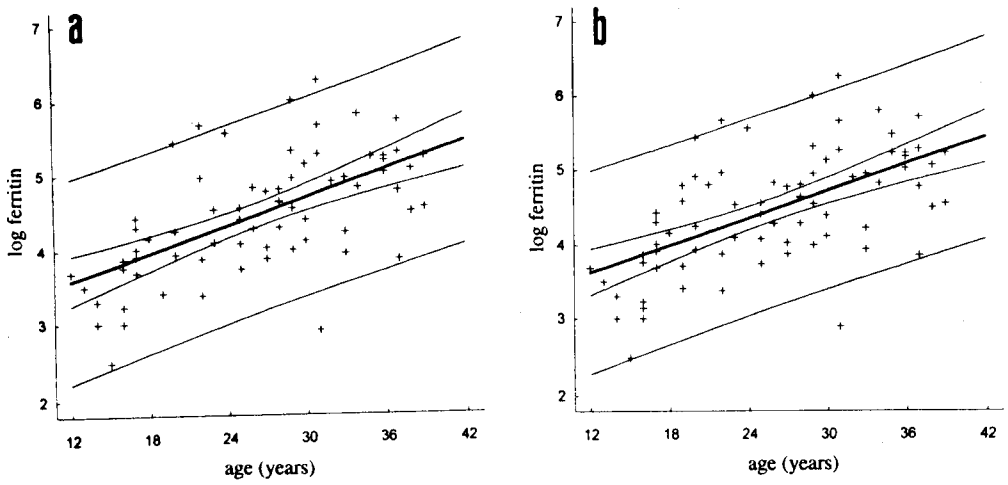

Fig.2 Linear regression of log of serum ferritin and age for males under 40 years from the study of Vicente et al (7) (2a) and from the 2a and the present study together (2b). Plot of the least square straight line, the 95%confidence bands, and the 95% prediction bands.

values in females over 40 years and males under 40 years of age. The previous linear regression model relating the log of serum ferritin and age in males under 40 years of age (Fig.2a) did not change with the inclusion of the present data (Fig.2b). Although not apparent in the figures, the previous model for females over 40 (Fig.3a) had a slight, but statisticaly significant, increase in the slope of the regression line with the inclusion of the present data (Fig.3b). This alteration, however, did not have any important impact on the established reference values used.

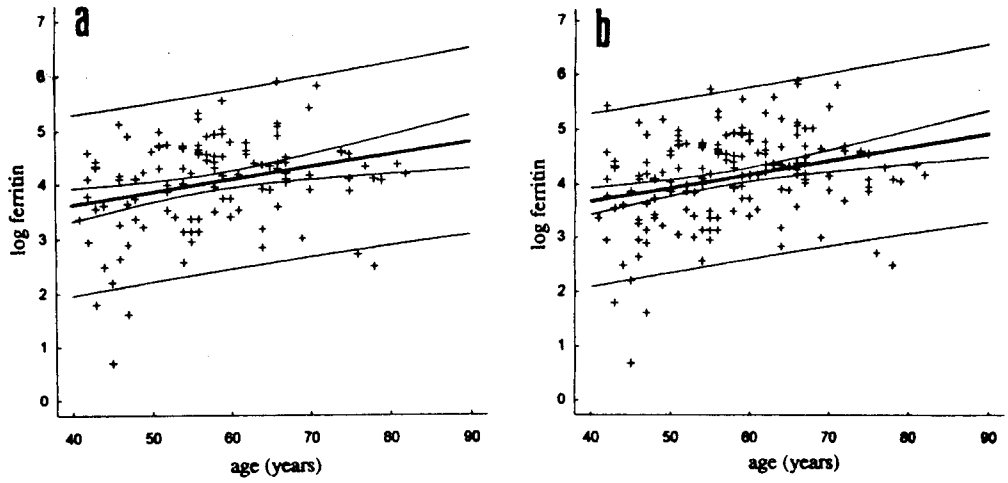

Fig.3 Linear regression of log of serum ferritin and age for females over 40 years from the study of Vicente et al (7) (3a) and from 3a and the present study together (3b). Plot of the least square straight line, the 95%confidence bands, and the 95% prediction bands.

Alcohol intake

On the whole group of subjects, significantly higher serum ferritin values were found in those with higher daily alcohol levels. In the present sample however, such effect could not be distinguished from the sex and age effects, for the distribution of these variables was not similar in the different groups of subjects according to the alcohol level: subjects with higher daily alcohol intake levels were mainly males and older females. To test further if the increased serum ferritin levels were related with hepatic alterations induced by alcohol intake, we have examined

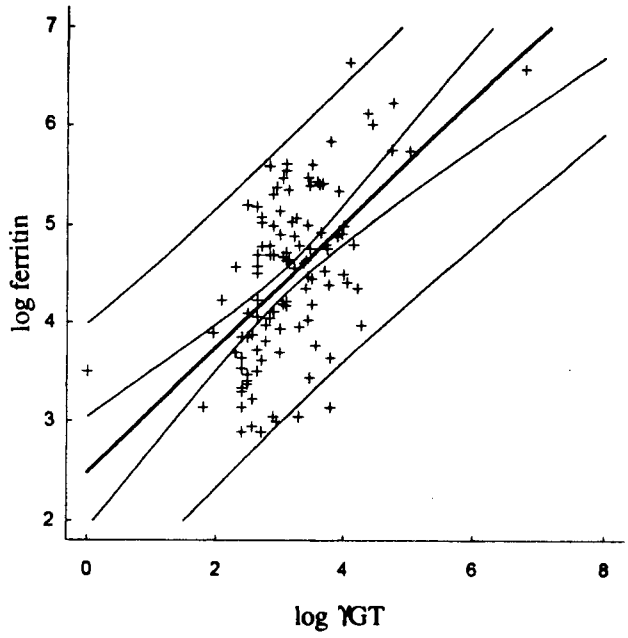

Fig.4 Linear regression of log of serum ferritin and γ-GT concentration for the whole sample. Plot of the least square straight line, the 95%confidence bands, and the 95% prediction bands.

in all subjects the concentrations of serum gamma-glutamyl transferase, used classically as a marker of alcohol abuse (10). In general, no significant correlation was found between γ-GT and the level of alcohol intake. Individual analysis of this parameter, however, revealed that all subjects with abnormally high serum ferritin values also had abnormally high γ-GT values. The correlation between γ-GT and serum ferritin values (Fig.4) was highly significant ($r=0.57$; $p<0.000001$).

Region of origin

Using the Kolmogorov-Smirnov two sample test, significant differences were noted in the distribution of serum ferritin values between the populations from Refoios and that from the south (Castelo de Vide). Subjects from the south had more frequently lower serum ferritin values whereas subjects from Refoios had more frequently higher values. This difference was observed both in males and females. No significant differences in the distribution of serum ferritin values were found between the population from Refoios and the other northern populations. To better characterize the differences found between the populations from the two regions (north and south), we compared the distribution of serum ferritin values between the whole group of subjects from the three populations in the north and the group of subjects from the south, eliminating, as much as possible, the effects of sex, age and alcohol intake (see materials and methods). Confirming the results of our previous study (7), after correction of these variables, still significant differences in the distribution of serum ferritin values in

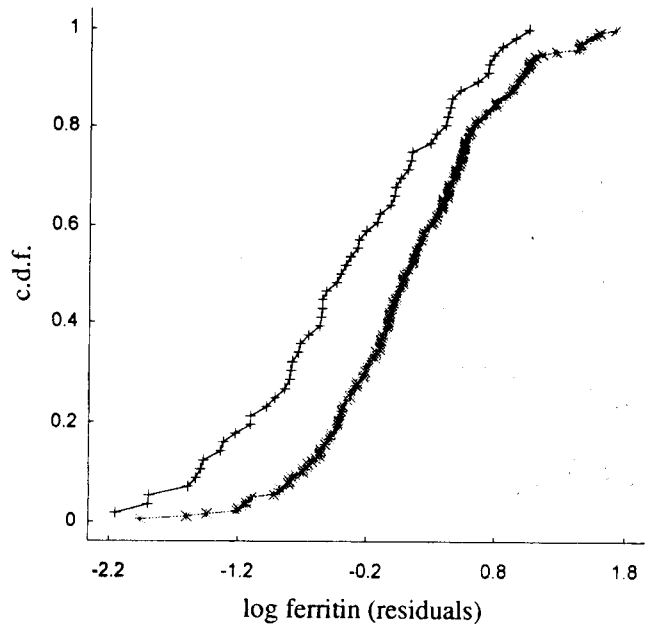

Fig.5 Sample cumulative distribution frequencies (c.d.f.) of the serum ferritin values corrected for age and alcohol intake (see M & M) in females from Refoios (+) and Castelo de Vide (×).

females were observed between the two regions, subjects from the north having more frequently higher values than subjects from the south (Fig.5).

Screening of Iron Overload and Deficiency

Taking the reference values previously established for the portuguese population (7,8), 15 subjects (13%) were found to have at least one of the iron parameters out of the 95% confidence limits for the normal population (Table 2). Six subjects had abnormally low transferrin saturation values (subjects 1-6 in Table 2). Hemoglobin

Table 2. *Relevant biochemical and hematological data in 15 subjects with abnormal values screened from the population from Refoios- Ponte de Lima*

Subject	Sex	Age (yrs)	Transferrin Saturation (%)	Serum Ferritin (ng/ml)	Hemoglobin (g/dl)	gamma-glutamyl transferase (U/l)
1	M	59	**12**	56	16.3	**31**
2	F	46	**11**	19	14.8	**13**
3	F	49	**11**	68	14.6	**22**
4	F	47	**13**	18	13.3	**15**
5	F	16	**13**	33	13.4	**1**
6	M	16	**17**	23	14.5	**6**
7	M	46	**69**	270	16.1	**33**
8	F	62	**63**	201	14.0	**18**
9	F	16	**60**	38	15.1	**11**
10	F	25	**61**	88	14.0	**31**
11	M	41	**65**	**763**	16.9	**59**
12	M	29	n.a.	**711**	15.9	**885**
13	M	45	48	**503**	16.3	**116**
14	M	66	36	**453**	15.3	**78**
15	M	63	24	**406**	15.7	**84**
Controls*						
(n=226)	M				14.8 (13.2,16.4)	(11,50)
(n=256)	F				13.2 (11.6,14.8)	(7,32)
(n=157)	M		39 (19,62)	**		
(n=193)	F		34 (15,59)	**		

* Control values are indicated as the mean and/or (95% confidence limits)
Abnormal values are indicated in bold
** See Table 1
n.a.= not available M=males F=females

concentration as well as serum ferritin values were within the normal range in all of them. No evidence of inflamation or malignant disease was found in these subjects; they are currently being followed up. Following the combined criteria of abnormally high serum ferritin and/or transferrin saturation values, nine subjects had biochemical evidence of iron overload. Four subjects (subjects 12-15 in Table 2) were found to have abnormally high serum ferritin values together with abnormally high γ-GT, suggesting that alcohol abuse may have been responsible for some of the increased serum ferritin concentrations observed. Subject 12 also had a clinical picture of liver cirrhosis of probable alcoholic origin. Abnormally high transferrin saturation values were found in 5 subjects: in 4 of them (subjects 7-10 in Table 2), the high transferrin saturation was the only abnormality found; the other one (subject 11 in Table 2) had additionally abnormally high serum ferritin values. Using the combination of raised serum ferritin concentration and raised percentage transferrin saturation to detect homozygoty for IH (9), subject 11 was considered a suspect case. He was an apparently healthy 41 years old male with no signs or symptoms related to iron overload. This subject did not agree in performing a confirmatory liver biopsy. Thus, liver iron content was assessed by Magnetic Ressonance Imaging using gradient echo sequences (9) and signal ratios corresponding to 100 μmol/g of dry liver weight were obtained. This level is diagnostic of homozygous hemochromatosis (11).

DISCUSSION

The present results reinforce the importance of establishing appropriate local reference values for the biochemical tests used in the screening of iron overload, given the significant regional variations which may occur, largely dependent on environmental factors like the alcohol consumption habits, but also possibly as a result of the genetic background of the populations.

Serum ferritin values in the screening of hemochromatosis

We confirm here that serum ferritin values in normal females and younger males in the portuguese population are higher than those reported in other studies in the literature (reviewed in 8). We still don't know whether this difference is the reflection of different habits of the population, particularly the generally observed high regular

alcohol intake. A more correct analysis of the influence of alcohol consumption on serum ferritin values independently of sex and age differences would require the inclusion of a far greater number of males who do not drink and younger females who drink more heavily. Such a sample is not easy to find for it does not correspond to the usual social habits of the portuguese population. For practical purposes, when interpreting the finding of a high serum ferritin value, the problem of the effect of alcohol may be partially overcome with the determination of γ-GT concentration. Although this marker has a low specificity for alcohol abuse in general, its high sensitivity justifies its use as a possible indicator in individual subjects (10). History of alcohol abuse and abnormally high liver enzymes, however, do not in any circumstance rule out the possibility of the influence of the hemochromatosis gene in the pathogenesis of iron overload. This point is particularly relevant in countries like Portugal where the high prevalence of both alcoholic liver disease and hemochromatosis makes highly probable the combined effect of both disorders.

Based on the differences found in the distribution of serum ferritin values between different regions in the country one could adress the issue of whether we should determine different reference values for a person coming from the north or the south of Portugal but we think it is premature to do that at this stage. This study must be extended to other regions of Portugal and also to recent and past portuguese emigration communities before deciding whether the finding of regional differences is fortuitous or whether iron-related parameters accompany other genetic characteristics of populations.

The prevalence of hemochromatosis in the north of Portugal

Estimations of the prevalence of IH made by studies of the phenotypic expression in the general population from many different countries have generally failed to validate the highest predictions based on HLA genotypes (12-17). This discrepancy may be explained by variations in the real prevalence of IH in different geographic regions, as clearly demonstrated in the different studies conducted in Sweden (18) or by the different methods in selecting populations. The present results are an indication that the real prevalence of the hemochromatosis gene in this region in the north of Portugal corresponds to the estimated high prevalence based on family studies with HLA typing. We have previously estimated, by HLA genotypes, a gene frequency of 0.14 that corresponds to a frequency of homozygotes and heterozygotes of 0.019 and 0.24, respectively (7). Biochemical evidence of iron overload was found in the present

population in a frequency of 0.080 (9/112 subjects). Using the combined criteria of abnormal serum ferritin and transferrin saturation values to detect homozygous IH, we found one patient in a sample of 112 subjects. The diagnosis was confirmed by the demonstration of excessive liver iron by MRI using gradient echo sequences (9). The other subjects had either transferrin saturation values greater than 60% with no increased serum ferritin (four subjects) or increased serum ferritin values only (five subjects).

It is well known that there are great difficulties in prevalence studies for genetic hemochromatosis in normal populations, because of the lack of simple and reliable tests to discriminate between heterozygotes, mildly affected homozygotes, alcohol abusers and even normal people. In the present study we did not consider as IH cases those subjects with high serum ferritin values and normal transferrin saturation values (probably secondary to alcohol abuse) neither subjects with abnormally high transferrin saturation and normal serum ferritin values. However, transferrin saturation may be raised in the absence of a significant increase in storage iron in the early stages of IH (19). That could be the case in some young subjects in this study who must therefore be followed-up to detect any increase in iron stores with time. If more of those cases are confirmed as IH in the future, then the real prevalence of the disease in this particular population may be even higher than initially estimated.

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PARTE II

ASPECTOS HEMATOLÓGICOS E IMUNOLÓGICOS:

Interacção entre o metabolismo do ferro e as células do sistema imunológico

CAPÍTULO 6

IDIOPATHIC HEMOCHROMATOSIS AS A WINDOW INTO THE STUDY OF THE IMMUNOLOGICAL SYSTEM IN MAN: CONFIRMED A NOVEL CORRELATION BETWEEN CD4/CD8 POPULATIONS AND IRON ABSORPTION

IDIOPATHIC HEMOCHROMATOSIS AS A WINDOW INTO THE STUDY OF THE IMMUNOLOGICAL SYSTEM IN MAN: CONFIRMED A NOVEL CORRELATION BETWEEN CD4/CD8 POPULATIONS AND IRON ABSORPTION

(submitted for publication)

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ABSTRACT

In the present study we report a serial investigation of the numbers of the peripheral blood cells: erythrocytes, polymorphonuclear neutrophils, total lymphocytes, T lymphocyte subpopulations (CD2, CD4, CD8), B lymphocytes and monocytes, in a group of 21 patients with hemochromatosis during the time of intensive phlebotomy treatment, i.e., from iron overload until the beginning of iron deficiency.

A remarkable individual stability of all blood cell populations studied was found in all patients. Patients differed in their relative proportions of CD4+ and CD8+. Each individual's CD4/CD8 ratio, as well as the absolute numbers, remained unaffected with time, confirming the existence of a strict homeostatic regulation of the relative numbers of the two major peripheral T lymphocytes. A significant positive correlation between CD4/CD8 ratios and the amount of iron mobilised by phlebotomy was found during this study. A novel correlation between the relative proportions of CD4+ and CD8+ cells and iron absorption is confirmed by the follow-up of iron re-entry in the serum transferrin pool in the treated patients.

INTRODUCTION

The demonstration of the existence of a strict regulation of the expansion of the two major thymus-derived populations has been the subject of considerable interest and extensive analysis in experimental animals (1-10). In man, models for the study of this question are understandably limited and have been generally confined to groups of patients with manifest immunological or inflammatory (11-14) diseases. Motivated by an interest in investigating a postulated physiological link between the immunological system and the regulation of the metabolism of iron (15,16), we have been studying patients with idiopathic hemochromatosis. Idiopathic or hereditary hemochromatosis (IH) is an HLA-linked autossomal recessive iron overload disease (17,18) characterised by a failure of the still unknown mechanism(s) regulating iron absorption. As a result of this failure, patients accumulate iron first in the transferrin pool and later in critical organs resulting in diverse pathologies of the liver (cirrhosis), pancreas (diabetes), joints (arthritis), heart (arrithmias), hypophysis (hypogonadism), etc. The treatment consists in the weekly removal of 400-500 cc of blood enabling the serial analysis of peripheral blood cell populations in single subjects.

In a recent preliminary report of the results of a serial study of peripheral blood T cell populations in IH patients, we presented evidence that individuals appear to have individually "set" normal or abnormally high CD4/CD8 ratios (19) that could be related to the iron entry into the transferrin pool. In the present paper we confirm the existence of a novel correlation between the relative numbers of CD4 and CD8 lymphocytes and iron absorption and illustrate further the existence of a remarkable individual stability of T lymphocyte populations, distinct from the greater variation seen in monocytes and B lymphocytes.

MATERIAL AND METHODS

Patients

A group of 21 patients (17 males and 4 females) aged from 23 to 68 years with the diagnosis of hemochromatosis were regularly followed-up during the whole intensive phlebotomy treatment. The diagnosis of hemochromatosis was based on previously described criteria (20) including transferrin saturation or serum ferritin values abnormally elevated in comparison to local reference values (20,21) and the

confirmatory histological examination of parenchymal-cell iron overload in a liver biopsy (22). Relevant patient data are summarized in Table 1. Eleven patients were

Table 1 - *Clinical data at the time of diagnosis, time of intensive treatment and amount of iron mobilised by phlebotomies (stores) in the patients*

Patient nº	Sex*	Age (yrs	Tansferrin Saturation (%)	Serum Ferritin (ng/ml)	Family history of IH	Signs or symptoms	Time of intensive treatment (months)	Stores (g)	CD4 / CD8 ratio
1	M	66	109	857	yes	yes	30	16.0	3.6
2 **	M	38	92	1500	yes	no	19	9.8	2.0
3	F	69	61	573	no	yes	5	1.8	2.2
4	F	40	44	748	no	yes	5	3.2	2.5
5	M	35	68	388	yes	no	7	4.3	1.5
6 a	M	51	100	3600	yes	yes	22	13.4	3.0
7	M	36	98	880	yes	no	18	8.7	3.3
8	F	53	90	288	yes	no	7	2.1	2.3
9 b	M	56	54	472	no	yes	4	2.5	0.8
10 b	M	43	87	1920	yes	no	12	9.0	1.4
11	M	39	100	526	yes	no	7	4.7	1.2
12	F	34	93	345	yes	no	9	4.5	1.5
13	M	23	80	432	yes	no	14	6.0	3.7
14 b	M	30	62	620	no	no	5	2.9	1.2
15 b	M	38	100	1560	yes	yes	34	17.4	3.1
16 c	M	42	53	953	no	yes	3	1.6	1.9
17 d	M	70	86	3459	no	yes	20	13.1	2.3
18 d	M	65	92	752	no	yes	3	2.0	2.5
19 d	M	43	57	527	no	no	6	3.7	4.0
20 d	M	50	51	412	no	yes	5	3.0	1.5
21 d	M	68	53	462	no	yes	3	1.6	0.8

* M=male F=Female
** Splenectomised

a . patient kindly refered to us by Dr. José Manuel Lopes
b . patients kindly refered to us by Dr. José Fraga
c . patient kindly refered to us by Dr. Jorge Mota
d . patients kindly refered to us by Dr. Guilherme Macedo

diagnosed on the basis of clinical signs or symptoms of iron overload (patients 1,3,4,6,9,15-18,20,21, Table 1), 3 were diagnosed as a result of a liver biopsy performed because of abnormally elevated liver enzymes (patients 10,14,19, Table 1), and 7 were asymptomatic and were diagnosed in the course of family studies (patients 2,5,7,8,11,12,13, Table 1). The latter started therapy at younger ages. Intensive

treatment consisted of a program of regular weekly phlebotomies until depletion of iron stores was achieved. Iron depletion was considered when there was a fall in haemoglobin levels with transferrin saturation or serum ferritin values lower than 10% and 10 ng/ml respectively. The final assessment of iron stores by quantitative phlebotomy (see below) confirmed an iron overload of more than 4g in 11 of the 21 patients studied (Table 1). All patients in this group (with more than 4g of iron in store) had a family history confirming the HLA linked transmission of IH, except one patient (patient 17 in Table 1) who had no HLA identical siblings. The latter case, however, had clinical manifestations of the disease (hepatomegaly, diabetes, hepatocarcinoma) and iron stores (13g) compatible with the diagnosis of homozygoty for idiopathic or hereditary hemochromatosis. All these 11 cases were thus confirmed as IH cases.

Controls

The data collected from a group of 19 regular (every 3-5 months) blood donors (15 males and 4 females aged between 20 and 58 years) from Santo António General Hospital Blood Bank were analysed retrospectively for the variations in red blood cell and total leukocyte counts over a period of two years of blood donation.

Hematological, Biochemical and Immunological Parameters

Hematological and biochemical parameters of iron metabolism were determined by standard techniques at regular intervals during phlebotomy treatment: whole blood cell counts were done in a Coulter JS automatic cell counter; serum iron (SI) and total iron binding capacity (TIBC) were determined in an Hitachi automatic analyser; transferrin saturation was calculated from the ratio of SI and TIBC; the serum ferritin determinations were done by micro plate immunoenzymatic assay (Abbott Diagnostics, Wiesbaden-Delkenheim). Peripheral blood mononuclear cell phenotyping was also performed in 11 patients at regular intervals during the period of intensive treatment by immunocytochemistry by the APAAP method (23) using monoclonal antibodies (from Dakoppats, Denmark) against CD2, CD4, CD8, CD19 and monocytes (not classified according to CD nomenclature). Based on the results previously obtained in a preliminary study of the CD4+ and CD8+ populations in IH patients (19), these were divided in two groups according to CD4/CD8 ratios: those

with ratios greater than 2.9 were considered to have "abnormally high ratios" and those with ratios equal or lower than 2.9 were considered normal.

Assessment of iron mobilised by phlebotomies and iron re-entry in the serum transferrin pool.

The amount of iron mobilised by phlebotomies was calculated at the end of intensive treatment from the total amount of haemoglobin removed by phlebotomies with a correction for the amount of iron absorbed during the treatment period, estimated as 3 mg daily (24). After the end of intensive treatment, iron re-entry was assessed by regular determinations of serum transferrin saturation and serum ferritin.

Statistical Methods

For purposes of statistical analysis the square root transformation was used for transferrin saturation and the logarithm transformation was used for serum ferritin. In each case the goal was to achieve an empirical distribution compatible with the normal (Gaussian) assumptions. The coefficients of variation were used to analyse the variability in cell counts. This calculation was done only when more than 3 determinations were available. The modifications of the different parameters with time were also analysed by linear regression analysis (25). Group means were compared by the Student's t test. Linear regression analysis was also used to define the relashionship between each pair of variables. The data were analysed using the Statgraphics - Statistical Graphics System - STSC.

RESULTS

1. Stability of Peripheral Blood Cell Counts

1.1 Group *versus* Individual Analysis

The average values, the range intervals and the coefficients of variation (c.v.) of the total red and white blood cells in individual patients and controls are shown in Table 2. The individual variation is evidenced by the c.v. calculated from all the values determined in each subject. The variation in a group is evidenced by the total c.v. calculated from the mean values of all the subjects in that group.

<u>Erythrocytes</u> (Table 2). The erythrocytes constituted a remarkably stable peripheral blood cell population showing individual coefficients of variation from 2%

Table 2 - *Variation in erythrocyte and leukocyte counts* ***in IH patients during intensive phlebotomy treatment and in controls*** *during a period of regular blood donations*

PATIENTS

			Erythrocytes ($10^6/mm^3$)				Leukocytes ($10^6/ml$)			
Nº	Time of study in months	n	Start value	Mean value	Min--Max	C.V.	Start value	Mean value	Min--Max	C.V.
1	30	85	3.9	3.8	2.9-5.5	8%	3.5	4.0	2.2-5.3	13%
2*	19	38	3.2	3.5	3.2-3.9	4%	12.8	9.8	8.0-12.8	13%
3	5	9	4.7	4.3	3.7-4.8	9%	3.8	4.2	3.8-4.6	7%
4	5	21	3.5	3.0	2.6-3.5	8%	4.1	4.1	3.3-5.1	11%
5	7	26	4.0	3.7	3.0-4.2	6%	2.0	3.5	2.0-4.6	6%
6	22	71	4.6	3.7	3.3-4.6	6%	5.6	6.3	3.2-9.2	18%
7	18	38	4.9	4.1	3.7-4.8	5%	6.9	5.3	4.0-7.7	16%
8	7	17	4.3	3.8	3.4-4.8	9%	4.2	3.6	2.1-5.3	20%
9	4	10	4.0	3.7	3.3-4.0	8%	3.8	4.0	3.3-5.3	14%
10	12	34	5.3	4.4	3.6-5.3	7%	5.3	4.2	2.3-6.7	19%
11	7	14	5.4	4.3	3.9-4.7	7%	6.4	5.8	4.6-7.0	13%
12	9	23	4.5	3.7	3.2-4.5	8%	6.3	6.8	5.1-9.3	19%
13	14	21	4.6	4.2	3.7-4.6	5%	6.4	7.0	4.2-10.2	21%
14	5	17	4.1	3.6	3.1-4.1	8%	7.9	7.9	6.3-9.8	13%
15	34	56	4.1	4.0	3.4-4.6	5%	5.3	5.4	3.8-6.8	12%
16	3	8	4.3	3.6	3.3-4.3	9%	6.0	5.4	3.8-6.4	18%
17	20	40	4.2	3.9	3.4-4.6	7%	4.2	3.5	2.5-4.8	16%
18	3	11	4.3	3.4	3.1-4.3	10%	5.5	5.0	4.4-5.5	7%
19	6	13	4.2	3.8	3.5-4.2	6%	8.1	5.9	4.7-8.1	16%
20	5	11	4.2	3.6	3.1-4.2	10%	6.2	5.1	3.8-6.2	14%
21	3	15	4.7	3.3	2.9-3.7	8%	4.4	3.9	2.8-5.0	18%
Mean			4.3**	3.8**			5.7**	5.1**		
Total range and c.v.					2.6-5.5	9%			2.0-12.8	25%

CONTROLS

			Erythrocytes ($10^6/mm^3$)				Leukocytes ($10^6/ml$)			
Nº	Time of study in months	n	Start value	Mean value	Min--Max	C.V.	Start value	Mean value	Min--Max	C.V.
1	13	5	5.2	4.7	4.5-5.2	7%	7.9	6.4	5.5-7.9	14%
2	14	5	5.1	4.8	4.7-5.1	4%	9.0	10.2	7.3-13.6	23%
3	25	5	4.3	4.5	4.3-4.8	4%	13.3	10.0	7.5-13.3	23%
4	22	5	5.0	4.8	4.6-5.1	5%	5.0	8.1	5.4-10.8	24%
5	24	5	4.3	4.4	4.3-4.6	3%	6.7	6.0	4.1-6.7	18%
6	19	5	4.4	4.7	4.4-5.0	6%	6.1	6.0	4.5-7.9	21%
7	23	5	3.9	4.1	3.9-4.2	2%	7.2	6.4	5.5-7.2	13%
8	26	5	4.6	4.5	4.3-4.6	2%	6.6	6.8	5.4-8.1	15%
9	23	7	4.9	5.0	4.9-5.2	2%	10.5	10.7	8.2-12.6	16%
10	21	6	4.7	4.8	4.5-5.0	4%	7.6	7.7	6.8-9.1	10%
11	30	5	4.6	4.8	4.6-4.8	2%	8.1	8.4	7.0-9.8	12%
12	24	5	4.3	4.6	4.3-4.8	5%	7.7	8.4	7.2-10.5	17%
13	25	6	5.0	5.0	4.6-5.1	5%	6.1	5.8	5.0-7.5	16%
14	26	7	4.7	4.7	4.3-4.9	5%	7.2	7.6	6.7-10.4	17%
15	24	6	4.7	4.9	4.5-5.5	8%	8.8	9.1	6.0-11.3	19%
16	25	5	4.3	4.2	3.7-4.5	7%	7.6	7.1	5.8-7.9	12%
17	17	5	5.2	5.2	5.1-5.3	2%	11.1	11.8	9.5-15.7	20%
18	26	5	4.9	4.7	4.5-4.8	3%	8.4	7.5	7.1-8.1	6%
19	25	5	4.4	4.4	4.0-4.8	6%	6.4	7.6	6.4-8.8	20%
Mean			4.7	4.7			8.0	8.0		
Total range and c.v.					3.7-5.5	6%			4.1-15.7	22%

n = number of determinations
c.v. = coefficient of variation
* = this patient was splenectomised and therefore was not included for the calculation of mean values
** = significantly different from controls ($p<0.002$)

to 8% in controls and from 4% to 10% in patients who were phlebotomised weekly until iron depletion. Furthermore, the variation amongst different subjects, as evidenced by the total coefficients of variation in the patient or control groups (9% and 6% respectively), were within the range of the individual variations (Table 2).

Total white blood cell counts (Table 2). The total leukocyte counts showed some variation. This variation, however, was more marked amongst different subjects in a group, both in patients and controls (with total coefficients of variation of 25% and 22% respectively), than in most individual subjects (in patients the individual c.v. varied from 6% to 21% and in controls from 6% to 24%). This was particularly evident in the patient group, that included 9 patients with mean leukocyte counts lower than $4.5 \times 10^9/l$ (patients 1,3,4,5,8, 9,10,17,21) contributing to the higher variation in this group. None of the patients showed an individual coefficient of variation greater than 21%, and most of them (11/21) had coefficients of variation of less than 15%.

Leukocyte subpopulations (Fig.1). Unfortunately, no data for the different leukocyte subpopulations were available in the controls. In the patients, however, analysis of the relative variability in the leukocyte subpopulations showed that the cell population with the greatest individual variation was the population of monocytes

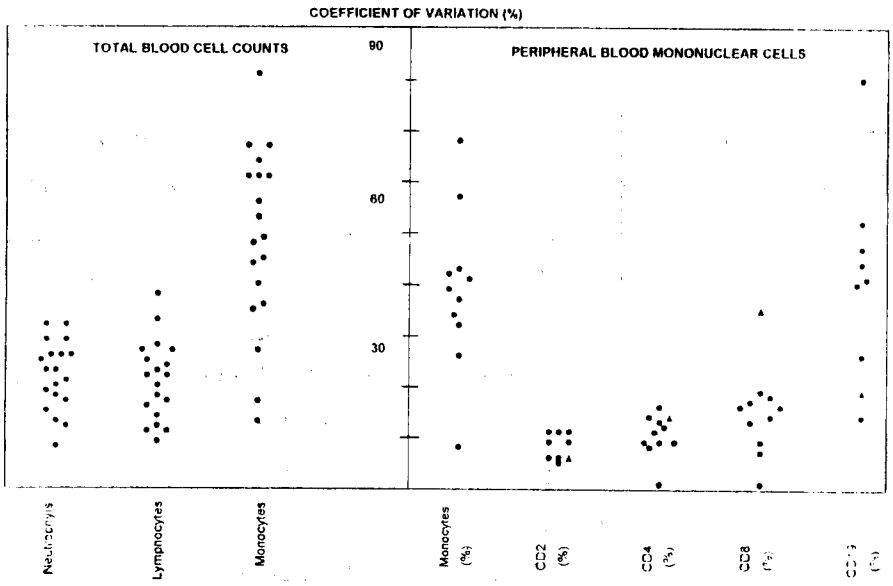


Fig.1 Coefficients of variation of the numbers of total blood cell counts (left pannel) and of the percentages of PBMC populations (%) in hemochromatosis patients during the time of intensive treatment. Each point represents one patient. The patient represented by a triangle in the right pannel is splenectomised.

(Fig.1). Here, in contrast with the other cell populations, there was a greater individual than group variation, i.e., the coefficients of variation in all except 3 of the 19 individuals analysed, were greater than 35% whereas the total coefficient of variation among the subjects in the group was 31%.

Lymphocyte sets (Fig.1). As shown also in Fig.1, a remarkable low variation was observed in the relative proportions of T lymphocyte subsets: the coefficients of variation of the percentages of CD2+, CD4+ and CD8+ cells were constantly lower than 20%. Only the splenectomised patient had a variation in the percentages of CD8+ cells of 35%. In contrast, the coefficients of variation of the percentages of CD19+ cells was greater than 40% in most of the patients (6/9).

1.2 Individual and Group Analysis *versus* Time

The modifications of the different parameters with time was further analysed by regression analysis of the serial determinations of each parameter against the time of treatment for each individual patient and also for the whole group. A decrease or increase of the parameters with time was only considered if there was a significant negative or positive slope of the regression line (at the 0.05 level of significance). If the slope was not significantly different from zero the parameter was considered stable. As shown in Fig. 2 the number of peripheral blood cells remained remarkably stable during treatment . This result was also observed when analysing each patient individually (data not shown). A stability of the overall number of lymphocytes during phlebotomy treatment was also observed for all the lymphocyte populations examined. As seen in Fig.3, there was no increase or decrease with time in the numbers of T (CD2+, CD4+ and CD8+) or B (CD19+) lymphocytes. In the present group of patients, however, two subgroups could be distinguished on the basis of the relative proportions of the T cell subsets as previously reported (19): 6 had abnormally high CD4/CD8 ratios (patients 1,6,7,13,15 and 19 in Table 1); the remaining 15 had CD4/CD8 ratios within the normal range. The CD4/CD8 ratios were maintained stable during treatment whether the patients had abnormally high or normal ratios at the beginning of treatment. The remarkable stability of the peripheral blood cell population numbers contrasted with the manifest significant ($p<0.0001$) decrease of transferrin saturation and serum ferritin with time and the progression of iron depletion (Figs 4,5) consequent to the weekly serial removal of large volumes of blood.

Fig.2 Stability of the peripheral blood cell populations in 21 hemochromatosis patients during repeated phlebotomy: for each cell population a regression was run between the serial individual determinations and the standardized time of intensive treatment. Time of intensive treatment was standardized by calculating at each date the ratio between the number of phlebotomies previously carried out and and the total number of phlebotomies required for reaching iron depletion. This variable varies thus from 0, corresponding to the begining of intensive treatment, to 1 corresponding to the end.

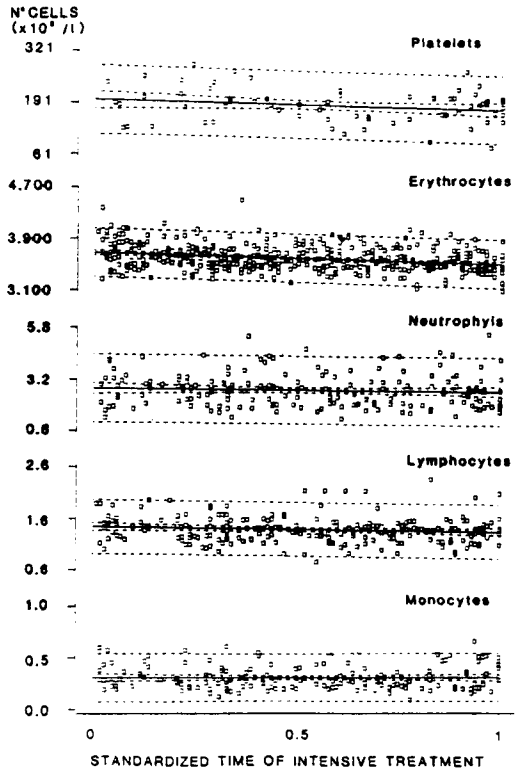
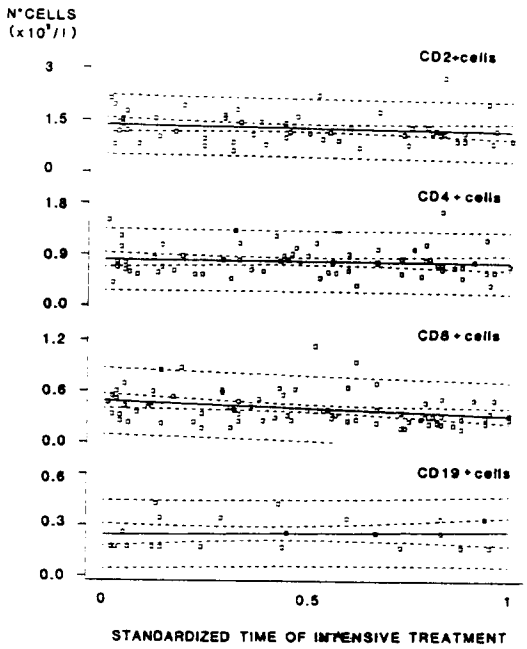
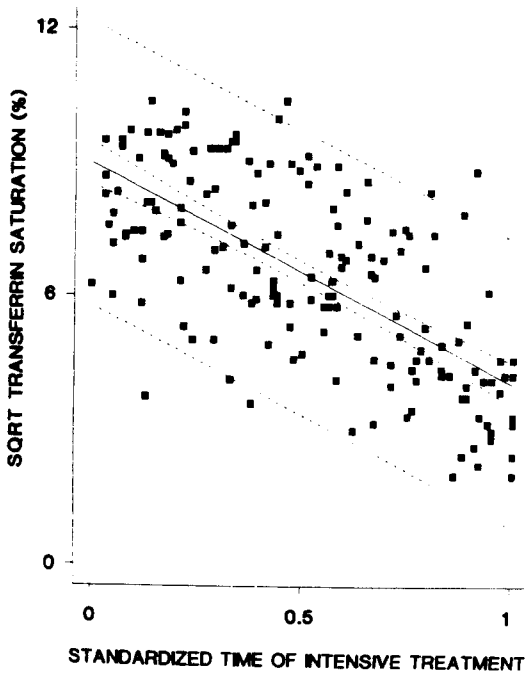


Fig.3 Stability of the different lymphocyte subpopulations in 11 hemochromatosis patients during repeated phlebotomy: for each lymphocyte subset a regression was run between the serial individual determinations and the standardized time of intensive treatment (see legend to Fig.2).





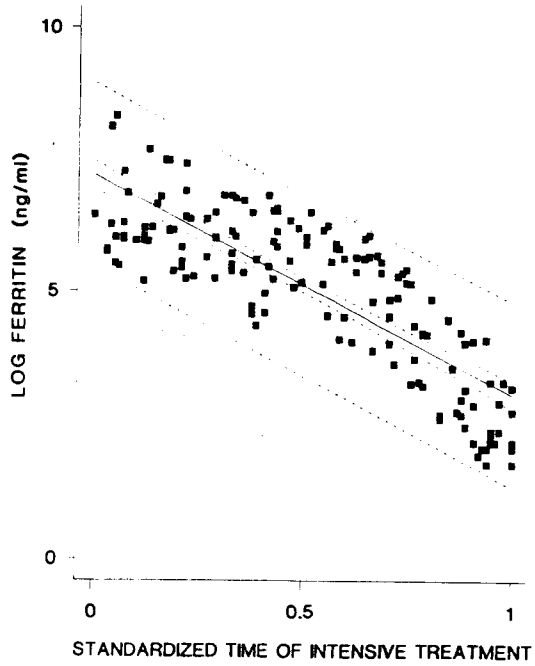


Fig.4 Therapeutic reduction of transferrin saturation in 21 hemochromatrosis patients: regression of the square root transformation of transferrin saturation values from serial individual determinations on the standardized time of intensive treatment (see legend to Fig.2).

Fig.5 Therapeutic reduction of serum ferritin in 21 hemochromatosis patients: regression of the log of serum ferritin values from serial individual determinations on the standardized time of intensive treatment (see legend to Fig.2).

2. Different CD4/CD8 ratio profiles relate to correction of the iron balance

In the course of the iron depletion process it was noted that patients with CD4/CD8 ratios higher than 2.9 required a longer course of phlebotomy treatment to achieve it than patients with CD4/CD8 ratios close to 1. The mean amount of iron mobilised by phlebotomies in patients with high CD4/CD8 ratios ($x=10.9\pm5.6$) was significantly higher ($p=0.004$) than in patients with normal ratios ($x=4.4\pm3.5$). This led us to focus our attention on the analysis of the possible correlation between the CD4/CD8 ratio profiles and iron stores evaluated by the amount of iron mobilised by

phlebotomies. Only three variables correlated significantly with the amount of iron mobilised by phlebotomies: transferrin saturation (r=0.69;p=0.0005), serum ferritin (r=0.67;p=0.0009) and the CD4/CD8 ratio (r=0.47;p=0.032). No correlation was found with any of the other cell populations tested, namely erythrocytes, neutrophils, lymphocytes, monocytes and platelets (data not shown). No correlation was found between the amount of mobilised iron and the age at diagnosis when the whole group of patients was analysed. If the analysis included only the group of IH patients who mobilised more than 4g of iron, a significant correlation between age and mobilised iron could be observed (r=0.63; p=0.04). After correction of the amount of iron mobilised for the effect of age in the latter group (considering as dependent variable the residuals of the correlation between mobilised iron and age) the correlation coefficient between this variable and the CD4/CD8 ratio increased to 0.64 (p=0.04).

3. The percentage of CD8+ but not CD4+ cells correlates significantly with the correction of iron balance (Fig. 6)

To further characterise the contribution of CD4+ or CD8+ cells to the correlation found between the CD4/CD8 ratios and the correction of iron balance, we analysed the correlation between the percentages of CD4+ or CD8+ cells and the amount of mobilised iron, after correction for the effect of age (Fig.6). The amount of iron

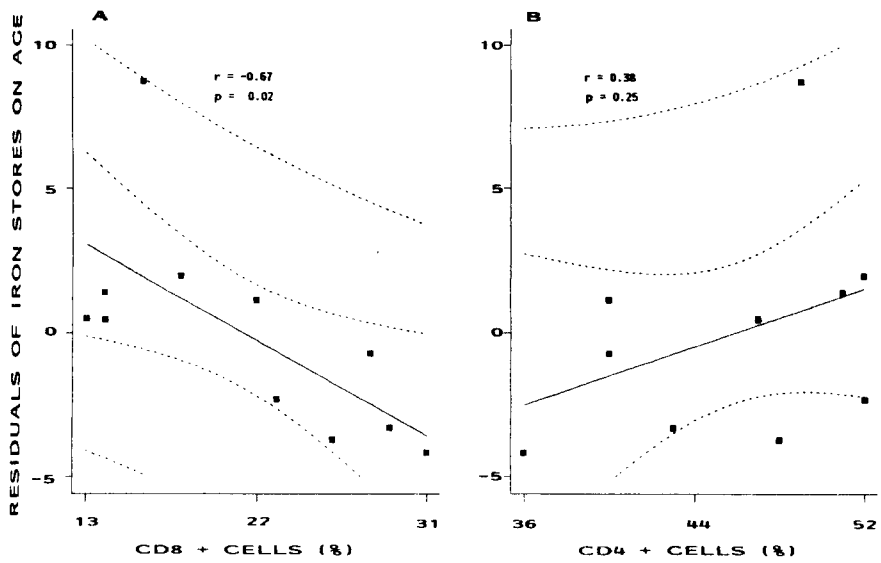


Fig.6 Correlation between iron stores and the relative proportions of CD4+ and CD8+ lymphocytes after correction for the effect of age: regression of the corrected iron stores (residuals of the linear regression of iron stores on age) on the percentages of CD4+ (A) and CD8+ (B) cells in 11 IH patients.

mobilised by phlebotomy correlated significantly with CD8+ cells (r= -0.67; p=0.02) but not with CD4+ cells (r=0.38;p=0.254). These findings enabled us to conclude that it is the percentage of CD8+ cells and not of CD4+ cells that is responsible for the observed statistically significant correlation of the CD4/CD8 ratio with the amount of iron mobilised by phlebotomies in IH patients.

4. CD4/CD8 ratios and reloading of the serum transferrin pool after correction of the excessive iron balance

To examine the possibility that one reason for the significant correlation between the protracted iron removal and high CD4/CD8 ratios was a higher rate of iron absorption, we followed serum ferritin levels and serum transferrin saturation in those patients with "high" or "normal" CD4/CD8 ratios during the first two years of maintenance phlebotomy, i.e. after iron depletion had been reached. Patients in maintenance therapy usually follow a program of one phlebotomy every 3-4 months. If a marked increase in transferrin saturation (to values greater than 60%) is consistently noted during the first year, then a program of one phlebotomy every 1-2 months is followed. This happened so far in all the patients with abnormally high CD4/CD8 ratios, not in the patients with normal ratios. As can be clearly seen in Fig.7, patients with high CD4/CD8 ratios (Fig.7.A) showed a sharp increase during the first year (slope=6.810; p=0.00000), after which the serum transferrin values remained stable, possibly as a result of the necessary therapeutic intervention. In contrast, patients with normal CD4/CD8 ratios (Fig 7.B) showed a significantly lower increase along the two years' period (slope=0.860; p=0.00034). Transient high values were found in one patient in the last group (patient 14 in Table1), at a time when he was not following the phlebotomy program proposed. After treatment was resumed, transferrin saturation values returned to normal.

DISCUSSION

The results of the present serial study of hemochromatosis patients during the course of phlebotomy treatment are of relevance to two aspects of the physiology of the immunological system in man: the homeostasis of the cells circulating in peripheral blood; and the interaction between the cells of the immune system and iron metabolism.

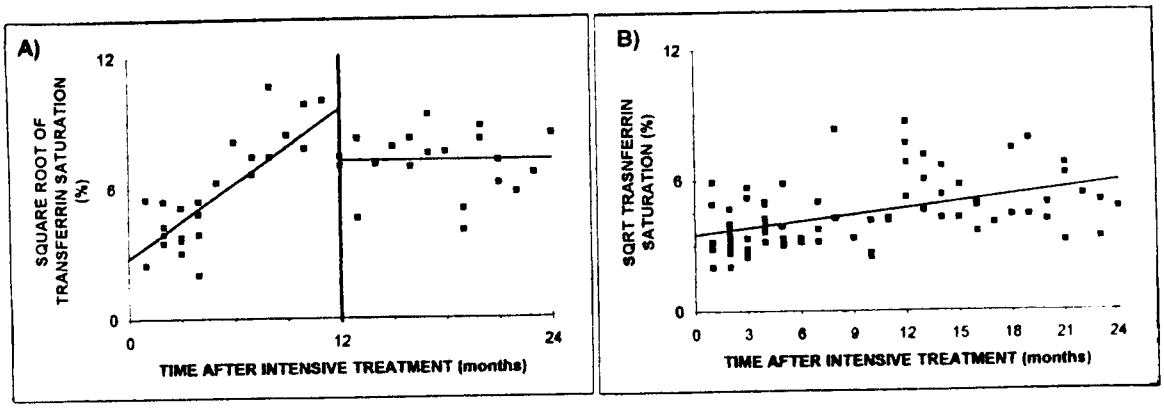


Fig.7 Increase in transferrin saturation in 21 hemochromatosis patients after the establishment of iron depletion by intensive treatment: regression of the square root transformation of transferrin saturation values from serial individual determinations on the time after intensive treatment was completed in patients with high(A) or normal(B) CD4/CD8 ratios. Each point represents one determination. The vertical line drawn at 12 months in pannel A serves as a reminder that, for ethical reasons, the number of maintenance phlebotomies in the second year had to be increased in order to control the rapid iron reentry into the transferrin pool seen in the patients with high CD4/CD8 ratios (pannel A). The resulting difference between the first and second year of maintenance treatment created the need to use two distinct regression equations. In patients with normal CD4/CD8 ratios (pannel B) the frequency of maintenance phlebotomies was not changed during the 24 months observation period and therefore the same regression equation was used throughout.

Homeostasis of the cells circulating in peripheral blood

The number of white blood cells circulating in human peripheral blood is thought to have great variability under physiological conditions (26). However, as first shown by Bernard Shaw in 1934, in the study of one subject for one year, an individual's range of leukocyte counts is not as great as that of a population, making the total leukocyte count an individual and not a group character (27). The present results, based on an analysis of 40 subjects along periods of time that varied from 3 months to 3 years, confirm the existence of some variability in white blood cell counts between different subjects and demonstrate, using for the first time antigen differentiation markers, a remarkable stability of all blood cell populations in each individual (Table 2). The lower average erythrocyte counts in patients during treatment in comparison to the starting values, shown in Table 2, are the result of the inclusion of values at the end of treatment when anemia was provoked. This also resulted in a higher range of erythrocyte values in some patients. In any individual patient, however, the slope of

the regression of erythrocyte counts with time was not significantly diferent from 0, denoting a remarkable stability of erythropoiesis along the treatment time. This stability of erythrocyte numbers is of particular relevance in view of the fact that during the time of study, patients were removing about 1/10 of their blood volumes every week, becoming depleted of iron at the end of the study. This is a further demonstration of the autonomy of erythropoiesis as a vital function dependent exclusively on iron stores, having acquired in evolution a degree of independence from possible variations in external environmental iron supplies (28). The distinction between the nature of a maintenance function turned to the internal environment, expressed by the erythrocyte counts, and a function directed to respond to external stimuli, represented by the leukocyte counts, is probably well documented in the different coefficients of variation observed in erythrocyte or leukocyte counts both in the control and the patient groups (Table 2).

Most importantly, however, the serial removal of blood in patients being treated for iron overload provided an unique opportunity of demonstrating the homeostasis of peripheral T cell pools in man. The relative proportions of both CD4+ and CD8+ T cell subsets showed a very low variability in contrast with the variations observed in B cells (CD19+) or monocytes.

A number of recent studies of peripheral T cell populations in experimental animals have consistently documented a remarkable stability of the relative expansion of the CD4+ and CD8+ populations (1-6). The present results demonstrate, to our knowledge for the first time, the existence of a similar homeostasis in man. Preliminary results of the TCR V chain usage in the patients is showing that the individual stability extends to the T cell repertoire (J M Cabeda, unpublished observations).

Perhaps the most novel contribution of the present study relates to the finding of a correlation between the relative expansion of the CD4+ and CD8+ populations and iron metabolism. The implications of this finding is discussed separately. The fact that Idiopathic Hemochromatosis is an autossomic recessive disease linked to MHC class I antigens supports the need of clarifying further the possible abnormalities in the regulation of the expansion of the CD8+ population in the context of the MHC.

Novel correlation of T cells with iron metabolism

The novel finding of a highly significant correlation of high or normal CD4/CD8 ratios with iron stores and iron absorption may have two distinct interpretations: 1.

the size of the CD8+ cell pool, or of some of its components, may itself contribute to the regulation of iron absorption. 2. The abnormally high mucosal uptake and transfer of iron seen in IH has as one of its principal consequences the regulation of the size of the CD8+ cell pool in the periphery. The fact that the CD8+ cell pool is largely associated with epithelial cells and that IH is an MHC class I linked disease supports indirectly both alternatives. The therapeutic decrease of iron stores or of circulating iron stores does not correct the high rate of iron entry at the intestinal level (29). The fact that with the progressive removal of iron stores there are no changes in the relative proportions of CD4+ and CD8+ cells in the blood favours the interpretation that the primary defect is of an immunological nature to do with the size of the CD8+ cell pool. The impossibility of obtaining serial intestinal biopsies in parallel with the weekly therapeutic phlebotomies weakens the clarification of that point.

The fact that iron re-entry occurs faster in the first year after completion of the intensive phlebotomy course in patients with high CD4/CD8 ratios strengthens the evidence for a definitive statistically significant correlation between the two systems and hints at a possible equally significant biological correlation. It does not herald the cause-effect direction of the correlation. The definitive evidence to decide whether iron entry regulates the size of the CD8+ pool or the size of the CD8+ pool regulates the signaling for iron entry must await similar studies in experimental animals. Independently of cause or effect, the present findings have considerable clinical bearing. The full clinical expression of IH is known to be heterogeneous. Sex and age are two major factors known to influence the accumulation of iron in tissues. A considerable heterogeneity is observed, however, in clinical presentation and in the development of iron overload in patients, independently of sex and age (30,31). This clinical experience has raised the question of the existence of other undefined factors that could influence iron absorption. We suggest that the present results place T lymphocyte populations among the players to be considered. A recent report of defective TNF-α production by mononuclear cells from IH patients (32) constitutes a separate piece of evidence favouring the consideration of the existence of an inextricable link between the immunological system and the regulation of iron metabolism, tentatively illustrated in Fig.8.

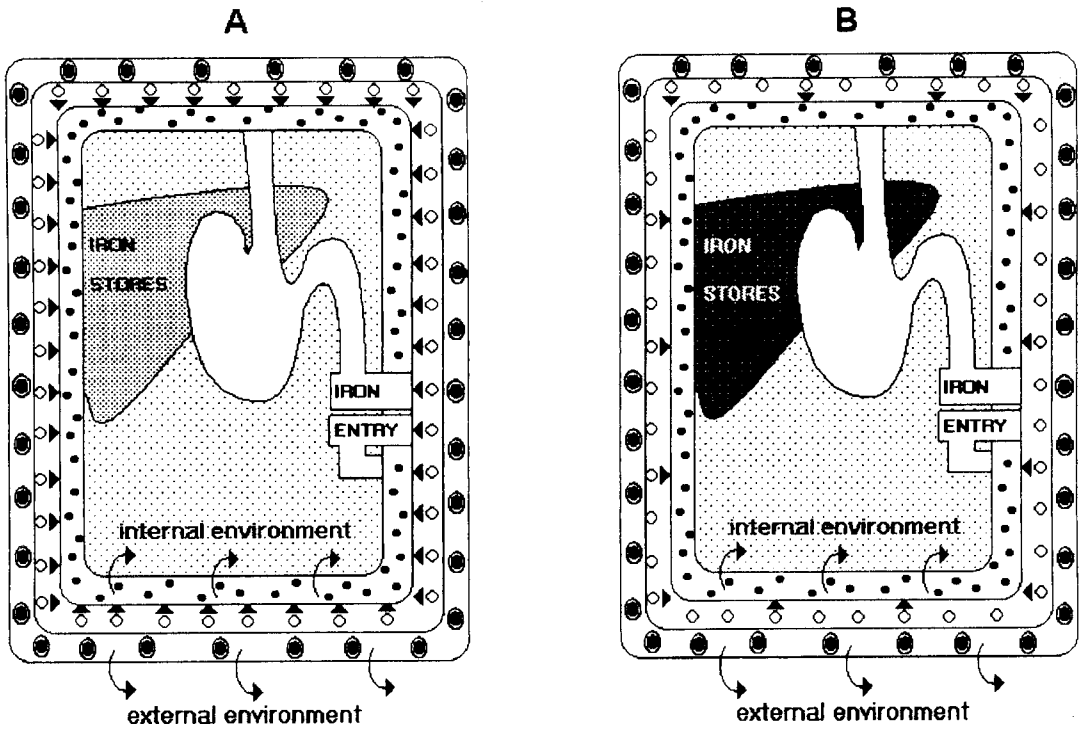


Fig 8. Diagramatic representation of the principal conclusions reached in the present study. Erythrocyte counts (•), even in the face of weekly removal of 1/10 of an individual's blood volume, remained stable. This stability might reflect the relative autonomy of erythropoiesis, a vital function which, as such, should remain independent from possible variations in the external environmental supply of iron. In contrast, marked fluctuations (with coefficients of variation greater than 35%) were seen in monocytes and B lymphocyte populations (represented by) as perhaps might be expected of cell populations with a key role in the response to external antigens. Surprisingly, with the exception of one splenectomised patient where CD8+ cell fluctuations were seen, the relative proportions of CD8+ (▼) and CD4+ (○) T cells manifest a great stability. This stability appears to extend to the Vß chain usage (J.M. Cabeda, unpublished observations). Its physiological significance remains unclear. In addition, distinct patterns of CD4/CD8 ratios were found that could be related to iron stores and rate of iron entry into the transferrin pool in IH patients: Patients with abnormally high CD4/CD8 ratios (panel B) have a higher iron entry and consequently increased iron stores than patients with CD4/CD8 ratios close to 1 (panel A). This finding may substantiate a postulated role for T lymphocytes in the regulation in the metabolism of iron (16).

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CAPÍTULO 7

RELATIVE IMPACT OF HLA PHENOTYPE AND T CELL SUBSET RATIOS IN PREDICTING IRON OVERLOAD

RELATIVE IMPACT OF HLA PHENOTYPE AND T CELL SUBSET RATIOS IN PREDICTING IRON OVERLOAD

(submitted for publication)

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ABSTRACT

Idiopathic Hemochromatosis is an hereditary iron overload disease linked to HLA. The clinical expression of hemochromatosis is known to be influenced by sex and age, but additional factors must account for the notorious heterogeneity of expression of this disease independently of sex, age and HLA phenotype. The present study attempts to clarify some of these additional factors, with basis on an exhaustive statistical analysis of data collected from 55 patients with hemochromatosis followed at the hemochromatosis outpatient clinic at the Porto Hospital Geral de Santo António. Thirty seven probands were refered to the centre from clinicians in other central or peripheral hospitals in the region from 1986 to 1992. Eighteen additional patients were identified by family studies. The statistical analysis focused on three groups of variables: the first group included variables reflecting the clinical expression of the disease; the second group represented the biochemical and hematological values at the time of diagnosis; the third group consisted of the independent variables sex, age, HLA phenotyope and the CD4/CD8 ratios. Independence between categorical variables was analysed by the Chi-square test with Yates's correction or the exact Fisher test if small numbers were present. Group means were compared by the Student's t test or by one way analysis of variance. Correlation between variables was analysed by linear regression. The relationship between a dependent continuous variable and several independent (continuous or categorical) variables was analysed by multiple linear regression with dummy variables. The results demonstrate that the relative expansion of the two main T cell subsets expressed by the CD4/CD8 ratios

combined with the HLA phenotype, constitutes a powerful additional factor predicting the clinical expression and progression of the disease. The present findings substantiate further the postulate that T cells have a role in the regulation of iron metabolism.

INTRODUCTION

It is becoming increasingly evident that the "clinical scene" of diagnosis of idiopathic haemochromatosis (IH) is changing (1). This change is in part the result of a greater awareness of the disease (2,3) and of a growing interest in iron associated pathology among clinicians (4,5) and, above all, the result of the existence of an established link between the disease and HLA (6,7). HLA phenotyping, by permitting the detection of cases through family screening has led to the identification of cases much before the classical clinical symptoms develop (8). To a certain extent, the latter development has exposed further the ignorance of the factors underlying the heterogeneity in presentation and progression of the disease (2).

We have recently found that IH patients can be subdivided in distinct groups differing in CD4/CD8 ratios with distinct responses to intensive phlebotomy treatment (9,10).

The present study consists of an extensive comparative analysis of the relative impact of factors known for sometime to influence presentation and course of the disease, namely, sex and age, with the CD4/CD8 ratios and HLA phenotype. The results substantiate the existence of a highly significant association between CD4/CD8 ratios, HLA phenotype and clinical progression of the disease.

MATERIAL AND METHODS

Patients

Fifty five patients (43 males and 12 females aged from 16 to 70 years) with the diagnosis of hemochromatosis who have been referred to the hemochromatosis outpatient clinic at the Porto Hospital Geral de Santo António, either for family studies or for treatment, were included in the present study. Inclusion criteria were a liver biopsy demonstrating parenchymal cell iron overload and biochemical evidence of iron overload, i.e., transferrin saturation and serum ferritin values abnormally high according to local reference values (11). Exclusion criteria were the presence of either hemolytic disease (even if associated with hereditary hemochromatosis) or any other iron loading anemias, porfiria cutanea tarda, or transfusional iron overload. History of

alcohol abuse (more than 60g/day for more than 5 years) or chronic liver disease associated with hepatitis B or C markers were not exclusion criteria for the purpose of this study. The availability of such a high number of patients from a relatively small area of the country represents a working clinical network between gastroenterologists at the Porto Central Hospitals (J Fraga and G Macedo) and gastroenterologists or clinicians from other specialities in central or peripheral hospitals. The number of patients, region and clinician involved in the recruitment are summarized in Table 1.

Table 1. *Number of patients from central and peripheral hospitals participating in study*

Nr. of Patients	Hospital Central	Hospital Peripheral	Speciality	Clinician
16	S. João - Porto		Gastroenterology	Fraga, J
8	St. António - Porto		Gastroenterology	Macedo, G
1	St. António - Porto		Internal Medicine	Mendonça, T
1	St. António - Porto		Internal Medicine	Correia, J
1	St. António - Porto		Neurology	Martins, A
4		Vale do Sousa	Gastroenterology	Soares, JM
2		V.Nova de Gaia	Internal Medicine	Fortuna, AM
1		Matosinhos	Gastroenterology	Mota, J
1		Braga	Hematology	Queirós, L
1		Famalicão	Internal Medicine	Garcia, JR
1	University-Coimbra		Internal Medicine	dos Santos, P

Thirty seven probands were referred to this Centre from 1986 to 1992: 18 presented with clinical manifestations of hemochromatosis; 16 were diagnosed by liver biopsy in the course of investigating a chronic liver disease, unexplained hepatomegaly or persistently elevated liver enzymes; and 3 were suspected by the casual finding of abnormal serum ferritin and transferrin saturation values. Family studies were systematically done in all first degree relatives of the probands with determination of transferrin saturation, serum ferritin and HLA typing as previously described (12). In family members with biochemical evidence of iron overload a confirmatory liver biopsy was proposed. From 18 newly identified family patients, 4 refused liver biopsy. Homozygoty for IH was assumed in these by HLA identity with the respective probands. In patients who had no HLA identical siblings, the diagnosis

of hereditary, HLA linked, hemochromatosis could neither be confirmed nor ruled out.

Clinical manifestations and biochemical data at the time of diagnosis were assessed by interview and/or a written questionaire completed by the referring physicians. Biochemical parameters and complete hematological counts were assessed in each visit by standart techniques. Additionally, since 1988, the CD4/CD8 ratios were systematically determined in all patients by immunocytochemistry and/or by FACS analysis (FACScan Becton & Dickinson) using monoclonal antibodies against CD4 and CD8 T cell antigens (Becton & Dickinson). According to previously published data from our laboratory, CD4/CD8 ratios higher than 2.9 were considered abnormal (9). Immunological phenotype was not available in 10 patients whose last visit had taken place more than four years ago. HLA phenotyping was done at the time of the family studies, or individually if the patient had no family members available for the study.

Of the 55 patients included: 4 died before completing treatment; 3 refused treatment; 18 were being treated at local centres by the referring physicians; and 30 were treated at this Centre. Of these, 21 have completed intensive treatment and are now in maintenance therapy. The intensive treatment consisted in the weekly removal of 400-500 ml of blood until iron depletion, that was considered complete at the first signs of iron deficiency, i.e., when there was a fall in hemoglobin levels with transferrin saturation and serum ferritin values lower than 10% and 10 ng/ml respectively. In these patients, mobilised iron stores were calculated from the total amount of hemoglobin removed by phlebotomies (taking the volume of blood and the hemoglobin concentration for calculation) with a correction for the amount of iron absorbed during the treatment period, estimated as 3 mg daily (13). In one patient this calculation was not done for he resumed intensive treatment before depletion of the iron stores. Data on iron stores from patients already treated in other centres were not included for it was not possible to follow the same rigorous criteria for their estimation.

Statistical Analysis

The statistical analysis of the data consisted in the identification of variables that could be related to the clinical or biochemical expression of the disease.

The analysis focused on three groups of variables which are listed in Table 2. The first

Table 2. *List of variables used in the statistical analysis*

I. Clinical expression of the disease

a. Levels of Clinical presentation:
1=asymptomatic; 2=liver disease; 3= Clinical hemochromatosis

b. Manifestations: diabetes, cirrhosis/fibrosis, skin pigmentation, cardiomiopathy, arthropathy

c. Iron stores (estimated by quantative phlebotomy)

II. Biochemical and hematological variables

Serum iron (SI), total iron binding capacity (TIBC), transferrin saturation, serum ferritin, hemoglobin, mean corpuscular volume, leucocyte count.

III. Factors influencing the clinical and laboratory variables

a. Sex and age
b. HLA phenotype: 1=nor A3 nor B7; 2=A3 or B7; 3=A3 and B7
c. CD4/CD8 ratio

group included variables reflecting the clinical expression of the disease, i.e., the clinical presentation at the time of diagnosis, the clinical manifestations of the disease and the iron stores calculated by the amount of iron mobilised by phlebotomy. Three levels were set for the variable "clinical presentation". Accordingly, each patient was assigned to one of these 3 levels: 1. no signs or symptoms of iron overload; 2. pre-fibrotic liver disease or liver cirrhosis or fibrosis with no diabetes, no hipogonadism nor severe arthropathy; 3. liver cirrhosis or fibrosis with two or more of the following manifestations: diabetes, gonadal failure or severe arthropathy. The second group of variables represented the biochemical and hematological values at the time of diagnosis: serum iron (SI), total iron binding capacity (TIBC), transferrin saturation, serum ferritin, hemoglobin, mean corpuscular volume (MCV) and total leukocyte count. The third group consisted of the independent variables analysed as possible factors influencing the clinical and laboratory variables, i.e.: sex, age, the CD4CD8 ratio and the HLA phenotype. The variable "HLA phenotype" divided the patients in three levels according to the expression of the antigens A3 and B7, known to be the only HLA antigens significantly associated with IH in this country (14,15): Level 1

includes patients who express neither A3 or B7, level 2 includes patients who express A3 or B7, and level 3 includes patients who express A3 and B7 in the same haplotype.

Because serum ferritin values show a lognormal distribution, the logaritmic transformation was used for the analysis of this variable. The other laboratory variables were not transformed. The variables sex and the clinical manifestations of the disease were categorical variables.

Independence between categorical variables was analysed by the Chi-square test with the Yates's correction. Whenever small numbers were present the exact Fisher test was used. Group means were compared by the Student's t test or by one way analysis of variance whenever two, or more than two, independent samples were being analysed, respectively. To study the linear relationship between continuous variables, correlation coefficients were estimated. To study the relationship between a dependent continuous variable and several independent (continuous or categorical) variables the multiple linear regression with dummy variables was performed (16).

All testing was performed at the level of significance of 0.05. Data were analysed using the Statgraphics- Statistic Graphics System - STSC.

RESULTS

1. Definition of clinical groups at diagnosis

Twenty one patients (38%) were asymptomatic at diagnosis. Most, but not all, of these (81%) were diagnosed in the course of family studies. Of the symptomatic patients, 19 (35%) presented with a clinical picture of hemochromatosis and 15 patients (27%) were diagnosed in the course of investigating a chronic liver disease (with or without cirrhosis or fibrosis) or persistently elevated liver enzymes, with no other manifestations that could call the doctor's attention for the diagnosis of hemochromatosis. History of alcohol abuse was present in 47% of the clinical hemochromatosis patients and in 60% of the patients with liver disease only. Within each group no significant differences in the clinical and biochemical manifestations were observed between patients with or without history of alcohol abuse (data not shown). In addition to history of alcohol abuse, 2 patients were also seropositive for the B hepatitis virus antigen and 2 had circulating anti-C hepatitis virus antibodies.

The clinical manifestations of patients at diagnosis are illustrated in Fig.1 in which the present results are compared with those from one other recent published series (1). No significant differences were found between the two series. The most frequent presenting symptoms were arthralgia (33%), weakness (29%) and abdominal pain

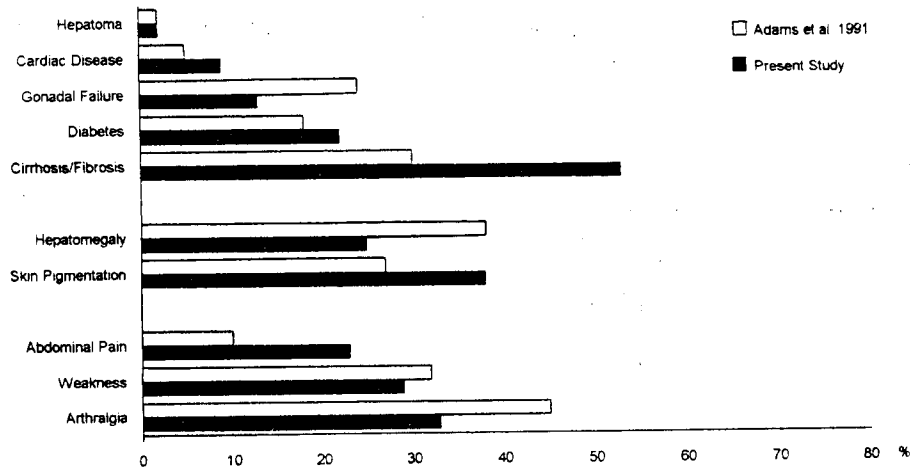

Fig .1 Clinical manifestations of hemochromatosis patients in the study in comparison with a recently published series from Adams et al (1). :n=93 n=55

(23%). The predominant physical findings were skin pigmentation (38%) and hepatomegaly (25%). Ascites was present in 5 patients (9%). Cirrhosis or fibrosis was present in 53% of the patients; diabetes in 22%; gonadal failure in 13%; cardiac disease in 9%; one patient had hepatoma.

The laboratory findings at diagnosis of the patients grouped according to the clinical presentation are summarized in Table 3. Patients with clinical hemochromatosis had significantly higher iron stores (mean=15g; range=13.4-17.4) than the other two groups ($p<0.0001$). Iron stores in patients with liver disease only (mean=3.1g; range=1.6-9.0), were in the same range as those of asymptomatic patients (mean=4.9g; range=2.1-8.7). Patients with clinical hemochromatosis also had significantly higher serum ferritin values, although, in the whole group, serum ferritin values were not correlated with the levels of iron stores. Clinical hemochromatosis

Table 3. *Laboratory findings at diagnosis in patient subgroups according to clinical mpresentation[a] and significance of the differences between groups[b]*

	Patient Subgroup			
	1 (n=21)	2 (n=15)	3 (n=19)	p value
Male/Female	13/8	13/2	17/2	0.07
Age (Years)	35±11	50±12	49±11	0.0001
SI (μg/dl)	187±24	199±64	191±31	n.s.
TIBC (μg/dl)	230±43	263±55	220±37	0.0119
Transferrin Saturation %)	83±15	76±25	89±16	n.s.
Ferritin (g/dl)	510±588	977±757	1584±1108	0.0000
Hemoglobin (g/dl)	14.5±1.1	14.3±1.7	13.0±1.5	0.0011
Mean Corpuscular Volume(μ^3)	96±5	99±6	98±5	n.s.
Leukocytes($x10^9$/l)	6.0±1.4	5.9±1.8	5.7±1.7	n.s.
Iron Stores (g)	4.9±2.1	3.1±2.3	15.0±2.1	0.0000

a. See table 2
b. analysed by Chi-square (Male/Female) and ANOVA (all the other variables)
c. arithmetic means (±SD) for all variables except ferritin (geometric mean)
ns. not significant

patients had significantly lower TIBC and hemoglobin values. High MCV values were almost constantly seen in hemochromatosis patients at diagnosis, 39% of the patients having values higher than $100\mu^3$. Although asymptomatic patients seemed to have lower MCV values, the difference was not significant. The leukocyte counts showed some interindividual variation, as previously described (9); 20% of the patients had counts lower than $4.5x10^9$/l. No correlation was observed, however, between leukocyte counts and any of the clinical or laboratory variables tested.

2. Distribution according to sex and age

Sex

As expected, the male/female ratio (M/F) in the present group of patients was significantly different (p=0.04) when comparing probands (M/F=32/5) with family members (M/F=11/7). Interestingly, the relative frequency of probands and family

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members did not follow exactly the relative frequency of symptomatic and asymptomatic patients: 3 probands who were diagnosed by chance in the course of investigating an incidental illness were asymptomatic; one family member was already symptomatic at the time of the screening. The ratio between symptomatic and asymptomatic patients (S/A) was significantly different (p=0.02) between males (S/A=30/13) and females (S/A=4/8). No significant differences were observed in transferrin saturation values between males (mean=83%) and females (mean=82%) nor in any of the other laboratory parameters except serum ferritin that was higher in males (geometric mean=1266 ng/ml) than in females (geometric mean=949 ng/ml). This difference was statistically significant (p=0.035). Although markedly lower iron stores were found in females (mean=2.9g) than in males (mean=6.9g), this difference was not statistically significant.

Age

The age distribution of males and females according to the clinical presentation is shown in Fig.2. Given the low frequency of symptomatic females, it is not possible to compare the age distribution between males and females in the symptomatic groups. In the group of asymptomatic patients no significant difference could be seen in age distribution between the two genders (Fig.2). For this reason, further analysis of the effect of age on the other variables was done including all patients.

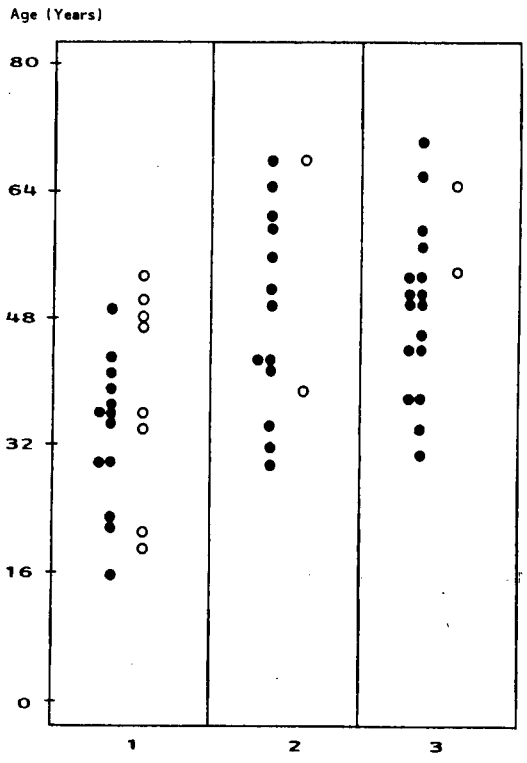

Fig.2 Age distribution of male (●) and female (o) hemochromatosis patients according to the clinical presentation: 1=no signs or symptoms of disease; 2=liver disease only; 3=clinical picture of hemochromatosis

As shown in Table 3, patients differed significantly (p=0.0001) in age according to the mode of presentation. The mean age in symptomatic patients was similar whether they presented with clinical hemochromatosis (mean=49 years) or not (mean=50 years) but was lower in asymptomatic patients (mean=35 years). The only manifestations that were significantly more common in older (>40 years) than in younger (<40 years) patients were cirrhosis (p=0.008) and skin pigmentation (p=0.028). The other manifestations could not be related to age. The influence of age on the laboratory parameters was analysed by simple regression analysis. Age was significantly correlated with serum ferritin (p=0.009) with the low correlation coefficient of 0.36. No other variable was found to be correlated with age.

3. CD4/CD8 ratios in groups of symptomatic patients

The influence of CD4/CD8 ratios on the clinical and biochemical expression of hemochromatosis was first assessed by analysing symptomatic patients only. The reason for this selection was primarily to eliminate the major factor influencing disease expression, i.e., early diagnosis by family studies. The variables tested are summarized in Table 2. As shown in Table 4, the frequency of abnormally high CD4/CD8 ratios (≥2.9) was significantly higher in patients with clinical hemochromatosis than in patients with liver disease only. By simple regression analysis a significant correlation was found between CD4/CD8 ratios and both iron stores (r=0.70; p=0.008) and transferrin saturation (r=0.41; p=0.028). None of the other variables tested was correlated with the CD4/CD8 ratios.

4. HLA phenotype in groups of symptomatic patients

To avoid bias of certain HLA haplotypes found in large families (for instance, one family had 5 HLA identical siblings, one proband and 4 asymptomatic) the family cases were excluded of this analysis. In general HLA phenotype was significantly correlated with the clinical and biochemical expression of the disease. As also shown in Table 4, the frequency of clinical hemochromatosis was significantly higher in patients who had both antigens A3 and B7. The mean iron stores as well as the mean serum ferritin values analysed by one way analysis of variance showed significant differences according to the HLA phenotype (p=0.017 and p=0.018 respectively): patients with both A3 and B7 had higher values than patients with only one of these antigens; the latter patients had higher values than patients who had neither the A3

nor the B7 antigens. No other variables were related to HLA including the CD4/CD8 ratios.

Table 4. *Summary of statistical analysis to test the significance of the differences[a] found in groups with different levels of clinical presentation, HLA phenotype and CD4/CD8 ratio.*

Categorical variable	Level	Level of clinical presentation 2	3	Stores (g)	Ferritin (ng/ml)	Transferrin Saturation (%)
HLA phenotype	**1**	6	4	2.5±0.6[c]	827±778	73±25
	2	6	5	8.6±6.3	1236±958	86±21
	3	0	9	15.3±3.0	1940±1091	87±18
		(p_c=0.013)		(p=0.017)	(p=0.018)	n.s.
CD4/CD8 ratio	**≥ 2.9**	1	9	10.9±5.6	1249±1010	91±21
	< 2.9	13	5	4.0±3.3	745±935	79±20
		(p_c=0.006)		(p=0.0027)	n.s.	n.s.

a. analysed by Chi-square (pc) and ANOVA (p)
b. see text and Table 2
C. arithmetic means (±SD) for all variables except ferritin (geometric mean)
n.s. not significant

5. Iron stores as a function of HLA and CD4/CD8 ratio in all clinical groups

As described, iron stores, measured by quantitative phlebotomy, correlated not only with presentation at diagnosis, but also with the CD4/CD8 ratios and the HLA phenotype in the clinical groups 2 and 3. To establish a predictive model for iron stores depending on the other variables and to establish the relative impact of each of the variables in all cases, i.e., symptomatic and asymptomatic, a multiple linear regression with dummy variables was run for the data from the 20 phlebotomized patients taking as dependent variable the iron stores and as independent variables: sex, age, the clinical presentation, the CD4/CD8 ratio and the HLA phenotype. The final regression equation obtained was:

$$\text{Iron stores} = 9.536 + 3.117\ CD4/CD8 - 8.523\ HLA_a - 5.936\ HLA_b - 2.409\ S1\ CD4/CD8 - 2.35\ S2\ CD4/CD8$$

HLA_a indicates the absence of the antigens A3 and B7, HLA_b indicates the presence of only one of the antigens A3 or B7, S1 indicates the absence of symptoms and S2 indicates the presence of liver disease with no clinical picture of hemochromatosis. The R-squared for the full regression was 0.816. The global significance level was 0.0001 and the significance levels for the regression coefficients were 0.0004 for the CD4/CD8 ratio, 0.0007 for HLA_a, 0.0073 for HLA_b, 0.0005 for S1CD4/CD8 and 0.0075 for S2CD4/CD8. As already indicated by the equation, the positive linear relationship between iron stores and the CD4/CD8 ratio is modified by the HLA phenotype and the clinical presentation at diagnosis: the absence of one or the two HLA antigens A3 and B7 (HLA_b and HLA_a respectively) decreases the intercept of the regression line between iron stores and the CD4/CD8 ratios, whereas the absence of symptoms or of the clinical picture of hemochromatosis (S1 and S2 respectively) decreases the slope of the regression. To illustrate better the relative impact of HLA and the clinical presentation on the relationship between CD4/CD8 ratios and iron stores we calculated, from the general equation, 6 representative linear regression models according to different HLA phenotypes and clinical presentation. These models (illustrated in Fig. 3) provide a clear representation of the relative impact of HLA phenotype and the CD4/CD8 ratios in predicting iron overload.

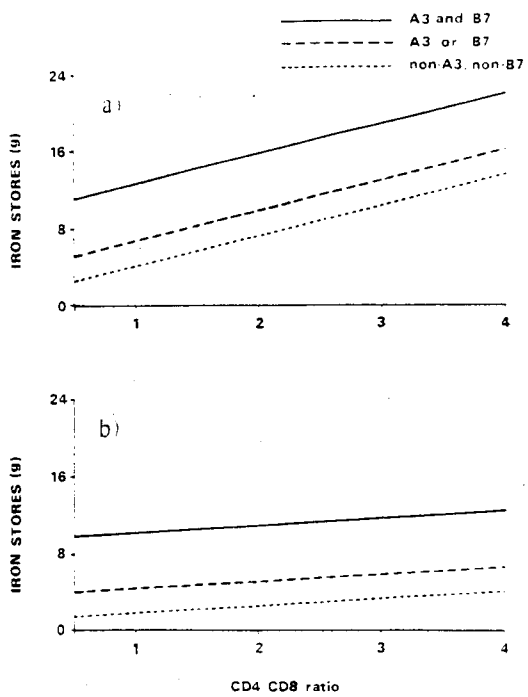

Fig.3 Predicted iron stores in hemochromatosis patients as a function of their CD4/CD8 ratios and depending on the HLA phenotype. The regression line models were calculated from the general equation obtained by multiple regression analysis with dummy variables (see text). The models represented here correspond to the ones obtained in patients with the clinical picture of hemochromatosis (a) and in asymptomatic patients (b).

DISCUSSION

It is now well accepted that the clinical scene of hemochroma-

tosis is changing (1). Less severe or even asymptomatic forms of the disease are increasingly more frequent making difficult the differential diagnosis between the primary and some secondary forms of iron overload like, for instance, alcoholic liver disease. It is becoming also evident that excess iron in a clinical setting can be implicated in the co-pathogenesis of disease processes beyond genetic hemochromatosis. These include malignant transformation (17-21), heart disease (5,22), antigenicity in microorganisms (23-26), to mentione a few. Consequently, the identification of factors contributing to the regulation of iron metabolism is acquiring increasing importance.

Work from this group has developed for some time round the postulate that the immunological system could have as one major task the surveillance of potentially toxic products of the circulation of the blood, in particular iron, and thus contribute to the regulation of iron metabolism (27-29). It is now well documented that cytokines such as Tumor Necrosis Factor (TNF) have as one of their biological effects the decrease of serum iron levels (30). An earlier piece of work from Gordeuk et al. has demonstrated that peripheral blood mononuclear cells from HH patients have a defective TNF-α production in vitro (31). That finding is attractive for two reasons: 1. a failure of TNF-α production could contribute to the patients' inability to regulate serum iron levels; 2. the TNF-α gene has been located in chromosome 6. No link to HLA phenotype was reported in that study. In a previous serial study of patients undergoing phlebotomy treatment we demonstrated that some patients consistently had abnormally high CD4/CD8 ratios which could be related to the response to iron removal and iron reloading after treatment, suggesting a relashionship between the relative expansion of the two main T cell subsets and iron absorption (9,10). The present findings demonstrate that, in addition, differences in HLA phenotype combined with the relative expansion of the two main T cell subsets influence the severity of iron overload. This combination can now be used as predictive of the clinical expression of the disease. As previously discussed by Simon (32), an association between HLA type and iron overload states is not merely of theoretical interest because accurate identification of susceptible individuals may be important for preventing not only primary but also secondary forms of iron overload. So far, studies of the prevalence of the hemochromatosis associated-HLA alleles in secondary forms of iron overload have been inconclusive for the question of the implication of the IH gene in those situations. The existence of a discrepancy between the theoretical frequency of the IH gene in the general population and in some secondary iron

overload states has led to the speculation that an "increased absorption allele of weak expression and high prevalence could exist beside the hemochromatosis allele and perhaps at the same locus" (32). A putative abnormality of CD8+ cells to expand in patients with hemochromatosis is an additional plausible regulatory factor of iron metabolism, taking in consideration the additional established fact that the expansion of this subset of T cells occurs in the context of MHC class I antigens (33,34).

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CAPÍTULO 8

DISCUSSÃO GERAL E CONCLUSÕES

O METABOLISMO DO FERRO NA PERSPECTIVA CLÍNICA ACTUAL

O ferro participa em reacções bioquímicas fundamentais do metabolismo celular, nomeadamente em reacções de oxidação-redução (*Da Silva & Williams, 1991*), e por isso adquiriu nos organismos superiores um papel-chave em funções biológicas vitais, tais como no transporte de electrões na cadeia respiratória, como cofactor enzimático e na distribuição de oxigénio aos tecidos. Paradoxalmente, é um catalizador da produção de radicais livres de oxigénio que podem induzir a peroxidação lipídica com consequente injúria tecidular, sendo por isso um tóxico potencial (*Bacon et al, 1988; Bacon & Britton, 1990*). O ferro existe normalmente nos tecidos apenas ligado a proteínas e, portanto, numa forma não tóxica (*Fairbanks & Beutler, 1991*). No entanto, em doentes com sobrecarga de ferro, nos quais a capacidade de fixação pela transferrina é excedida, pode-se encontrar ferro no plasma sob a forma de complexos de baixo peso molecular, com capacidade para acelerar a formação de radicais livres (*Grootveld et al, 1989*). As consequências clínicas do excesso de ferro em doentes com hemocromatose são hoje bem conhecidas (*Niederau et al, 1985; Basset et al, 1986*). Embora não esteja formalmente demonstrado nesses doentes qual o mecanismo responsável pela toxicidade, há já evidência, em modelos experimentais de sobrecarga de ferro, que a toxicidade resulta da peroxidação lipídica "in vivo" (*Bacon et al, 1988; Bacon & Britton, 1990*).

A importância dada, na prática clínica geral, às duas faces opostas da biologia do ferro i.e., como nutriente essencial ou como tóxico, é cronològicamente desfasada. Nos anos 60 e 70 dominou a preocupação pelo deficiente aporte nutricional de ferro quer em países em desenvolvimento quer em países desenvolvidos, como os Estados Unidos e a Suécia, tendo-se assistido à generalização de programas de fortificação dos alimentos com ferro (*Cook et al, 1976*). Essa atitude é hoje controversa porque está demonstrado que a absorção de ferro fornecido em concentrações elevadas, numa forma altamente biodisponível, pode exceder a quantidade necessária e tornar-se prejudicial (*Fairbanks & Beutler, 1991*). Já no final dos anos 80 era manifesta a preocupação pelos efeitos adversos que a suplementação indiscriminada da dieta com ferro podia ter nos indivíduos heterozigóticos para o gene da hemocromatose (*Olsson et al, 1988; Lynch et al,1989; Nichols & Bacon,1989*), que se sabe poderem ter uma desregulação da absorção do ferro (*Lynch et al, 1989*). A controvérsia gerada à volta dos perigos da suplementação alimentar ganha hoje ainda mais relevo perante a evidência que a sobrecarga de ferro pode estar implicada no prognóstico de doenças

malignas (*De Sousa & Potaznik, 1984; Potaznik et al, 1987; Hann et al,1990)* e até associada a um maior risco de desenvolvimento de neoplasias (*Stevens et al, 1988; Fraquelli et al, 1992*) e de enfarte de miocárdio (*Sullivan, 1989; Salonen et al, 1992*). Neste contexto importa cada vez mais investigar os mecanismos, ainda desconhecidos, de regulação da absorção e acumulação de ferro no organismo.

A HEMOCROMATOSE COMO MODELO DE ESTUDO DA INTERACÇÃO ENTRE O METABOLISMO DO FERRO E O SISTEMA IMUNOLÓGICO

O trabalho presente foi desenvolvido com base no postulado que o sistema imunológico pode contribuir para a regulação do metabolismo do ferro (*De Sousa, 1978, 1981, 1989; De Sousa et al, 1982, 1988, 1991*). A escolha da hemocromatose idiopática como modelo de estudo deveu-se a: (1) existir na HI um defeito primário de absorção do ferro; (2) não se associar a factores que interfiram no sistema imunológico, tais como a transfusão sanguínea ou a esplenectomia, ambos presentes em modelos de hemocromatose secundária; (3) e ser uma doença associada ao MHC (*Simon et al, 1977, 1987; Cartwright et al, 1979; Jouanolle et al, 1990*). Às vantagens teóricas deste modelo juntou-se ainda a antecipação dos benefícios na saúde pública que um estudo deste tipo teria, ao passar necessariamente por um trabalho de despiste da doença na população conduzindo ao diagnóstico e tratamento precoces com consequente prevenção das manifestações clínicas da doença. Para obter o material clínico necessário ao estudo foi, assim, desenvolvido um programa de despiste sistemático da doença, quer por meio de estudos familiares quer por estudos populacionais, para os quais tivemos necessidade de definir valores de referência locais para os parâmetros do metabolismo do ferro (*Capítulos 3, 4 e 5*). Os doentes identificados foram tratados por meio de flebotomias intensivas (semanais) e a sua vigilância regular permitiu-nos obter dados seriados sobre a evolução dos parâmetros hematológicos, bioquímicos e imunológicos ao longo do tratamento (*Capítulos 6 e 7*). Os resultados obtidos serão discutidos focando três aspectos que constituem contribuições novas para o conhecimento da hemocromatose e para a compreensão de alguns mecanismos ainda não esclarecidos do metabolismo do ferro: 1. A prevalência elevada da hemocromatose no norte de Portugal e a importância de definir valores de referência locais para o metabolismo do ferro. 2. A demonstração da existência de uma grande estabilidade das populações de células circulantes no sangue periférico,

em particular dos linfócitos T, e a sua relação com a absorção de ferro. 3. A associação do fenótipo HLA com o desenvolvimento de sobrecarga de ferro. No decorrer deste estudo verificámos uma elevada frequência de leucopenias constitucionais e macrocitose associada à doença (*Capítulo 6*), ambas ocorrendo independentemente de possíveis anomalias na função hepática. Nenhuma correlação foi encontrada entre essas alterações e os outros parâmetros clínicos, hematológicos e imunológicos descritos. Estes achados estão actualmente a ser objecto de investigação não sendo por isso discutidos neste trabalho.

O GENE DA HEMOCROMATOSE EM PORTUGAL E VALORES DA FERRITINA SÉRICA NA POPULAÇÃO

A hemocromatose idiopática, apesar de autossómica recessiva, é uma das doenças genéticas mais comuns na população caucasiana (*Motulsky, 1979*) com frequências de heterozigóticos estimadas entre 8 e 13% em várias populações (*Beaumont et al, 1979; Olsson et al, 1984; Karlsson et al, 1988; Edwards et al, 1988; Leggett et al, 1990; Velati et al, 1990; Wiggers et al, 1991*). Neste estudo foi encontrada uma frequência da doença superior a qualquer outro estudo anteriormente publicado. Com base na tipagem HLA de 136 membros familiares de 15 doentes com hemocromatose calculamos na população do norte de Portugal uma frequência génica de 14%, correspondente a frequências de homozigóticos e heterozigóticos de 1.9% e 24% respectivamente. Em estudo posterior no qual se incluiram 385 membros familiares de 30 doentes na mesma região os resultados foram sobreponíveis (*Porto, dados não publicados*). Esta alta prevalência estimada com base em estudos familiares com tipagem HLA, embora surpreendente, foi substanciada por estudos de despiste da doença em indivíduos normais de populações restritas no norte de Portugal, como por exemplo na população de Refoios-Ponte de Lima, onde numa amostra de 112 indivíduos identificámos 6 com sobrecarga de ferro, num dos quais já se confirmou o diagnóstico de HI.

A razão para a alta prevalência do gene da hemocromatose não é clara, tendo-se já especulado a hipótese de existir uma vantagem selectiva do gene que, durante a evolução humana, teria funcionado como um alelo de "suplemento nutricional", permitindo aos portadores, nomeadamente às mulheres em idade reprodutiva, extrair mais ferro de uma dieta naturalmente pobre (*Cartwright et al, 1979; Rotter &*

Diamond, 1987; Kushner et al, 1988; Cox & Lord, 1989). Esta hipótese, no entanto, nunca foi demonstrada. Por outro lado, está descrito que na evolução humana o efeito fundador pode, pelo menos temporariamente, conduzir ao aparecimento de populações com frequências muito elevadas de certos traços genéticos que podem ser, em termos de selecção natural, não necessariamente vantajosos mas apenas neutros (*Diamond & Rotter, 1987*). Uma vez que não se conhece para o gene da hemocromatose a existência de uma forte pressão selectiva (como é o caso, por exemplo, do gene da drepanocitose em regiões onde a malária é endémica), é possível que o efeito fundador e a deriva genética tenham tido um papel importante na evolução do gene da hemocromatose (*Diamond & Rotter, 1987; Livingstone, 1992*) explicando assim a sua alta prevalência em certas regiões, como a que foi encontrada na população do norte de Portugal no presente trabalho. De acordo com essa hipótese, Simon e colaboradores propuzeram, com base num estudo de 609 haplótipos HLA ligados à hemocromatose, que o gene terá sido originado por uma mutação rara, se não única, que produziu um marcador ancestral (o haplótipo HLA:A3B7) que foi posteriormente espalhado geograficamente por migração das populações e possivelmente modificado por recombinações (*Simon et al, 1987*). A mutação única, no entanto, não explica a alta frequência e distribuição mundial do gene, a não ser que haja uma forte pressão selectiva, pois o risco de extinção de uma mutação única neutra, ou mesmo com uma pequena vantagem selectiva, é extremamente elevado (*Vogel & Motulsky, 1979*).

A frequência elevada de heterozigóticos poderá influenciar a distribuição dos valores da ferritina sérica numa população, já que os heterozigóticos poderão ter aqueles valores mais elevados que a população normal não portadora do gene (*Beaumont et al, 1979; Basset et al, 1981*). No estudo da ferritina sérica na população portuguesa encontrámos valores mais elevados que na maioria dos estudos anteriormente publicados. No mesmo estudo encontrámos também diferenças significativas entre populações do norte e do sul do país, com uma tendência para valores mais elevados nas populações do norte. Embora não possamos excluir que as diferenças encontradas relativamente a outros estudos possam ser reais (por exemplo devidas a diferenças nos hábitos alcoólicos), a nossa convicção é que elas se devem sobretudo à aplicação de diferentes métodos na escolha da amostra e na definição de valores normais. No nosso estudo os valores de referência foram definidos em amostras representativas de várias populações em regiões diferentes do país e os limites da normalidade foram definidos em ambos os sexos tendo em consideração a

dependência da idade. Após a correcção para os efeitos do sexo, idade e ainda da ingestão de álcool (por regressão múltipla e análise de distribuição de resíduos), manteve-se significativa a diferença entre as populações do norte e do sul. Esta correcção pareceu-nos particularmente importante na interpretação dos resultados pois estes três factores influenciam significativamente os valores da ferritina sérica e porque existiam diferenças nos hábitos de ingestão de álcool entre as duas populações. Assim, a diferença nos valores da ferritina sérica entre as populações do norte e do sul foi interpretada como a influência, pelo menos em parte, do gene da hemocromatose que demonstrámos ser frequente nas populações do norte. É de notar, contudo, que esta conclusão se baseia no estudo de três populações do norte e uma do sul de Portugal. A extensão do estudo a outras populações do país bem como de outras comunidades portuguesas de emigração antiga ou recente é indispensável para determinar se o achado de diferenças regionais é apenas um dado fortuito ou se é, de facto, o reflexo das características genéticas da população.

LINFÓCITOS T E SOBRECARGA DE FERRO

A análise seriada dos parâmetros hematológicos e bioquímicos dos doentes com hemocromatose durante o tratamento por flebotomias intensivas revelou dois aspectos importantes na fisiologia do sistema imunológico no homem: a homeostasia dos linfócitos T circulantes e a sua relação com a absorção de ferro.

Estudos realizados em animais experimentais documentam a existência de uma estabilidade da expansão relativa das subpopulações de células T (*Rocha et al, 1983, 1984, 1989; Miller & Stutman, 1984; Rocha, 1987; Bell et al, 1987; Freitas & Rocha, 1993*). A vigilância regular de doentes com hemocromatose durante o tratamento por flebotomias intensivas permitiu-nos descrever, pela primeira vez no homem, que as proporções relativas de células T CD4+ e CD8+ se mantêm estáveis à periferia. Esta estabilidade persiste apesar da contínua remoção de grandes quantidades de sangue que, em certos doentes estudados, ultrapassaram 10 vezes o volume sanguíneo original. Adicionalmente observámos uma correlação significativa entre as proporções relativas das subpopulações de linfócitos T e a absorção de ferro. Essa correlação foi estabelecida de dois modos. Por um lado, a percentagem de células CD8+ estava inversamente relacionada com o nível de sobrecarga de ferro medido por meio de flebotomias quantitativas. Por outro lado, a reentrada de ferro

após tratamento, avaliada pela subida da taxa de saturação da transferrina, ocorreu mais rapidamente nos doentes com baixas proporções de células CD8+. Concluimos, assim, que o perfil das células T no sangue periférico, em particular a baixa proporção de células CD8+, se correlaciona com a entrada de ferro no organismo. No entanto, não é possível, apenas com os dados obtidos neste estudo, definir se é a baixa proporção de células CD8+ que condiciona a maior entrada de ferro ou se é a maior absorção de ferro que vai condicionar o defeito de expansão das células CD8+. O facto de as proporções de células se manterem estáveis apesar, do tratamento e da consequente baixa dos níveis de ferro, não serve para esclarecer o sentido da correlação uma vez que o aumento de absorção do ferro a nível intestinal também não é corrigido pelo tratamento (*Marx, 1979*). Este ponto poderia ser esclarecido por estudos de modulação das células CD8+. Por razões éticas, tal não pode ser feito em modelos humanos, mas poderá sê-lo em modelos animais. Qualquer que seja o sentido da correlação, estes resultados apontam para uma ligação definitiva entre as células do sistema imunológico e o metabolismo do ferro.

MHC E SOBRECARGA DE FERRO

Está hoje bem aceite que a expressão clínica da hemocromatose é heterogénea podendo ser influenciada por vários factores que incluem o sexo, a idade e certamente outros ainda desconhecidos (*Nichols & Bacon, 1989*). O facto de termos demonstrado a existência de uma relação entre a expansão das células CD8+ e o grau de sobrecarga de ferro nos doentes com hemocromatose, que é uma doença geneticamente ligada ao MHC, levou-nos a formular a hipótese que o fenótipo HLA poderia, de algum modo, estar relacionado com a expressão da doença, uma vez que está bem estabelecido que as células CD8+ são activadas e proliferam no contexto do MHC da classe I (*Zijlstra et al, 1990; Koller et al, 1990*). Não foram ainda descritos quaisquer estudos em que tenha sido avaliada a relação entre a expressão clínica da doença e o fenótipo HLA. A ausência de estudos nesse sentido é, de certo modo, compreensível, uma vez que domina o conceito, baseado em estudos da associação da doença com os haplótipos HLA definidos não só serologicamente mas também por meio de marcadores de ADN (*Yaouanq et al, 1992*), que essa associação resulta "apenas" de uma proximidade física entre o gene putativo da hemocromatose e o locus A do HLA no cromossoma 6. Segundo Simon e colaboradores, os alelos HLA

não podem eles próprios ser responsáveis pela hemocromatose, dada a multiplicidade de possíveis haplótipos associados à doença em contraste com a uniformidade clínica e histológica quer a doença esteja ou não associada ao antigénio A3 (*Simon et al, 1987*). Os nossos resultados contrariam a opinião estabelecida já que apresentámos evidência que o fenótipo HLA influencia o nível de sobrecarga de ferro e, consequentemente, a expressão clínica na hemocromatose: os doentes portadores do haplótipo A3B7 (haplótipo que está significativamente associado à doença na população portuguesa) tinham desenvolvido maior sobrecarga que os doentes portadores de haplótipos contendo os antigénios A3 ou B7 mas não no mesmo haplótipo e estes, por sua vez, uma sobrecarga maior que os doentes que não possuem os antigénios A3 nem B7. Em todos estes casos os níveis de sobrecarga de ferro estavam correlacionados com a razão CD4/CD8, ou seja, para o mesmo haplótipo HLA os doentes com razões CD4/CD8 anormalmente elevadas tinham uma maior sobrecarga que os doentes com razões normais. Assim concluímos que o grau de sobrecarga de ferro depende do efeito combinado das proporções relativas de linfócitos T e do fenótipo HLA.

Vários estudos procuraram estabelecer se os casos de hemocromatose secundária se associam também com os antigénios HLA ligados à hemocromatose idiopática. Essas associações têm de facto sido descritas em várias situações: na anemia sideroblástica refractária idiopática (*Cartwright et al, 1980; Barron et al, 1989*), na porfiria cutânea tarda (*Edwards et al, 1989*), na β-talassemia, e na sobrecarga de ferro em doentes em hemodiálise (revisão em *Simon, 1985*). Dado que a β-talassemia ou a hemodiálise são situações comuns, para que se possa estabelecer a associação com o alelo da hemocromatose é necessário que essa associação seja consistente com a prevalência do alelo na população em geral (*Simon, 1985*). No entanto, a frequência do HLA-A3 encontrada naquelas duas situações tem sido muito superior à que seria de esperar nas populações estudadas. Para explicar essas discrepâncias Simon postulou, numa base puramente especulativa, que poderia existir, para além do alelo da hemocromatose, outro alelo "de absorção" ligado ao HLA-A3 com alta prevalência e fraca expressão, requerendo para se exprimir, de outras condições favoráveis à sobrecarga secundária (*Simon, 1985; Yaouanq & Simon, 1988*). A hipótese aqui formulada de que a expansão de células CD8+, no contexto do próprio fenótipo HLA, influencia a sobrecarga de ferro, poderá servir para o esclarecimento dessas situações.

IMPLICAÇÕES NA PATOGÉNESE DA HEMOCROMATOSE

Neste trabalho demonstrou-se, em doentes com hemocromatose, uma correlação entre a proporção de células CD8+ circulantes e a sobrecarga de ferro, que depende ainda da combinação com o fenótipo HLA. Estes dados sugerem que uma anomalia na expansão das células CD8+, no contexto do MHC, pode constituir um mecanismo importante na patogénese da hemocromatose, podendo influenciar a severidade clínica da doença. Não ficou claro onde se situa a anomalia responsável pela maior absorção de ferro: tanto é possível que uma alteração no HLA vá influenciar a expansão das células CD8+, como um defeito primário nas células CD8+ possa ser influenciado pelo HLA. Nenhuma destas hipóteses põe em causa o padrão de transmissão da doença tal como está actualmente estabelecido, nem o carácter haplotípico do marcador HLA. O mecanismo que poderá levar à alteração da absorção é nesta altura desconhecido mas poder-se-á especular uma acção mediada por citoquinas com efeito no epitélio duodenal, área onde a expansão de células CD8+ parece estar correlacionada com a expansão à periferia (*Reimão et al, dados não publicados*). O facto de nem todos os doentes apresentarem, de forma global, alterações nas proporções de células CD8+ à periferia, aponta para o facto de a anomalia poder ser característica de subpopulações de células CD8+ e de haver de algum modo possibilidade de modulação dessa anomalia, o que poderia explicar a heterogeneidade clínica da doença. Resultados de um estudo do perfil de utilização de famílias das regiões variáveis do receptor da célula T indicam que existem anomalias na expressão de algumas dessas famílias e que, pelo menos numa delas, a anomalia pode estar relacionada com a expressão clínica da doença (*Cabeda et al, manuscrito em preparação*). É de realçar a importância destes resultados na prática clínica uma vez que, ao contribuirem para o esclarecimento da heterogeneidade clínica dos doentes, poderão ter implicações no tratamento e prevenção da sobrecarga de ferro, não só na hemocromatose idiopática mas possivelmente também na hemocromatose secundária.

CONCLUSÕES

Em resumo, os resultados obtidos e analizados neste trabalho permitem tirar as seguintes conclusões:

1. A hemocromatose idiopática é uma doença com uma alta prevalência no norte de Portugal, sendo estimado, com base em estudos familiares com tipagem HLA uma frequência génica de 0.14, correspondente a frequências de homozigóticos e heterozigóticos de 0.019 e 0.24 respectivamente.
2. Os estudos de despiste da hemocromatose realizados até à data na população em geral permitem validar as estimativas de prevalência da doença obtidas anteriormente com base na tipagem HLA.
3. Na população portuguesa, tal como noutras populações descritas em vários países, os antigénios HLA A3 e B7 são significativamente mais frequentes nos doentes com hemocromatose que na população em geral. O único haplótipo HLA que está significativamente associado à hemocromatose em Portugal é o haplótipo A3B7.
4. Leucopenia e macrocitose são alterações hematológicas comuns nos doentes com hemocromatose, independentemente de possíveis alterações consequentes a insuficiência hepática.
5. Os doentes com hemocromatose diferem entre eles nas proporções relativas de células T CD4+ e CD8+, uma grande parte deles apresentando razões CD4/CD8 anormalmente elevadas. Em cada indivíduo, a razão CD4/CD8 é mantida estável no decurso do tratamento por flebotomias intensivas.
6. A percentagem de linfócitos CD8+ está relacionada com o nível de sobrecarga de ferro (doentes com razões CD4/CD8 altas têm uma maior sobrecarga que os doentes com razões normais) e com a reacumulação de ferro após o tratamento intensivo (os doentes com razões CD4/CD8 altas atingem ràpidamente valores elevados de saturação da transferrina após o tratamento, o mesmo não acontecendo com os doentes com razões normais). Estes achados constituem evidência para uma correlação entre as proporções de células CD8+ e a absorção do ferro.
7. A correlação entre a sobrecarga de ferro e as razões CD4/CD8 nos doentes com hemocromatose é influenciada pelo haplótipo HLA, ou seja, para o mesmos valores de razões CD4/CD8, os doentes portadores do haplótipo A3B7 têm uma maior sobrecarga que os doentes portadores de haplótipos contendo os antigénios

A3 ou B7 mas não no mesmo haplótipo e estes, por sua vez, uma sobrecarga maior que os doentes que não possuem os antigénios A3 nem B7.

8. Com base nos pontos 6, 7 e 8, conclui-se que a expansão de linfócitos CD8+ à periferia, no contexto do MHC, pode estar directamente relacionada com a absorção de ferro. Uma anomalia nesse processo poderá constituir a base patogénica da hemocromatose idiopática.
9. As conclusões apresentadas suportam manifestamente o postulado de que as células do sistema imunológico têm um papel importante na regulação do metabolismo do ferro.

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SUMMARY AND CONCLUSIONS

Idiopathic Hemochromatosis (IH) is an HLA-linked, autossomal recessive disease of iron metabolism in which a defect of the still unknown mechanism regulating iron absorption leads to the progressive accumulation of this metal in various tissues with eventual tissue damage and organ disfunction. Although IH is recognised nowadays as a common disease of easy diagnosis and easy treatment, important aspects of this disorder are still unknown, namely the factors that may influence the individual variability in the clinical expression of the disease and the mechanisms underlying the defect in the regulation of iron absorption.

In 1986 we have started a program of screening for IH in Portugal including both family and population studies. Family studies with HLA typing were systematically done and have permitted the demonstration of the association of the disease with the haplotype A3B7 in this country (*Chapter 2*). In the course of the screening program it became apparent the need to establish reference values for the biochemical parameters of iron metabolism in the country since there are no universal cut-off values established for that purpose. This is particularly relevant for serum ferritin, given the great variability in normal values described in several studies. A novel method for the establishment of local reference values depending on sex and age was developed and is described in *Chapter 3* in comparison with other studies in the literature. The importance of establishing appropriate local reference values for the metabolism of iron is also discussed in *Chapter 4* where we analyse the influence of some factors in those values, namely: sex, age, level of regular alcohol intake and region of origin. As a result of the family studies we found a high prevalence of the putative IH gene in the north of the country (*Chapter 4*) that was later confirmed by studies of the phenotypic expression in subjects from the normal population, namely in a study conducted at Refoios-Ponte de Lima (*Chapter 5*).

Motivated by an interest in studying the interaction of the metabolism of iron with the cells of the immunological system, we examined serially the peripheral blood cell populations, including the relative proportions of lymphocyte subsets, in individual IH patients during the course of phlebotomy treatment (*Chapter 6*). This study provided a unique opportunity of demonstrating, for the first time in man, a remarkable stability of the total numbers and relative proportions of T lymphocytes at periphery. In the course of that study we were faced with the new findings of a high frequency of constitutional leukopenia among IH patients and the constant observation of macrocytic red blood cells, both occuring independently of phlebotomy treatment or the development of hepatic failure. The most relevant finding is this study was the

demonstration that a subgroup of patients had abnormally high CD4/CD8 ratios, an abnormality that was correlated with the clinical behaviour of patients, namely, the response to iron removal and iron reloading after treatment. To better characterise the clinical behaviour and degree of iron overload in IH patients according to their CD4/CD8 ratios we have done an exhaustive statistical analysis also including as variables the clinical presentation at diagnosis and the HLA phenotype (*Chapter 7*). In that study we demonstrate that the CD4/CD8 ratios, combined with the HLA phenotype, constitute a powerful factor predicting the clinical expression and progression of the disease. The implications of a possible anomaly of the expansion of CD8+ cells (in the context of the MHC) on the pathogenesis and clinical heterogeneity of Idiopathic Hemochromatosis is discussed (*Chapter 8*).

From the data described in this work the following conclusions were drawn:

1. Idiopathic Hemochromatosis is a disease with a high prevalence in the north of Portugal, having been estimated, by family studies with HLA typing, a gene frequency of 0.14, corresponding to homozygote and heterozygote frequencies of 0.019 and 0.24 respectively.
2. Studies for the screening of hemochromatosis done so far in normal subjects from the general population have confirmed the high prevalence estimated by family studies in this region.
3. In the portuguese population, such as in other populations in the rest of the world, antigens HLA A3 and B7 are significantly more frequent in patients than in the normal population. The only HLA haplotype significantly associated with hemochromatosis in this country is the haplotype A3B7.
4. Leukopenia and macrocytosis are hematological alterations frequently observed in hemochromatosis independently of treatment or hepatic disease.
5. Patients with hemochromatosis differ in their relative proportions of CD4+ and CD8+ T cells, many of them showing abnormally high CD4/CD8 ratios. In each patient the CD4/CD8 ratio is maintained stable in the course of intensive phlebotomy treatment.
6. The relative proportion of CD8+ cells is correlated with the degree of iron overload (patients with high CD4/CD8 ratios have higher iron stores than patients with normal or low ratios) and the reloading of iron after treatment (patients with

high CD4/CD8 ratios show a fast increase in their transferrin saturation values after treatment, the same not occuring in patients with normal or low ratios).

7. The correlation between iron stores and CD4/CD8 ratios is influenced by the HLA phenotype, i.e., for equivalent CD4/CD8 ratios, patients with the haplotype A3B7 have higher iron stores than patients with haplotypes carrying A3 or B7 but not on the same haplotype and these ones have higher stores than patients who do not express neither A3 nor B7.

8. Based on 5, 6 and 7 it can be concluded that the expansion of CD8+ cells, in the context of MHC, may be directly related to iron absorption. An anomaly in that process may constitute the pathogenic basis of Idiopathic Hemochromatosis.

9. These conclusions support the postulate that the cells of the immunological system have an important role in the regulation of iron metabolism.

RÉSUMÉ ET CONCLUSIONS

L'Hémochromatose idiopathique (HI) est une maladie du métabolisme du fer, autosomique, récessive, liée au système HLA, dans laquelle une anomalie du mécanisme régulateur de l'absorption du fer, d' origine encore inconnu, mène à l'accumulation tissulaire progressive de ce métal avec éventuelle dysfonction des organes. Bien que la HI soit reconnue à présent comme une maladie usuelle, de diagnostic et traitement faciles, des aspects importants de cette maladie sont encore malconnus, notamment les facteurs qui peuvent agir sur la variabilité individuelle dans l'expression clinique de la maladie et des mécanismes qui soulignent cette anomalie-là dans la régulation de l'absorption du fer.

En 1986, nous avons commencé un programme de dépistage de la HI au Portugal, qui a été fait de deux façons: dépistage familial et dépistage populationnel. Chez le premier, la recherche a été développée systématiquement d'après le groupage HLA et cela nous a permis démonstrer que, dans ce pays, la maladie est associée au haplotype A3B7 (*Chapitre 2*). Pendant le programme, le besoin d' établir des valeurs de référence pour les paramètres biochimiques du métabolisme du fer est devenu évident une fois que des valeurs universelles d'exclusion n'existaient pas. Ceci devient particulièrement avantageux pour la ferritine sérique, à cause de la grande variation des valeurs normales déjà décrite dans des études divers. Une méthode nouvelle pour l'établissement des valeurs de référence locale, comprenant la variation sexe/âge a été développée et décrite dans le *Chapitre 3* en comparaison avec d'autres études publiées. L'importance d'établir des valeurs de référence locale convenables pour le métabolisme du fer est aussi matière de discussion dans le *Chapitre 4*, où nous analysons l'influence de quelque facteurs sur ces mêmes valeurs, c'est-à-dire: sexe, âge, moyenne de consomption d'alcool et région d'origine. Comme résultat des études familiales nous avons trouvé une haute prévalence du gène putative de la HI au nord du pays (Chapitre 4), ce qui a été confirmé plus tard par des études d'expression phénotypique chez des individus de la population normale, nommément dans une étude accomplie à Refoios-Ponte de Lima (*Chapitre 5*).

Motivés par l'intérêt à étudier l'interaction du métabolisme du fer avec les cellules du système immunitaire, nous avons examiné les populations cellulaires du sang périphérique, aussi que les proportions relatives des sous-populations lymphocytaires, dans les malades avec HI pendant le déroulement du traitement par des saignées regulières (*Chapitre 6*). Avec cette étude nous avons eu l'opportunité unique de démontrer, pour la première fois chez l'homme, un équilibre remarquable soit dans les

nombres totaux soit dans les proportions relatives des lymphocytes T à la périphérie. Pendant cette étude, nous nous sommes trouvés avec ces découvertes d'haute fréquence de leucopénie constitutionnelle parmi des malades avec HI et l'observation constante de cellules sanguines rouges macrocytiques, tous les deux ayant lieu indépendamment du traitement par des saignées ou du développement de la dysfonction hépatique.

La découverte la plus importante dans ce travail a été la démonstration qu'un sous-groupe de malades présentait des ratios CD4/CD8 extrêmement élevés et que cette anomalie était en rapport avec le comportement clinique des malades, nommément la réponse au prélèvement du fer et fer recharge après traitement. Pour une meilleure caractérisation du comportement clinique et niveau d'accumulation de fer chez les malades avec HI, d'après leurs ratios CD4/CD8, nous avons fait une analyse statistique minutieuse, en y considérant aussi la présentation clinique au moment du diagnostic et le phénotype HLA comme variables (*Chapitre 7*). Dans notre étude nous avons démontré que les ratios CD4/CD8 combinés avec le phénotype HLA devient une facteur puissante pour la prévision de l'expression clinique de la maladie et sa progression. Il a été aussi objet de discussion la probabilité et les implications d'une anomalie dans l'expansion des cellules CD8+ (dans le contexte du MHC) chez la pathogénie et hétérogénie clinique de l'hémochromatose idiopathique (*Chapitre 8*).

Ayant en considération les données obtenues, nous pouvons conclure que:

1. La prévalence de l'Hémochromatose Idiopathique est très élevée au nord du pays, selon les estimations basées sur les études familiales. D'après la groupage HLA la fréquence du gène est de 0,14, correspondant aux fréquences d' homozygotes et d' hétérozygotes de 0,019 et 0,24 respectivement.

2. Le dépistage de l'hémochromatose accompli chez des individus normaux dans la population en général, a confirmé l'haute prévalence estimée par les études familiales dans cette région.

3. Dans la population Portugaise, tel que dans les populations du reste du monde, les antigènes HLA A3 et B7 sont significativement plus souvent détectés chez les malades que dans la population normale. Dans le pays, le seul haplotype significativement associé à l'hémochromatose est le haplotype A3B7.

4. La leucopénie et la macrocytose sont d'altérations hématologiques souvent observées dans l'hémochromatose et sont indépendantes du traitement ou de l'affection du foie.

5. Des malades avec hémochromatose présentent des différences dans les proportions relatives de cellules T CD4+ et CD8+. Beaucoup d'eux ont des ratios CD4/CD8 anormalement élevés. Dans chaque malade le ratio CD4/CD8 a été maintenu presque constant pendant le traitement intensif par des saignées.

6. La proportion relative des cellules CD8+ est en rapport avec le niveau d'accumulation du fer (des malades avec ratios élevés de CD4/CD8 ont des stockages de fer plus agrandis que ceux qui révèlent des ratios normaux ou inférieurs) et la recharge de fer après la thérapeutique (malades avec des valeurs élevées de CD4/CD8 montrent une augmentation plus rapide de leur saturation de transferrine après le traitement; le même n'arrive pas chez ceux qui ont des ratios normaux ou bas).

7. Le rapport entre le stockage du fer et les ratios de CD4/CD8 souffre l'influence du phénotype HLA, c'est à dire, pour les valeurs similaires ou équivalentes de CD4/CD8, les malades avec le haplotype A3B7 montrent des stockages de fer plus élevés que les malades avec haplotypes portant A3 ou B7, mais pas dans le même haplotype, et ceux-ci ont des stockages supérieurs aux malades qui n'exhibent ni l'haplotype A3 ni le haplotype B7.

8. Basés sur les points 5, 6 et 7 nous pouvons conclure que l'expansion de la population des cellules CD8+ dans le contexte de MHC peut être directement rapporté à l'absorption du fer. Une anomalie dans ce processus peut être le principe ou la base de l'hémochromatose idiopathique.

9. Ces conclusions vont renforcer le postulat que les cellules du système immunitaire jouent un rôle essentiel dans la régulation du métabolisme du fer.

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