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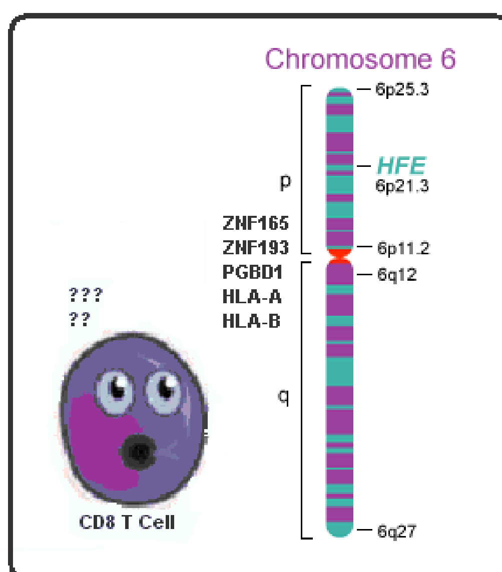
Universidade do Porto – Instituto de Ciências Biomédicas de Abel Salazar

Curso de Mestrado Integrado em Medicina

A study of 147 extended haplotypes carrying the C282Y HFE mutation: a novel approach to explain the involvement of the MHC-class I region in the setting of CD8+ T lymphocyte numbers in humans.



Sandra Isabel Alves Morais

Porto, 2010

Instituto de Ciências Biomédicas de Abel Salazar

Universidade do Porto

A study of 147 extended haplotypes carrying the C282Y HFE mutation: a novel approach to explain the involvement of the MHC-class I region in the setting of CD8+ T lymphocyte numbers in humans.

TESE ELABORADA NO ÂMBITO DO CURSO DE MESTRADO INTEGRADO EM MEDICINA DO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR DA UNIVERSIDADE DO PORTO

Orientadora: Professora Doutora Graça Porto

Porto, 2010

Agradecimentos

Gostaria de expressar os meus mais sinceros agradecimentos a todos os que, directamente ou indirectamente, contribuíram para a realização deste projecto.

À minha orientadora Professora Doutora Graça Porto pelo voto de confiança por me ter dado a oportunidade de realizar este projecto, por todos os ensinamentos, pelo apoio, paciência, atenção e constante disponibilidade demonstrada.

À Professora Doutora Margarida Lima pelo empenho e disponibilidade demonstrada e pelos esclarecimentos fornecidos.

À Professora Doutora Maria Berta Martins da Silva pela disponibilidade e interesse demonstrados e pelos esclarecimentos fornecidos.

À Mónica Costa por todo o apoio a nível laboratorial, paciência e amizade demonstradas.

À Andreia Bettencourt por todo o apoio a nível laboratorial, pela ajuda a nível da análise estatística e paciência demonstrada.

À Dra. Eugénia Cruz gostaria de agradecer toda a disponibilidade, conselhos e sugestões.

Ao pessoal do Laboratório de Imunogenética e do grupo IRIS (IBMC) por toda a amizade e apoio.

Aos meus pais e irmãos, por serem as pessoas que mais acreditam em mim, obrigada por toda a compreensão e apoio.

Ao Gonçalo, por ter estado sempre do meu lado a apoiar-me e a motivar-me, obrigada por toda a ajuda e carinho.

À Alexandrina e Eduarda, amigas sempre presentes, obrigada por toda a motivação.

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Capítulo I

Planeamento e submissão de tese

Trabalho Académico de Investigação

CHARACTERIZATION OF HAPLOTYPES AAT AND GGG IN A POPULATION OF INDIVIDUALS HOMOZYGOUS FOR THE C282Y MUTATION IN HFE GENE: CONTRIBUTION TO THE STUDY OF PENETRANCE OF HEREDITARY HEMOCHROMATOSIS.

Área de Investigação: Sobrecarga de Ferro – Hemocromatose e HFE

Curso de Mestrado Integrado em Medicina
ICBAS/UP – HSA/CHP

Disciplina de Iniciação à Investigação Clínica (DIIC)
Responsável: Prof. Doutora Margarida Lima

Aluno: **SANDRA MORAIS**

Orientadora: Prof. Doutora Graça Porto

Ano Lectivo: 2008 / 2009 e 2009 / 2010

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SERVIÇOS DO CHP ONDE O ESTUDO VAI SER REALIZADO

Serviço de Hematologia Clínica, HSA/CHP.

INSTITUIÇÕES INTERVENIENTES, ALÉM DO CHP

IBMC/UP.

DATA LIMITE: Início em Agosto/Setembro de 2009.

VERSÃO do estudo

Novo X

CLASSIFICAÇÃO do estudo

Trabalho Académico de Investigação (Mestrado Integrado) X

DESENHO do estudo

Quanto ao alvo do estudo

Humanos X

Quanto aos Países / Instituições envolvidas

Nacional X Multicêntrico X

Quanto às características do estudo

Transversal X Analítico X
Observacional X

Quanto à natureza do estudo

Clínico X Laboratorial X

Quanto à existência de grupo controlo

Não controlado X

Quanto à selecção dos indivíduos

Não aleatório X ("conveniência")

Quanto ao conhecimento

Aberto (não cego) X

Quanto à fase do estudo (se EC)

Não aplicável X

Outros aspectos

Saída amostras p/ instit. privadas .... Não
Consentimento informado escrito .... Sim
Realização de estudos genéticos .... Sim
Realização de inquéritos / questionários ..... Não
Realização de entrevistas .... Não
Recolha de dados clínicos .... Sim

DATAS previstas

Início: Agosto / Setembro de 2009.
Conclusão: Maio / Junho de 2010.

INDICADORES de produção

Apresentação da proposta de projecto nas Jornadas de Iniciação à Investigação Clínica (JIIC) 2009
Organização das JIIC 2010
Apresentação dos resultados nas JIIC 2010 e em Congresso da Especialidade

ORÇAMENTO do estudo

Total: 6869,20 Euros

FINANCIAMENTO do estudo

Total: 4000 Euros
Bolsa de Iniciação à Investigação Clínica / Roche Farmacêutica

TÍTULO | TITLE

CHARACTERIZATION OF HAPLOTYPES AAT AND GGG IN A POPULATION OF INDIVIDUALS HOMOZYGOUS FOR THE C282Y MUTATION IN HFE GENE: CONTRIBUTION TO THE STUDY OF PENETRANCE OF HEREDITARY HEMOCHROMATOSIS

INSTITUIÇÕES, DEPARTAMENTOS E SERVIÇOS | INSTITUTIONS AND DEPARTMENTS

Centro Hospitalar do Porto (CHP), Hospital de Santo António (HSA) (HSA/CHP)

Department of Medicine, Service of Clinical Hematology

Instituto de Biologia Molecular e Celular (IBMC) da Universidade do Porto (UP) (IBMC/UP)

Department of Molecular Evolution

Centro de Genética Preventiva e Predictiva (CGPP)

UNIDADES DE INVESTIGAÇÃO | RESEARCH UNITS

Instituto de Biologia Molecular e Celular (IBMC) da Universidade do Porto (UP) (IBMC/UP)

EQUIPA DE INVESTIGAÇÃO | RESEARCH TEAM

Aluna | Student

- **Sandra Morais**: Aluna do Curso de Mestrado Integrado em Medicina, ICBAS/UP e HSA/CHP, Disciplina de Iniciação à Investigação Clínica.

Orientadora | Project supervisor

- **Prof. Doutora Graça Porto**: Médica, Especialista de Imunohemoterapia, Assistente Hospitalar Graduada, Serviço de Hematologia Clínica, HSA/CHP; Professora Catedrática do ICBAS/UP; Investigadora do Serviço de Hematologia Clínica do HSA/CHP e do IBMC/UP.

Supervisora | Discipline supervisor

- **Prof. Doutora Margarida Lima**: Médica Especialista em Imunohemoterapia; Investigadora do Serviço de Hematologia Clínica do HSA/CHP e da UMIB/UP; Professora Convidada do ICBAS/UP, responsável pela Disciplina de Iniciação à Investigação Clínica.

Outros investigadores | Other investigators

- **Susana Almeida:** Licenciada em Ciências do Meio Aquático (Biologia Marinha) pelo ICBAS/UP. Aguarda data de defesa de Mestrado pela FMUP; Geneticista Molecular no CGPP, IBMC/UP (responsável pelas extracções de DNA e pelo diagnóstico de HFE).

- **Mónica Costa:** Técnica Superior de Anatomia Patológica licenciada pela ESTSP. A desenvolver mestrado de Biologia Molecular e Celular pela universidade de Aveiro. Actualmente possui bolsa de Técnica de Investigação no IBMC/UP.

- **Luís Correia:** Licenciado em Bioquímica pela FCUP e mestrado em Controlo de Qualidade pela FFUP; Gestor de Qualidade do CGPP, IBMC/UP.

- **Jorge Vieira:** Investigador do grupo de Evolução Molecular do IBMC/UP.

Funções e responsabilidades | Functions and responsabilities

- À aluna competirá a elaboração, redacção e apresentação da proposta do trabalho académico de investigação; solicitar e recolher a informação clínica adicional aos médicos responsáveis pelos doentes; execução dos estudos laboratoriais; analise e tratamento estatístico dos dados; interpretação e apresentação dos resultados; redacção do trabalho académico de investigação.
- À Orientadora caberá a função de orientação da execução do projecto e de supervisão científica.
- Aos outros investigadores:
  - Susana Almeida – Recolha das amostras biológicas e extracção do DNA das mesmas.
  - Mónica Costa – Orientação/apoio na execução dos estudos laboratoriais.
  - Luís Correia – Apoio na solicitação e recolha da informação clínica adicional aos médicos responsáveis pelos doentes e auxilio a nível de questões internas/institucionais.
  - Jorge Vieira – Apoio na análise e tratamento estatístico dos dados e na interpretação dos resultados.

Tempo afecto ao projecto

Aluno: 30% durante cerca de 10 meses

Orientador: 10% durante 10 meses

Outros investigadores:

- Susana Almeida: 10% durante 5 meses
- Mónica Costa: 15% durante 3 meses
- Luís Correia: 10% durante 6 meses
- Jorge Vieira: 10% durante 4 meses

Total: 1 Pessoa a 30% durante 10 meses + 1 pessoa a 10% durante 10 meses + 1 pessoa a 10% durante 6 meses + 1 pessoa a 10% durante 5 meses + 1 pessoa a 10% durante 4 meses + 1 pessoa a 15% durante 3 meses = 5,95 pessoas*mês

DESENHO DO ESTUDO | STUDY DESIGN

- Estudo nacional, multi-institucional, observacional, transversal, analítico, clínico e laboratorial feito em seres humanos.

CALENDARIZAÇÃO

Duração / Duration

10 meses

Datas de início e conclusão / Starting and closing dates

Agosto /Setembro de 2009 a Maio/Junho de 2010

Cronograma de execução / Chronogram of execution

Janeiro a Abril de 2009	Elaboração da proposta.
Maio 2009	Entrega da proposta – Submissão à aprovação institucional.
Junho 2009	Apresentação da proposta nas Jornadas de Iniciação à Investigação Clínica
Julho a Setembro de 2009	Preparação para a implementação do projecto. Solicitação de informação clínica adicional aos médicos responsáveis pelos doentes. Apresentação da proposta em reunião do Serviço de Hematologia Clínica.
Outubro a Dezembro de 2009	Execução dos estudos laboratoriais.
Janeiro a Março de 2010	Recolha, análise e tratamento estatístico dos dados.
Abril a Maio de 2010	Interpretação dos resultados.
Junho 2010	Apresentação dos resultados nas Jornadas de Iniciação à Investigação Clínica.

A. PLANO CIENTÍFICO | SCIENTIFIC PLAN

1. Introdução e estado da arte | Introduction and state of the art

Hereditary hemochromatosis (HH) is a primary inherited disorder of iron metabolism with progressive iron loading of parenchymal cells of the liver and other organs, which may eventually lead to tissue damage and dysfunction, generally by the fourth to fifth decades of life (Porto G et al, 2008; Feder JN et al, 1996). Clinical consequences of iron accumulation include cirrhosis of the liver, hepatocellular carcinoma, diabetes, heart failure, arthritis and hypogonadism (Cardoso CS et al, 2001).

Independently of the presenting clinical picture, HH should be suspected in any subject with clinical and/or laboratory evidence of severe iron overload, in the absence of other known iron loading conditions such as chronic liver disease or hematological disorders. Typically, both transferrin saturation (TfSat) and serum ferritin are abnormally high and normally represent the first phenotypic manifestation of HH (Porto G et al, 2008).

With origin in populations of northern European, over 90% of HH patients are homozygote's to C282Y mutation in HFE gene, a non-classical MHC class I gene 5 Mb telomeric to HLA-A at chromosome 6 (6p22.1) (Feder JN et al, 1996), while only about 1 in 200 people in the general population have this genotype (Jackson HA, 2001).

Type 1 HH, also called HFE-related HH or classic HH, is the most common form of inherited iron overload and one of the most common hereditary metabolic diseases in Caucasians (Cardoso CS et al, 2001). It is characterized by an autosomal recessive pattern of inheritance and is associated with mutation of the HFE gene located on chromosome 6 (Franchini M, 2006).

In spite of the generally reported high frequency of the C282Y mutation in most European derived populations and the finding that most homozygotes for the C282Y mutation of HFE show a common biochemical phenotype characterized by high transferrin saturation, the proportion of patients with a fully developed phenotype found in clinical practice seems to be very low (Porto G et al, 2008).

Previous studies showed that patients with high CD4/CD8 ratios display a faster re-entry of iron into the serum transferrin pool after intensive phlebotomy treatment, reaching abnormal transferrin saturation values more rapidly than patients with normal CD4/CD8 ratios (Reimão R et al, 1991). The abnormalities in lymphocyte populations were systematically found in CD8+ T cells and the association with total body iron stores could also be reflected in the total lymphocyte count (Porto G et al, 2008). A significant inverse relationship of total blood lymphocyte counts and severity of iron overload in HH patients with HFE C282Y homozygosity was also described (Barton JC et al, 1995).

HH is clinically very heterogeneous, varying from a simple biochemical abnormality to a full-blown picture of devastating iron overload disease (Porto G et al, 2008) and recent large population screenings are revealing that the clinical penetrance of HH may be lower than previously thought (Jackson HA, 2001). Heterogeneity observed in iron accumulation and associated clinical presentation may be partially explained by gender, age and environmental factors (Ryan E et al, 1996). There are also some genes, like those already associated with iron-storage disease such as transferrin, TfR2, ferroportin and ceruplasmin that could be good candidate genes at expression modifiers (Porto G et al, 2008). More recently there are growing evidences supporting the notion that other genes at the MHC class I region, possibly inherited with the ancestral C282Y containing haplotype are implicated in the clinical heterogeneity of HFE-associated HH (Kohgo Y, 2001).

By extended linkage disequilibrium analysis in the hemochromatosis gene region there was founded a highly significant association among the C282Y homozygous and the allele 8 of D6S105 microsatellite, 2Mb away from HFE gene. Moreover, male patients C282Y homozygous, with the two copies of the allele 8 of this microsatellite, were found to have significantly higher hepatic iron indices than those heterozygous or nullizygous for this allele. Thus, it was suggested that a gene modifying the phenotype of C282Y homozygotes may be localized around the area of D6S105. (Pratiwi R, 1999)

As mentioned previously, low numbers of CD8+ T cells, both in the peripheral blood and in the liver, were shown to be negatively correlated with total body iron stores (Porto G et al, 2008) and consequently have a more severe expression of HH (Reimão R et al, 1991). This immunophenotype is proposed to be a clinical marker of the severity of iron overload in HH patients (Cruz E et al, 2006). By sibpair analysis in hemochromatosis families it was shown that CD8+ T-lymphocyte numbers are genetically regulated by genes in the MHC region (Cruz E et al, 2004). More recently it has been shown that a genetic trait associated with the transmission of CD8+ T lymphocyte numbers is also demonstrated in the normal population and it is localized at the MHC class I region close to the D6S105 microsatellite marker (Vieira J et al, 2007).

The referred evidences led to the formulation of the hypothesis that a putative gene controlling CD8+ T cells numbers, localized on the MHC region, could be the same gene contributing to the clinical heterogeneity observed in HH (Porto G et al, 2008; Cruz E et al, 2008).

Previous studies performed in our group, using a small group of HH patients, have suggested that the that a highly conserved 500 Kb ancestral haplotype defined by the SNP markers PGBD1-A, ZNF193-A, ZNF165-T (AAT haplotype) marks the inheritance of "low" CD8+ T-lymphocyte numbers and predicts the development of a severe clinical expression of HH (in terms of iron overload and clinical manifestations) (Cruz E et al, 2008). In order confirm these studies, haplotype analysis will be done in the region 500Kb defined by the SNP markers PGBD1, ZNF193 and ZNF165, in a larger group of

previously characterized C282Y homozygous Portuguese HH patients. Once identified and described the different conserved haplotypes in that region, the results with be correlated with the phenotypic and clinical variables.

2. Problemas | Pitfalls

HH is clinically very heterogeneous, varying from a simple biochemical abnormality to a full-blown picture of devastating iron overload disease. Nevertheless, there is no a genetic marker that make us able to predict the clinical severity.

3. Hipótese de trabalho | Working hypothesis

Our general working hypothesis is that a putative gene controlling CD8+ T cells numbers, localized on the MHC region, is the same gene contributing to the clinical heterogeneity observed in HH. Specifically, we hypothesize that in the region of 500 kb defined by the SNP markers PGBD1, ZNF193, ZNF196, there may be a gene that affects inheritance of CD8+ T-lymphocyte numbers and predicts the development of a severe clinical expression of HH (in terms of iron overload and clinical manifestations).

4. Objectivos | Aims

General: To find new markers that could be used as more reliable prognostic variables in HH.

Specific: To confirm previous studies suggesting that in the region of 500 kb defined by the SNP markers PGBD1, ZNF193, ZNF196 there is a genetic marker predicting both the inheritance of CD8+ T-cell numbers and the severity of phenotypic expression in HH.

5. Implicações | Implications

The results of this study may have important implications not only for approaching the question of the penetrance of the hemochromatosis gene in Portuguese population but also to further narrow the region of interest to find a candidate gene involved in the setting of CD8+ T-lymphocyte numbers in humans.

6. Enquadramento, motivações e condições de suporte | motivations and support conditions

A Orientadora integra o IBMC/UP uma Instituição de Investigação da Universidade do Porto reconhecida pela FCT.

Têm grande experiência no estudo da HH: Desenvolvimento de tese de doutoramento sobre HH; Realiza a consulta de HH do serviço de Hematologia Clínica do HSA/CHP e consulta de diagnóstico genético de HH do CGPP;

Têm desenvolvido diversos projectos de investigação:

Porto G, Cruz E, Miranda HP, Porto B, Vasconcelos JC, Lacerda R, Roetto A, Daraio F and Bacelar C. Growth hormone (GH)-induced reconstitution of CD8+ CD28+ T lymphocytes in a rare case of severe lymphopenia associated with Juvenile Haemochromatosis and Turner's syndrome. Clinical Endocrinology Aug 2004, 61: 437-440.

Porto G, Cardoso CS, Gordeuk V, Cruz E, Fraga J, Areias J, Oliveira JC, Bravo F, Gangaidzo IT, MacPhail AP, Gomo ZAR, Moyo VM, Melo G, Silva C, Justiça B and de Sousa M. Clinical and genetic heterogeneity in hereditary haemochromatosis: association between lymphocyte counts and expression of iron overload. European Journal of Haematology Apr 2002, 67: 110-118.

Porto G, Cardoso CS, Gordeu, Cruz E, Fraga J, Areias J, Oliveira JC, Bravo F, Gangaidzo IT, MacPhail AP, Gomo ZA, Moyo VM, Melo G, Silva C, Justica B and de Sousa M. Clinical and genetic heterogeneity in hereditary hemochromatosis: association between lymphocyte counts and expression of iron overload. Eur J Haematol 2001, 67:110-8.

Porto G, Reimão R, Gonçalves C, Vicente C, Justiça B and de Sousa M. Haemochromatosis as a window into the study of the immunological system: A novel correlation between $CD8^{+}$ lymphocytes and iron overload. European Journal of Haematology 1994, 52: 283-290.

Tem redigido vários artigos de revisão:

Porto G and de Sousa M. Iron overload and immunity. World J Gastroenterol, Sep 2007, 21:4707-15.

Porto G, Cardoso CS, Macedo F, and Cruz E. Hereditary Hemochromatosis type I: Genetic, clinical and immunological aspects. Iron Metabolism and Disease. 2008: 435-460.

Tem coordenado inúmeros projectos de investigação:

- Correia AP, Pinto JP, Dias V, Mascarenhas C, Almeida S and Porto G. CAT53 and HFE alleles in Alzheimer's disease: a putative protective role of the C282Y HFE mutation. Neurosci Lett. Jul 2009, 3: 129-32.
- Porto B, Vieira R and Porto G. Increased capacity of lymphocytes from hereditary hemochromatosis patients homozygous for the C282Y HFE mutation to respond to the genotoxic effect of diepoxybutane. Mutat Res. Feb 2009, 19: 37-42.
- Cruz E, Whittington C, Krikler SH, Mascarenhas C, Lacerda R, Vieira J and Porto G. A new 500 kb haplotype associated with high CD8+ T-lymphocyte numbers predicts a less severe expression of hereditary hemochromatosis. BMC Med Genet. Nov 2008, 6: 97.
- Cruz E, Melo G, Lacerda R, Almeida S and Porto G. The CD8+ T-lymphocyte profile as a modifier of iron overload in *HFE* hemochromatosis: An update of clinical and immunological data from 70 C282Y homozygous subjects. Blood Cells, Molecules and Diseases, July-August 2006, 37: 33-39.

- Cruz E, Vieira J, Almeida S, Lacerda R, Gartner A, Cardoso CS, Alves H and Porto G. A study of 82 extended HLA haplotypes in HFE-C282Y homozygous hemochromatosis subjects: relationship to the genetic control of CD8+ T-lymphocyte numbers and severity of iron overload. BMC Med Genet. Mar 2006, 1: 16.

É coordenada ainda de diversos projectos de pós-graduação (dissertações de mestrado e teses de doutoramento).

Concomitantemente encontra-se a organizar o encontro anual da *International BioIron Society*: "IBIS 2009 Annual Meeting".

Motivações do aluno:

- Vontade em melhorar os conhecimentos sobre a patologia em questão (HH) quer da sua componente clínica quer da sua componente genética.
- Obter experiência em investigação clínica como premissa para aumento do interesse científico e desenvolvimento do espírito inquisitivo.
- Aperfeiçoamento e aprendizagem de técnicas e procedimentos laboratoriais.
- Reconhecimento pelos pares.

7. Desenho do estudo e metodologia | Study design and working

7.1. População em estudo | Study population

The population analyzed in this study will be composed by all the individuals homozygous for the C282Y mutation of the HFE gene (approximately 240 individuals) identified between 1997 and 2009 in the Centro de Genética Preventiva e Predictiva (CGPP), Porto. These subjects are Caucasians from the north and centre of Portugal and included subjects detected in the context of suggestive clinical picture of haemochromatosis, generally with related clinical manifestations, or detected accidentally after a routine test and generally asymptomatic or even family members detected in the context of systematic family screening programs.

7.2. Caracterização clínica | Clinical characterization

Clinical evaluation of the group of C282Y homozygous Portuguese patients was previously described in detail elsewhere (Porto G et al, 2001; Cruz E et al, 2006). They include both asymptomatic and symptomatic HH patients presenting with one or more of the following manifestations: liver cirrhosis /fibrosis, diabetes, arthropathy, hypopituitarism, skin pigmentation or cardiac abnormalities, and removed an average of iron stores by intensive phlebotomies. The clinical parameters used in the analysis will include: biochemical parameters of iron metabolism (TfSat and serum ferritin) determined at diagnosis by standard techniques as described (Porto G et al, 2001), total body iron stores (TBIS) determined by quantitative phlebotomies (Haskins D et al, 1952) and the presence of clinical manifestations related to HH. Whenever necessary, missing clinical data will be obtained by contacting

the physicians that accompanies those patients and sending them a formulary for clinical data registering (Attachment 1).

7.3. Caracterização imunológica | Immunological characterization

The immunological characterization of patients included the number of peripheral blood total lymphocytes. For the purpose of phenotypic characterization of patients, total lymphocyte numbers were considered “low” when they were $\leq 2{,}12\times10^6$/ml and were considered “high” when > $2{,}12\times10^6$/ml, as defined in previous studies of lymphocyte populations in hemochromatosis (Cruz E et al, 2006a; Cruz E et al, 2004; Cruz E et al, 2006b). These cut-off values were based on the median values of the parameters previously established on a control population from the north of Portugal (Cruz E et al, 2006a).

7.4. Caracterização genética | Genetic characterization

All subjects had been previously genotyped for HFE mutations (H63D and C282Y) and they are all homozygous for the C282Y mutation. Some HH patients were also previously genotyped for D6S105, PBGD1 and ZNF193. Genetic data have been partially published (Cruz E et al, 2004; Cruz E et al, 2008).

7.5. Definição do haplótipo | Haplotype definition

For the purpose of this study, extended haplotypes will be inferred using the program PHASE (http://www.stat.washington.edu/stephens/software.html), as described previously (Vieira J et al, 2007). Extended haplotypes will be defined with the information of the genotype of following markers: PGBD1 and ZNF193. The phase of length polymorphisms at microsatellite D6S105 was known from family studies, and this information will be used when running PHASE.

7.6. Desenho experimental | Experimental design

For the purpose of this study, some single nucleotide polymorphisms (SNPs) localized in the region around the microsatellite in the 6p21.3 region, and in the HLA-A region will be genotyped in all patients. The SNPs are localized in the following genes: piggyBac transposable element derived 1 gene (PGBD1) e zinc finger protein (ZNF) 193. The other SNPs, and respective characterization, used in this study are from some studies that still ongoing.

SNP genotyping will be performed by gene sequencing. Briefly, genomic DNA (gDNA) will be extracted from peripheral blood or stored. gDNA and amplicons containing the selected loci will be PCR-amplified using specific primers. Amplicons will be then electrophoresed and extracted from the gel whit the QIAquick Gel Extraction Kit (Quiagen). Sequencing reaction will be prepared with the Big Dye

Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and loaded in an ABI prism 310 Genetic Analyzer Sequencer (Applied Biosystems).

7.7. Análise estatística | Statistic analysis

Association studies between the total lymphocytes numbers and the genetic markers will be performed. Since each subject carries two haplotypes, the analyses will be performed considering the combination of the inherited conserved haplotypes. Finally, the combination of conserved haplotypes will be used to analyze its impact on the clinical expression of the disease, both in terms of the amount of iron mobilized by phlebotomies (TBIS) and the clinical manifestations.

For the statistical analyses that include total lymphocytes numbers, patients with clinical conditions known to influence those numbers (such as autoimmune or viral diseases) will be excluded.

The Chi-square test will be used to test the fitness of data to the normal distribution. Independence between categorical data was tested using Chi-square test. The Yates correction will be used when small samples (<5) were tested.

All statistical tests will be performed at 0.05 level of significance and all p values are two-sided.

Data will be analyzed by SPSS, version 17.

8. Tarefas | Tasks

8.1. Fase pré-projecto |Pre Project

- Literature review and project elaboration
    - Sandra Morais
    - January to March, 2009
- Identification of participants
    - Prof. Doutora Graça Porto
    - April to August, 2009

8.2. Fase projecto | Project

- Clinical data registering
    - Sandra Morais, under supervision of Prof. Doutora Graça Porto
    - September to December, 2009.
- Execution of laboratory exams
    - Sandra Morais, helped by Mónica Costa and Susana Almeida
    - September 2009 to March 2010
- Statistical analysis

- Jorge Vieira and Sandra Morais, under supervision of Prof. Doutora Graça Porto
    - January to March, 2010.
- Data interpretation and discussion / conclusions
    - All
    - April to May, 2010

9. Indicadores de produção científica | Scientific deliverables

The research proposal will be presented in the Clinical Hematology Service and at the "Jornadas de Iniciação à Investigação Clínica" (June 2009).

The results will be presented as oral communication or poster at the "Jornadas de Iniciação à Investigação Clínica" (June 2010).

Efforts will also be made in order to publish a paper in a peer-review journal.

10. Bibliografia

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2. Cardoso CS, Oliveira P, Porto G, Oberkanins C, Mascarenhas M, Rodrigues P, Kury F and De Sousa M. Comparative study of the two more frenquent HFE mutations (C282Y and H63D): significant different allelic frequencies between the North and South of Portugal. European Journal of Human Genetics. 2001, 9:843-848.
3. Cruz E, Melo G, Lacerda R, Almeida S, Porto G. The CD8+ T-lymphocyte profile as a modifier of iron overload in HFE hemochromatosis: an update of clinical and immunological data from 70 C282Y homozygous subjects. Blood Cells Mol Dis 2006a, 37:33-9.
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11. Kohgo Y. Hemochromatosis: Genetics, Pathophysiology, Diagnosis and Treatment. Edited by James C. Barton and Corwin Q. Edwards. Cambridge University Press, Cambridge, UK, 2000, J Gastroenterol 2001, 36:69.
12. Porto G, Cardoso CS, Gordeu, Cruz E, Fraga J, Areias J, Oliveira JC, Bravo F, Gangaidzo IT, MacPhail AP, Gomo ZA, Moyo VM, Melo G, Silva C, Justica B, de Sousa M. Clinical and genetic heterogeneity in hereditary hemochromatosis: association between lymphocyte counts and expression of iron overload. Eur J Haematol 2001, 67:110-8.
13. Porto G, Cardoso CS, Macedo F, and Cruz E. Hereditary Hemochromatosis type I: Genetic, clinical and immunological aspects. Iron Metabolism and Disease. 2008:435-460.
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15. Reimão R, Porto G and de Sousa M. Stability of CD4/CD8 ratios in man: new correlation between CD4/CD8 profiles and iron overload in idiopathic haemochromatosis patients. C R Acad Sci III. 1991, 313:481-7.
16. Ryan E, Byrnes V, Coughlan B, Flanagan A-M, Barrett S, O'Keane JC and Crowe J. Underdiagnosis of hereditary haemochromatosis: lack of presentation or penetration? Gut 2002, 51:108–112.

17. Vieira J, Cardoso CS, Pinto J, Patil K, Brazdil P, Cruz E, Mascarenhas C, Lacerda R, Gartner A, Almeida S, Alves H, Porto G. A putative gene located at the MHC class I region around D6S105 marker contributes to the setting of CD8+ T-lymphocyte numbers in humans. Int J Immunogenet 2007, 34:359-67.

B. QUESTÕES ÉTICAS | ETHICAL CONSIDERATIONS

This project is part of a HH study that was already approved by the ethical committee of Hospital.

Patients included in this study have previously signed the Informed Consent terms in use in the CGPP (Attachments 2 and 3).

C. PLANO FINANCEIRO | BUDGET AND FUNDING

1. Orçamento | Budget

Não haverá episódios (consultas, internamentos, etc.), exames, análises ou outros procedimentos no CHP.

Não haverá custos para o CHP.

	Custo estimado [1]	
	90 participantes [2] (2 primers – 180 amostras)	180 participantes [3] (2 primers – 360 amostras)
Despesas relacionadas com os estudos genéticos (primers, despesas de sequênciação, outros reagentes de laboratório, material de laboratório, etc.)	3234,6 Euros	6469,2 Euros
Transporte de amostras, material administrativo e outros consumíveis	100,00 Euros	100,00 Euros
Sub-Total	3334,60 Euros	6569,20 Euros
Poster	50,00 Euros	50,00 Euros
Inscrição aluno em Congresso da Especialidade	200,00 Euros	200,00 Euros
Organização das Jornadas de Iniciação à Investigação Clínica	50,00 Euros	50,00 Euros
Sub-Total	300,00 Euros	300,00 Euros

TOTAL	**3634,60 EUROS**	**6869,20 EUROS**

2. Financiamento | Funding

This project will be partially financed by Roche Farmacêutica (Bolsa de Iniciação à Investigação Clínica – Programa Roche/ICBAS/HSA 2008/2010).

[1] Custo aproximado de cada primer: 7,02 euros; Despesas relacionadas com a sequênciação: 10,95 euros.
[2] Financiamento solicitado – Bolsa de Iniciação à Investigação Clínica.
[3] Se forem encontradas outras fontes de financiamento para além da Bolsa de Iniciação à Investigação Clínica, serão estudados 180 indivíduos.

ABREVIATURAS e ACRÓNIMOS | ABBREVIATIONS

CGPP, Centro de Genética Preventiva e Predictiva

CHP, Centro Hospitalar do Porto

DIIC, Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS

ESTSP, Escola Superior de Tecnologia da Saúde do Porto

FCUP, Faculdade de Ciências da Universidade do Porto

FFUP, Faculdade de Farmácia de Universidade do Porto

FMUP, Faculdade de Medicina da Universidade do Porto

HH, Hemocromatose hereditária

HSA, Hospitalar de Santo António

IBMC/UP, Instituto de Biologia Molecular e Celular da Universidade do Porto

ICBAS/UP, Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

JIIC, Jornadas de Iniciação à Investigação Clínica

ANEXOS | ATTACHMENTS

Anexo 1: Formulário de registo de dados clínicos | Clinical data registering formulary

Anexo 2: Requisição de testes genéticos | Formulary to request genetic studies (CGPP)

Anexos 3 e 4: Termos de consentimento informado | Informed consents (CGPP)

PEDIDOS DE AUTORIZAÇÃO INSTITUCIONAL

HSA/CHP

Carta ao Presidente do Conselho de Administração

Exmo. Senhor Presidente do Conselho de Administração do Centro Hospitalar do Porto
Dr. Sollari Allegro

SANDRA ISABEL ALVES MORAIS, na qualidade de Aluna da Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS/HSA, vem por este meio, solicitar a Vossa Exa. autorização para realizar no Centro Hospitalar do Porto o Estudo de Investigação / Trabalho Académico acima mencionado, de acordo com o programa de trabalhos e os meios apresentados.

Data Assinatura

Carta à Presidente da Comissão de Ética

Exma. Senhora Presidente da Comissão de Ética para a Saúde do Centro Hospitalar do Porto
Dra. Luísa Bernardo

SANDRA ISABEL ALVES MORAIS, na qualidade de Aluno da Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS/HSA, vem por este meio, solicitar a Vossa Exa. autorização para realizar no Centro Hospitalar do Porto o Estudo de Investigação / Trabalho Académico acima mencionado, de acordo com o programa de trabalhos e os meios apresentados.

Data Assinatura

Carta ao Director do Departamento de Ensino, Formação e Investigação

Exma. Senhora Directora do Departamento de Ensino, Formação e Investigação do Centro Hospitalar do Porto
Prof. Doutora Margarida Lima

SANDRA ISABEL ALVES MORAIS, na qualidade de Aluna da da Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS/HSA, vem por este meio, solicitar a Vossa Exa. autorização para realizar no Centro Hospitalar do Porto o Estudo de Investigação / Trabalho Académico acima mencionado, de acordo com o programa de trabalhos e os meios apresentados.

Data Assinatura

PEDIDO DE FINANCIAMENTO

Bolsa de iniciação à investigação clínica

Carta ao Presidente do Conselho Directivo

Exmo. Senhor Presidente do Conselho de Directivo do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Prof. Doutor António Sousa Pereira

SANDRA ISABEL ALVES MORAIS, na qualidade de Aluno da Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS/HSA, vem por este meio, solicitar a Vossa Exa. a atribuição de 6869,20€ (seis mil oitocentos e sessenta e nove euros e vinte cêntimos) da Bolsa de Iniciação à Investigação Clínica da Roche Farmacêutica, SA, para financiamento do Estudo de Investigação / Trabalho Académico acima mencionado, de acordo com orçamento apresentado.

Data Assinatura

TERMOS DE RESPONSABILIDADE

Investigador Responsável

Termo de Responsabilidade do Investigador Principal e Responsável no CHP

Eu, abaixo assinado, MARIA DA GRAÇA BESSA GONÇALVES PORTO, Médica, Especialista de Imunohemoterapia do Serviço de Hematologia Clínica do HSA/CHP, na qualidade de Investigador Principal e Responsável no HSA/CHP, declaro assumir a liderança científica do Estudo de Investigação / Trabalho Académico acima mencionado, de acordo com o programa de trabalhos e os meios apresentados e com as normas internas da Instituição.

Data Assinatura

DECLARAÇÕES DE ALUNOS E ORIENTADORES / SUPERVISORES

Aluno

Declaração do Aluno

Eu, abaixo assinado, SANDRA ISABEL ALVES MORAIS, na qualidade de Aluna da Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS / CHP, declaro que durante a realização do Trabalho Académico acima mencionado, respeitarei as normas éticas e deontológicas, que a identificação dos doentes não será revelada e que os dados necessários para a realização do trabalho serão mantidos anónimos e não serão utilizados para qualquer outro fim.

Data Assinatura

Orientadores / Supervisores

Declaração do Orientador

Eu, abaixo assinado, MARIA DA GRAÇA BESSA GONÇALVES PORTO, Médica, Especialista de Imunohemoterapia do Serviço de Hematologia Clínica do HSA/CHP, na qualidade de Orientador de SANDRA MORAIS, Aluna da Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS/ CHP, declaro que me comprometo a acompanhar a aluna nas diferentes fases da realização do Estudo de Investigação / Trabalho Académico acima mencionado, responsabilizando-me por supervisionar a recolha e utilização dos dados necessários para a realização, bem como zelar pelo cumprimento das normas éticas e deontológicas, nomeadamente para que os dados utilizados na realização do referido trabalho sejam mantidos anónimos e não sejam utilizados para qualquer outro fim.

Data Assinatura

Declaração do Supervisor / Responsável pela Disciplina

Eu, abaixo assinado, MARGARIDA MARIA DE CARVALHO LIMA, Médica, Especialista de Imunohemoterapia do HSA/CHP, na qualidade de Professora Responsável pela Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS/ CHP, declaro que me comprometo a acompanhar a aluna SANDRA MORAIS, Aluna da Disciplina de Iniciação à Investigação Clínica do Curso de Mestrado Integrado em Medicina do ICBAS/ CHP nas diferentes fases de realização do Estudo de Investigação / Trabalho Académico acima mencionado.

Data Assinatura

TERMOS DE AUTORIZAÇÃO LOCAL

Serviços

Autorização do Director do Serviço de Hematologia Clínica

MANUEL CÉSAR ARAÚJO CAMPO, na qualidade de Director do Serviço de HEMATOLOGIA CLÍNICA do HSA/CHP, declaro que autorizo a execução do Estudo de Investigação / Trabalho Académico acima mencionado e comprometo-me a prestar as condições necessárias para a boa execução do mesmo, de acordo com o programa de trabalhos e os meios apresentados.

Data Assinatura e carimbo

Departamentos

Autorização do Director do Departamento de Medicina

JOSÉ LOPES GOMES, na qualidade de Director do Departamento de MEDICINA do HSA/CHP, declaro que autorizo a execução do Estudo de Investigação / Trabalho Académico acima mencionado e comprometo-me a prestar as condições necessárias para a boa execução do mesmo, de acordo com o programa de trabalhos e os meios apresentados.

Data Assinatura e carimbo

Capitulo II

Desenvolvimento de trabalhos

Durante o desenvolvimento do projecto este teve de ser alterado devido a resultados obtidos entretanto que nos fizeram enveredar por uma nova linha de pensamento.

Esses resultados confirmavam a elevada frequência do haplótipo AAT relativamente ao haplótipo GGG na população portuguesa de doentes com Hemocromatose Hereditária (HH) bem como confirmavam os resultados prévios obtidos por Cruz e colaboradores (*Cruz E, Whittington C, Krikler SH, Mascarenhas C, Lacerda R, Vieira J, Porto G: A new 500 kb haplotype associated with high CD8+ T-lymphocyte numbers predicts a less severe expression of hereditary hemochromatosis. BMC Med Genet 2008, 9:97.*) relativamente à associação observada entre estes haplótipos restritos, o número de linfócitos T CD8+ e a expressão clínica da doença, sugerindo que poderiam constituir marcadores de prognóstico importantes. Apesar dessa forte associação, os haplótipos SNP restritos AAT ou GGG não explicavam, contudo, toda a variabilidade existente no número de linfócitos T CD8+. De facto, uma proporção importante de doentes homozigóticos para o AAT (33.3%) tem número de linfócitos T CD8+ elevados, o que demonstra a necessidade de estudar outros marcadores genéticos na região na tentativa de melhor perceber a contribuição da região MHC na determinação do número de linfócitos T CD8+. Tendo em conta o grande desequilíbrio de ligação nessa região (*Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R, Jr., Ellis MC, Fullan A et al: A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 1996,*

13(4):399-408.), uma análise combinada com marcadores genéticos mais distantes, como os alelos e haplótipos HLA, é necessária.

Assim à luz dos novos resultados tornou-se pertinente alterar o titulo do trabalho de "**Characterization of haplotypes AAT and GGG in a population of individuals homozygous for the C282Y mutation in HFE gene: contribution to the study of penetrance of hereditary hemochromatosis.",** para um titulo mais abrangente e que melhor descreve todos os resultados obtidos: "**A study of 147 extended haplotypes carrying the C282Y HFE mutation: a novel approach to explain the involvement of the MHC-class I region in the setting of CD8+ T-lymphocyte numbers in humans."**

Capitulo III

Redacção de artigo científico para submissão

A STUDY OF 147 EXTENDED HAPLOTYPES CARRYING THE C282Y HFE MUTATION: A NOVEL APPROACH TO EXPLAIN THE INVOLVEMENT OF THE MHC-CLASS I REGION IN THE SETTING OF CD8+ T LYMPHOCYTE NUMBERS IN HUMANS

Sandra Morais*[1], Mónica Costa[2], Andreia Bettencourt[3], Eugénia Cruz[2,4], Susana Almeida[2], Berta M Silva[3] and Graça Porto[2,4,5]

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Abstract

Background: The numbers of peripheral blood CD8+ T lymphocytes are known to be genetically determined in the context of genes at the MHC-class I region. The present study analyses, for the first time, the inheritance of CD8+T lymphocytes in the context of extended haplotypes defined between *HFE* and HLA-B by segregation analysis in families of Hereditary Hemochromatosis (HH) patients. With this novel approach we aimed to clarify the relative impact of the HLA specificities or the whole haplotype on the genetic transmission of CD8+T lymphocytes in humans.

Subjects and Methods: A total of 71 unrelated C282Y homozygous HH patients and 61 of their family members were studied for extended haplotype analysis, performed by segregation analysis for the SNP markers on PGBD1, ZNF193 and ZNF165 (defining the restricted SNP haplotypes AAT and GGG) and for the HLA-A and -B loci, all correlated with the numbers of CD8+T lymphocytes. In addition, 123 DNA samples from an independent population of C282Y homozygous subjects were tested to confirm the frequencies of the SNP markers in the Portuguese HH population.

Results: The relative frequencies of the AAT and GGG haplotypes in the population of C282Y homozygous HH patients was 94.3% and 4.9% respectively. We confirmed the strong association of the most common HLA-A*03, -B*07 and the A3B7-AAT ancestral haplotype with the inheritance of low CD8+T lymphocytes. The HLA-A*03 was the only HLA allele increased in frequency in patients with high lymphocytes. The definition of extended haplotypes allowed us to construct a model with all patients' chromosomes classified as carrying a “low” or “high CD8+ trait” and to compare the distribution

of HLA alleles and haplotypes between the two groups. The results clearly showed a greater allelic and haplotypic variability in the “high” vs the “low CD8+ trait” group supporting the hypothesis of a different recombination history.

Conclusions: The study of extended haplotypes carrying the C282Y *HFE* mutation offers a new model to explain the contribution of the MHC region to the setting of CD8+T lymphocyte numbers. The results support the hypothesis of a putative still unidentified trait localized centromeric to HLA-A.

Background

Hereditary hemochromatosis (HH) is a primary inherited disorder of iron metabolism with progressive iron loading of parenchymal cells of the liver and other organs, which may eventually lead to tissue damage and dysfunction, generally by the fourth to fifth decades of life. Clinical consequences of iron accumulation include cirrhosis of the liver, hepatocellular carcinoma, diabetes, heart failure, arthritis and hypogonadism (reviewed in [1]). Independently of the presenting clinical picture, HH should be suspected in any subject with clinical and/or laboratory evidence of severe iron overload, in the absence of other known iron loading conditions such as chronic liver disease or hematological disorders. Typically, both transferrin saturation (TfSat) and serum ferritin are abnormally high and normally represent the first phenotypic manifestation of HH [1].

HH represents the most common form of inherited iron overload and one of the most common hereditary metabolic diseases in Caucasians [2]. It is characterized by an autosomal recessive pattern of inheritance associated with the C282Y mutation of the HFE gene, a non-classical MHC class I gene 5 Mb telomeric to HLA-A at chromosome 6 (6p21.3) [3-4].

With origin in populations of northern European, over 90% of HH patients are homozygotes to the C282Y mutation in the *HFE* gene. About 1 in 200 people in the general population have this genotype [5]. In Portugal, the distribution of the C282Y mutation varies from north to south of the country with allele frequency reaching the 6% in the north [2].

In spite of the generally reported high frequency of the C282Y mutation in most European derived populations and the finding that most homozygotes for

the C282Y mutation of HFE show a common biochemical phenotype characterized by high transferrin saturation, the proportion of patients with a fully developed phenotype found in clinical practice seems to be very low [1].

HH is clinically very heterogeneous, varying from a simple biochemical abnormality to a full-blown picture of devastating iron overload disease [1] and recent large population screenings are revealing that the clinical penetrance of HH may be lower than previously thought [5]. Heterogeneity observed in iron accumulation and associated clinical presentation may be partially explained by gender, age and environmental factors [6]. There are also some genes, like those already associated with iron-storage disease such as transferrin, TfR2, ferroportin and ceruplasmin that could be good candidate genes at expression modifiers [1]. More recently there are growing evidences supporting the notion that other genes at the MHC class I region, possibly inherited with the ancestral C282Y containing haplotype are implicated in the clinical heterogeneity of HFE-associated HH [7].

Previous studies showed that patients with high CD4/CD8 ratios display a faster re-entry of iron into the serum transferrin pool after intensive phlebotomy treatment, reaching abnormal transferrin saturation values more rapidly than patients with normal CD4/CD8 ratios [8]. The abnormalities in lymphocyte populations were systematically found in CD8+ T cells and the association with total body iron stores (TBIS) could also be reflected in the total lymphocyte count [1]. A significant inverse relationship of total blood lymphocyte counts and severity of iron overload in HH patients with HFE C282Y homozygosity was also described [9].

By extended linkage disequilibrium analysis in the hemochromatosis gene region it was found a highly significant association of the allele 8 of D6S105 microsatellite, 2Mb away from HFE gene, and the C282Y homozygous patients. Moreover, male patients, with the two copies of the allele 8 of this microsatellite, were found to have significantly higher hepatic iron indices than those heterozygous or nullizygous for this allele. Thus, it was suggested that a gene modifying the phenotype of C282Y homozygotes may be localized around the area of D6S105 [10].

As mentioned previously, low numbers of CD8+ T cells, both in the peripheral blood and in the liver, were shown to be negatively correlated with total body iron stores [1] and consequently have a more severe expression of HH [8]. This immunophenotype is proposed to be a clinical marker of the severity of iron overload in HH patients [11]. By sibpair analysis in hemochromatosis families it was shown that CD8+ T lymphocyte numbers are genetically regulated by genes in the MHC region, and for the first time was demonstrated in humans that CD8+ T lymphocytes are genetically determined in association with HLA [12]. More recently it has been shown that a genetic co-dominant trait associated with the transmission of CD8+ T lymphocyte numbers is also demonstrated in the normal population and it is localized at the MHC class I region close to the D6S105 microsatellite marker [13].

Previous studies performed in a group of 56 HH patients suggested that a highly conserved 500 Kb ancestral haplotype (around the microsatellite D6S105) defined by the SNP markers PGBD1-A, ZNF193-A, ZNF165-T (AAT haplotype) marks the inheritance of “low” CD8+ T lymphocyte numbers and predicts the development of a severe clinical expression of HH (in terms of iron

overload and clinical manifestations) [14]. The referred evidences led to the formulation of the hypothesis that a putative gene controlling CD8+ T cells numbers, localized on the MHC region, could be the same gene contributing to the clinical heterogeneity observed in HH [1, 14]. An important pending question is if the genetic modifier determining CD8+ T lymphocyte numbers is “the HLA itself” or if there another “still unidentified gene” transmitted in linkage with HLA and HFE, as suggested by the associations with AAT/GGG [14] and therefore a relevant genetic modifier.

So far, all association studies relating CD8+ T lymphocyte numbers with the MHC-class I region [12-15] were performed using information on particular alleles or small haplotypes only. In the present study we analyse, for the first time, the inheritance of CD8+ T lymphocytes in the context of extended haplotypes defined between *HFE* and HLA-B by segregation analysis in families of HH patients. With this novel approach we aimed to clarify the relative impact of the HLA specificities or the whole haplotype on the genetic transmission of CD8+ T lymphocytes.

Subjects and Methods

SAH Study Population

Patients

A total of 71 unrelated Hereditary Hemochromatosis (HH) patients, regularly followed-up at the Hemochromatosis Outpatient Clinic of Santo António Hospital (SAH) in Porto, were analyzed for the present study. This population consisted of 49 males (mean age 48 ± 14 years, range 6–75) and 22 females (mean age 45 ± 14 years, range 18–67). Clinical and genetic information from these subjects was available from their hospital clinical records. All subjects had been previously genotyped for *HFE* mutations and they were all selected by the sole criterion of being homozygous for the *HFE* C282Y mutation. Most HH patients (n=56) had been previously genotyped for HLA (on the loci HLA-A and HLA-B) and other SNP markers at the MHC-class I region (on the PGBD1, ZNF193 and ZNF165 genes) in the context of other previously published studies [12, 14-15]. The remaining 15 were selected for genotyping for the SNP marker on PGBD1 (assumed to be inherited in linkage disequilibrium with the other haplotypic SNP markers on ZNF193 and ZNF165) and for the HLA loci –A and –B for the purpose of the present study. Only 3 patients could not be SNP genotyped because there were not DNA samples available. The study was approved by the ethical committee of Santo António Hospital including an informed consent obtained from subjects according to the Helsinki declaration.

Family members

In order to define the extended haplotypes in the above described population of unrelated subjects, as well as to test the relationship between the extended haplotypes and the CD8+ T cell profile, data from patients' first degree relatives were also analyzed. For this purpose, we reviewed the genetic and phenotypic data from a total of 61 selected subjects belonging to 32 different families whose information was available on the hospital clinical database since it had been previously performed in the course of a family screening program for hemochromatosis. The selection of subjects was done in order to get informative cases for the purpose of: 1) genotype/phenotype correlations (on C282Y homozygous subjects only) and 2) for haplotype definition by segregation analysis (non-identical siblings or offsprings). The family population included 48 C282Y homozygous (28 males, mean age 35 ± 17 years, range 4–63, and 20 females, mean age 46 ± 14 years, range 19–65) and 13, non-C282Y homozygous, genetically informative relatives (10 males and 3 females). Information on HLA and SNP genotyping was available in all except 23 subjects who were therefore genotyped for the purpose of the present study using frozen DNA that had been previously stored with their informed consent for its use for research purposes. In 16 families it was not possible to obtain familiar information either because there were not informative cases available or because there was not stored DNA available. In these cases, the haplotype inference was done using the PHASE program (see below).

CGPP HH population

In order to confirm the expected frequency of the restricted SNP haplotypes AAT and GGG (as defined by Cruz and co-workers in 2008 [14]) in a larger Portuguese HH population, an additional independent sample of unrelated C282Y homozygous HH patients was genotyped for the haplotypic SNP marker on PGBD1, assumed to be inherited in linkage disequilibrium with the other haplotypic markers on ZNF193 and ZNF165 (see Table 1). This population consisted of a total of 123 patients (97 males, mean age 45 ± 14 years, range 17–74; 26 females, mean age 49 ± 14 years, range 25–74) that had been referred for the genetic diagnosis of HH at the Predictive and Preventive Genetic Center (CGPP) in Porto between 1997 and 2009, and they were selected by the sole criterion of being homozygous for the C282Y mutation. Seventeen of these (8 males and 9 females) were found to be identical siblings of other patients and therefore their information was not taken into account to estimate the frequency of the restricted SNP haplotypes. Frozen DNA samples were available from all subjects, and they all had been previously stored with the patients' informed consent to use for research purposes.

Control population

For the purpose of comparing the HLA allele and haplotype frequencies between HH patients and a representative normal Portuguese control population from the same geographical region, the published data [16], from a total of 7937 individuals (corresponding to 15874 HLA-A and -B alleles and 15874 HLA haplotypes) from the region of Porto, Portugal was used. These

data are available online at: “New Allele Frequency Database: http://www.allelefrequencies.net.

Genetic characterization of subjects

HFE genotyping

All subjects included in the study had been previously genotyped for the *HFE* mutations C282Y and H63D at the Predictive and Preventive Genetic Center, Porto (CGPP), between 1997 and 2009, by using the Haemochromatosis Strip Assay (Vienna Laboratory, Vienna, Austria) based on PCR amplification of the gene with subsequent detection of the mutations by allele-specific hybridization with oligonucleotide probes immobilized in test strips. Genotype information was available in their clinical records. All HH patients and their identical siblings were homozygous for the C282Y mutation. Family subjects selected as informative cases for haplotype definition were heterozygous for the C282Y mutation on *HFE*.

SNP genotyping

For the purpose of defining the restricted SNP haplotypes designated as “AAT” and “GGG” by Cruz and co-workers in 2008 [14] in the populations of HH patients from CGPP or from HSA who had not been previously characterized for these haplotypes (see populations descriptions above), 195 DNA samples were genotyped for the SNP rs1997660 in the piggyBac transposable element derived 1 gene (PGBD1) localized in the 6p21.3 region, assumed to be inherited in linkage with the other two SNPs that define the restricted haplotype, i.e. the SNP rs7206 in the zinc finger protein 193 (ZNF193) and the SNP

rs203878 in the zinc finger protein 165 (ZNF165). Since the GGG haplotype in HH patients is known to be much less conserved than the AAT haplotype [14], its presence was always confirmed by further testing all samples carrying the G-allele on PGBD1 for the other 2 SNPs on ZNF193 and ZNF165.

SNP genotyping of PGBD1 and ZNF193 were performed by Allele-Specific PCR (AS-PCR). AS-PCR consists of two PCR reactions each one using specific primers differing at 3' end matching one or the other nucleotide variant of the SNP. Positive controls (homozygous for the allele variant) and negative controls (without the allele) were used in all PCR reactions. The PCR products were electrophoresed and sample amplifications were compared with the specific controls. The two SNPs (rs1997660 on PGBD1 and rs7206 on ZNF193) gathered optimal conditions for AS-PCR. SNP genotyping of rs203878 on ZNF165 was performed by gene sequencing. Briefly, genomic DNA (gDNA) was extracted from peripheral blood or stored. gDNA and amplicons containing the selected loci were PCR-amplified using specific primers. Amplicons were then electrophoresed and extracted from the gel whit the QIAquick Gel Extraction Kit (Quiagen). Sequencing reaction was prepared with the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and loaded in an ABI prism 310 Genetic Analyzer Sequencer (Applied Biosystems).

HLA-A and -B genotyping

Seventy-three subjects had been previously genotyped for HLA-A and B loci [12, 15, 17-18] and this information was available in their clinical records. In the context of the present study, HLA-A and –B genotyping was performed in 70 additional DNA samples from the subjects described above. For this purpose,

DNA was amplified by polymerase chain reaction-sequence specific primers (PCR-SSP) for HLA class I alleles, loci A and B, based on standard well described methods [19].

Clinical characterization of subjects

Phenotypic data from the subjects included in study were available from their clinical records at Santo António Hospital. Clinical information from these patients was partially described in detail elsewhere [11, 20]. For the purpose of characterization of patients in terms of clinical expression, they were divided in two groups according to the presence or absence of HH related clinical manifestations. These included the presence of one or more of the following manifestations: liver cirrhosis /fibrosis, diabetes, arthropathy, hypopituitarism, skin pigmentation or cardiac abnormalities. As a measure of the severity of iron overload we used the estimation of Total Body Iron Stores (TBIS) determined by quantitative phlebotomies [21]. The patients' immunophenotype was defined by the total number of peripheral blood CD8+ T lymphocytes determined by FACS analysis as described in detail elsewhere [12]. For the purpose of analysis, a number of CD8+ T lymphocytes $\leq 0,41 \times 10^6$/mL is considered a "low" CD8+ profile, contrasting with the "high" profile when the CD8+ T lymphocyte numbers are above that limit. This cut-off value was defined based on the median value of the parameter previously established in a control population from north Portugal [12]. For the purpose of analysis of the CD8+ lymphocyte profile, some patients were excluded based on the following criteria: presence of lymphocytosis related with the age of the subjects (1 subject aged below 18

years), positive viral markers for hepatitis B virus (HBV) (n=1) and associated immunological disorders (n=1).

Extended haplotype definition

For the purpose of this study, extended haplotypes were defined by segregation analysis, by comparing the genotype information of the individuals homozygous for C282Y mutation with their informative family members. Extended haplotypes were defined with the genotype information of the following genetic markers: PGBD1, ZNF193 and ZNF165 and HLA-A and B locus. In subjects where no familiar information was available, the extended haplotypes were inferred using the program PHASE (http://www.stat.washington.edu/stephens/software.html), as described previously [13]. In few cases (n=10), information was missing for some particular alleles (on SNP markers – PGBD1, ZNF193 or ZNF165). In those cases, missing alleles were inferred using PHASE.

Statistic analysis

The comparison between the frequencies of AAT and GGG restricted SNP haplotypes between two independent patient samples was done using the Chi-square Test for a statistical significance value of $P < 0.05$. Comparisons between HLA allele and haplotype frequencies in different groups were done by Fisher Exact Test. The statistical significance of the tests was considered for a P value < 0.05 with further Bonferroni's correction for multiple comparisons. This was done by multiplying the Fisher test P values by the number of allelic or haplotype comparisons. To analyze the relative strength in HLA allele or

haplotype associations, the etiological fraction delta (δ) was calculated as described according to the formula d= (FAD-FAP)/(1-FAP), where FAD is the allele frequency in the diseased population and FAP the allele frequency in the control population [22-23]. Linkage disequilibrium between the alleles was estimated by the standard D value, where D is the difference between the observed and expected frequencies of the two alleles in the same chromosome. The significance of the association was tested using the Fisher test and the statistical significance was considered for a *P* value < 0.05. Data were analyzed by Statgraphics software (Statgraphics Graphics System, version 7.0).

To better understand the involvement of the MHC-class I markers on the setting of CD8+ T lymphocyte numbers, two different models were tested, where rules were generated for the prediction of the CD8+ T lymphocyte profile ("low" or "high" profile) in individual HH patients: 1) the first model assumes that the CD8+ T lymphocyte profile is predicted with information on HLA haplotypes, independently of the other SNP markers in the extended haplotypes; 2) and a second model where information on the restricted SNP markers is also included. The rules generated for each model were applied to the HH population under study and the predictive power (or sensitivity) as well as the specificity of each model were estimated. Sensitivity was determined by the formula: TP/(TP+FP) where TP means the number of true positive results and FP the number of false positive results. Specificity was determined by the formula: TN/(TN+FN) where TN means the number of true negative results and FN the number of false negative results.

Results

1. AAT and GGG restricted SNP haplotype frequencies in a representative sample of Portuguese HH patients

Previous studies performed by our group, using a population of 56 HH patients, have suggested that a highly conserved 500 Kb ancestral haplotype defined by the SNP markers PGBD1-A, ZNF193-A, ZNF165-T (AAT haplotype) marks the inheritance of "low" CD8+ T-lymphocyte numbers and predicts the development of a severe clinical expression of HH (in terms of iron overload and clinical manifestations). On the other hand, haplotype defined by the SNP markers PGBD1-G, ZNF193-G, ZNF165-G (GGG haplotype) marks the inheritance of "high" CD8+ T-lymphocyte numbers and predicts the development of a less severe clinical expression of HH [14].

In order to confirm the previous results in a larger and more representative population of Portuguese HH patients, the restricted SNP haplotypes were analyzed in the present SAH study population (n=71) and in an additional independent HH population designated here as CGPP population (see Subjects & Methods). The results are summarized in Table 1. In the SAH population we observed an haplotype frequency of 89.4% (n=127/142) for the AAT haplotype, 6.3% (n=9/142) for the GGG haplotype and 4.2% (n=6/142) for other haplotypes. A similar distribution was observed in the independent CGPP population where haplotype frequencies of 94.3% (n=232/246) for the AAT haplotype, 4.9% (n=12/246) for the GGG haplotype and 0.8% (n=2/246) for other haplotypes were observed. A comparison between the frequencies observed in the two populations did not show any statistical difference.

A summary of the clinical phenotype of HH patients in terms of the presence or absence of symptoms, the total body iron stores (TBIS) at diagnosis, and the average number of CD8+ T lymphocytes, is also shown in Table 1, in relation to the restricted SNP haplotypes. The results show that 53.5% of the patients with the AAT haplotype (n=31/58) were symptomatic patients, had on average 8.5 ± 4.7g of TBIS at diagnosis, and the average CD8+ T-lymphocyte count was 0.37 ± 0.2 x 10^6/mL. In contrast, only 12.5% (n=1/8) of the patients with the GGG haplotype were symptomatic, their average TBIS at diagnosis was 3.7 ± 3.3g and the average number of CD8+ T-lymphocytes was 0.58 ± 0.2 x 10^6/mL. These results confirm the high frequency of AAT relative to GGG in the Portuguese HH population and confirm the previous observations by Cruz and co-workers [14] of the association among those restricted haplotypes, CD8+ lymphocyte numbers and the clinical expression of the disease, suggesting that they could constitute important prognosis markers. In spite of its strong association, the restricted SNP haplotypes AAT or GGG do not explain, however, all the variability in CD8+ T lymphocyte numbers. In fact, an important proportion of AAT homozygous patients (33.3%) have high CD8+ counts, showing that other markers in the region are still necessary to clarify the contribution of the MHC region on the setting of CD8+ T lymphocyte numbers. Given the known extreme linkage disequilibrium in this region [3], a combined analysis with more distant markers, such as HLA alleles and haplotypes is warranted.

2. HLA allele and haplotype frequencies in C282Y carrying chromosomes

The frequencies of HLA-A and HLA-B alleles were estimated in the group of unrelated C282Y homozygous subjects from the SAH study population and compared with a control population representative of the Portuguese normal population from the same geographical region (see table 2). As expected [18, 24], after performing Bonferroni's correction for multiple comparisons, the HLA alleles A*03 and B*07 were the most commonly found in C282Y homozygous subjects highly significantly different from controls (0.408 for A*03 and 0.238 for B*07 *vs* 0.110 and 0.060 respectively in controls; all $P < 0.0001$). As also expected [18, 24] the strength of this association, as shown by the etiological fraction delta, is stronger for the HLA A*03 ($\delta = 0.335$) than the B*07 ($\delta = 0.189$). The HLA allele B*60 was also more common in the population of C282Y homozygous subjects as compared with the control population (0.014 *vs* 0.001; $P < 0.0001$), but the strength of this association, as shown by the etiological fraction delta, is very weak ($\delta = 0.014$) in comparison to those observed for the HLA alleles A*03 and B*07. To eliminate the eventual artificially lowered frequencies of other alleles that were due to the extra space occupied by HLA alleles A*03 and B*07, a prevalence's correction was done in our population according to the method described by Simon et al. (1987) [24]. Accordingly, the frequency of other HLA alleles was calculated by subtracting from the denominator the number of HLA-A*03 or –B*07 alleles, respectively, but no more significant associations were observed and therefore these data are not shown.

Segregation analysis by family studies, associated with haplotype inference by the program PHASE (see Methods), was done in order to define

the extended haplotypes associated with the *HFE* C282Y mutation in Portuguese HH patients. From a total of 76 C282Y homozygous individuals (the 71 unrelated patients from the SAH study population plus 5 haploidentical siblings in whom only the new haplotype was retained), a total of 147 extended haplotypes were defined. The results are summarized in Table 3 where the frequencies of the extended haplotypes are compared with the expected haplotype frequencies in the normal population (these expected values were estimated with information on HLA haplotypes only). The A3B7-AAT extended haplotype was the most common with a frequency of 0.177 ($P < 0.0001$ for the expected normal frequency). Other extended haplotypes, namely A2B7-AAT, A3B14-AAT, A3B27-AAT, A3B40-AAT, A32B40-AAT, A2B51-AAT and A3B51-AAT, were also more common in C282Y homozygous subjects as compared with the control population. However, the strength of association in these haplotypes, as shown by the etiological fractions delta, were very weak ($\delta<0.06$) in comparison to the much stronger association of the A3B7-AAT extended haplotype ($\delta=0.166$). In addition, the haplotype A3B7-AAT was the only one where the HLA alleles were in linkage disequilibrium ($P = 0.0223$). Like in the analysis of HLA alleles, a prevalence correction was performed to eliminate the eventual extra space occupied by the HLA haplotype A3B7 and no changes in results were observed (data not shown).

3. Relative impact of the HLA haplotype or the full extended haplotype on the setting of CD8+ T lymphocyte numbers

In order to test the relative impact of the HLA haplotypes or the full extended haplotype (including the information on restricted AAT or GGG

haplotypes) on the CD8+ T lymphocyte profile, we tested two different models where different rules were generated for the prediction of the CD8+ lymphocyte profile in individual patients. The first model was designed to test the hypothesis that the combination of particular HLA specificities alone can predict the CD8+ T lymphocyte profile (model A). The second model was designed to test the hypothesis that the CD8+ T lymphocyte profile can be better explained by the inclusion of genetic information of the SNP markers on an extended haplotype (model B).

3.1. Predicting the CD8+ T lymphocyte profile based on HLA haplotypes (Model A)

This model was tested assuming that the inheritance of CD8+ T lymphocyte numbers is mainly dependent on the HLA haplotypes alone. Based on the previous evidence by Vieira and co-workers [13] that alleles determining a high CD8+ T lymphocyte profile are dominant over the alleles determining a low profile, it was assumed that the HLA haplotypes observed in individuals with an unequivocal low CD8+ profile (arbitrarily established below 0,35 $x10^6$/mL) should always predict a low CD8+ profile. Using a sub-population of 37 subjects with unequivoval low CD8+ profiles, 29 HLA haplotypes were assigned: A3B7, A3B8, A3B14, A3B18, A3B27, A3B35, A3B40, A3B44, A3B51, A2B7, A2B14, A2B15, A2B27, A2B44, A2B51, A1B8, A1B15, A1B35, A1B44, A1B57, A11B8, A24B27, A24B35, A26B35, A26B40, A29B58, A32B18, A32B40, A33B14. As expected from the previous results by Cruz et al. (2006) [15] the large majority of these haplotypes (82.4%) carry the most common HLA alleles A*03, A*02 or A*01. We next generated a rule where all individuals who carry two of the

assigned haplotypes are expected to have a low CD8+ profile, while individuals who carry only one or none of these haplotypes are expected to have a high profile. We tested the model in a population of 84 C282Y homozygous subjects (65 probands and 19 family members, all having information on CD8+ T lymphocyte numbers) and the results show that this predictive model has a sensitivity of 95.8% (46/48 correctly predicted positive outcomes) but a specificity of only 66.7% (24/36 correctly predicted negative outcomes), in accordance with data previously published by Vieira et al. (2007) in a normal population where they could better predict correctly low numbers of CD8+ T lymphocytes than high numbers [13]. The finding of a low specificity of the predictive model may signify either those other independent factors are influencing the high CD8+ T cell numbers or that other markers in the region are better predictors, an hypothesis further explored with the next model.

3.2. Predicting the CD8+ T lymphocyte profile based on full extended haplotype information (Model B)

This model was designed to test if the inclusion of genetic information on the SNP markers on PGBD1-A, ZNF193-A, ZNF165-T on the extended haplotype could increase the sensitivity and/or specificity of the previous model. For that purpose, we used the previous rule on HLA haplotype information predicting a low CD8+ T lymphocyte profile and added a new rule where the presence of the restricted SNP haplotype GGG predicts a high CD8+ T lymphocyte profile. By applying this model to the same population of 84 C282Y homozygous subjects, the test sensitivity decreased from 95.8 to 91.7% and the test specificity increase from 66.7 to 69.4%. In spite of the slight increase in the

specificity of this model, it turned to be instead less sensitive, therefore it still does not explain the association, implying that a putative genetic trait determining the numbers of CD8+ T lymphocytes is not any of the tested markers but could be still another unidentified associated marker or a combination of more than one marker in the region. Work is on progress to further enlarge the region of interest to look for one or more candidate gene(s) by a deep sequencing study of the whole region including all HLA loci.

4. A new working model assigning a "low" or "high CD8+ trait" in each C282Y carrying chromosome

Globally, the results presented above support the hypothesis that the inheritance of CD8+ T lymphocyte numbers may be associated with an independent trait localized in the MHC-class I region in strong linkage with the other genetic markers tested but its precise localization is still elusive. As an approach to test that hypothesis, a new working model was designed where, based on the extended haplotype information plus the associated CD8+ lymphocyte profile, a putative genetic trait of "low" or "high" CD8+ T lymphocytes was assigned to each C282Y carrying chromosome from the study population. Based on the known distribution of CD8+ T lymphocyte numbers in the population [8, 11, 25] we considered that the putative genetic trait associated with CD8+ T lymphocyte numbers is transmitted in a co-dominant mode. For the purpose of constructing the model, all extended haplotypes observed in subjects with unequivocal low CD8+ T lymphocyte numbers (arbitrarily established below 0.35 $x10^6$/mL such as in model A) were assumed to be linked to a "low CD8+ trait". On the contrary, all extended haplotypes

observed in subjects with unequivocal high CD8+ T lymphocyte numbers (arbitrarily established above 0.45 x10^6/mL) were assumed to be linked to a "high CD8+ trait". Finally, in all subjects whose CD8+ lymphocyte numbers were between 0.35 and 0.45 x10^6/mL, we considered that they inherited a combination of a "low" with a "high CD8+ trait". In each of these individuals their extended haplotypes were compared with those previously assigned as "low" or "high" and classified accordingly. If they carried an extended haplotype that had not been assigned before, it was assigned as "low CD8+ trait" if he carried a "high CD8+ trait" in trans, and was assigned as "high CD8+ trait" if he carried a "low CD8+ trait" in trans. With this strategy, we were able to classify all the 147 extended haplotypes with 100% fitness.

Having constructed the model with all patients' chromosomes classified as carrying a "low" or "high CD8+ trait", we next tested the hypothesis that they could have had an independent recombination history. This was done by comparing the distribution of HLA alleles and haplotypes between the two groups of chromosomes (see next chapters).

5. HLA allele and haplotype frequencies in C282Y carrying chromosomes according to the CD8+ trait

In order to explore the recombination history of C282Y carrying chromosomes associated with a "low" or "high CD8+ trait", the frequencies of HLA-A and HLA-B alleles and HLA haplotypes were estimated in the 147 extended haplotypes from the C282Y homozygous patients studied, and compared, first with the expected frequencies obtained from the control

population and then compared between the two groups of chromosomes with a "low" or "high CD8+ trait" (results summarized in Tables 4-6).

5.1 HLA allele frequencies in C282Y chromosomes carrying a "low CD8+ trait"

As shown in Table 4, after applying a Bonferroni's correction for multiple comparisons, it was observed that, in chromosomes carrying a "low CD8+ trait", the HLA alleles A*02, A*03, A*24, A*29, B*07 and B*44 were significantly more common than in the control population (respectively 0.203, 0.456, 0.025, 0.013, 0.291 and 0.076 in patients' chromosomes *vs* 0.263, 0.110, 0.104, 0.053, 0.060 and 0.152 in controls; all $P < 0.005$). However, the strength of the association, as evaluated by the etiological fraction delta, was remarkably stronger for the HLA alleles A*03 and B*07, the association of A*03 being stronger than B*07 (δ-value=0.388 and 0.246, respectively). To eliminate the artificially lowered frequencies of other alleles that were due to the extra space occupied by HLA alleles A*03 and B*07, a prevalence's correction was done in our population according to the method described by Simon et al. (1987) [24]. Thus, the frequency of other HLA alleles was calculated by subtracting from the denominator the number of HLA-A3 or -B7 alleles, respectively. This corrected prevalence then allowed a more meaningful comparison between the frequencies of the HLA alleles in patients' chromosomes and in controls (see Table 4). With this correction performed, it is apparent that HLA alleles A*01 and B*40 are also significantly more common in chromosomes carrying a "low CD8+ trait" as compared with controls (0.279 and 0.161 *vs* 0.111 and 0.033

respectively; all $P < 0.005$) and that the association with the HLA allele A*01 is a strong one, as evidenced by the etiological fraction delta ($\delta = 0.189$).

5.2 HLA allele frequencies in C282Y chromosomes carrying a "high CD8+ trait"

In large contrast with the results observed in chromosomes carrying a "low CD8+ trait", after performing Bonferroni's correction for multiple comparisons in the "high CD8+ trait" group of chromosomes, we observed that only the HLA allele A*03 was significantly more common than in the control population (0.358 *vs* 0.110 respectively; $P < 0.0001$) with a strong association as evaluated by the etiological fraction delta ($\delta = 0.279$). Even after applying a prevalence's correction for the high frequency of HLA allele A*03, no other HLA allele appeared in significantly higher frequencies (data not shown). Curiously, the most common HLA-B allele in these chromosomes was not the B*07 (as expected by its higher frequency in HH in general) but the B*44 that is the most common allele in the normal population.

5.3 HLA haplotype frequencies in C282Y chromosomes carrying a "low CD8+ trait"

In terms of HLA haplotypes associated with a "low CD8+ trait", the A3B7 is the most common with a frequency of 0.203 ($P < 0.0001$). Other haplotypes were also significantly more common (see Table 5) but only the A3B7 haplotype had a strong association as revealed by the etiological fraction delta ($\delta = 0.192$). In these chromosomes, we confirmed the linkage disequilibrium between the HLA-A*03 and –B*07 alleles ($P = 0.0368$). A prevalence's correction was also

performed to compensate for the very high frequency of the haplotype A3B7, and after that, the HLA haplotypes A2B7 and A3B40 were also shown to be significantly more common in chromosomes carrying the “low CD8+ trait” as compared with controls (0.111 and 0.079 *vs* 0.0001 and 0.0001 respectively; all $P < 0.0001$), although only the A2B7 haplotype had a strong association as shown by the etiological fraction delta (δ-value=0.111).

5.4 HLA haplotype frequencies in C282Y chromosomes carrying a “high CD8+ trait”

Again, the results obtained for chromosomes carrying a “high CD8+ trait”, in terms of HLA haplotypes, largely contrast with those obtained for chromosomes carrying a “low CD8+ trait”. Although the HLA-A3B7 haplotype is also the most common, with a frequency of 0.151 ($P < 0.0001$), the strength of association is clearly lower (δ-value=0.140). Moreover, contrary to what is generally observed in C282Y carrying chromosomes, the linkage disequilibrium between HLA-A*03 and –B*07 is not confirmed ($P = 0.5552$). After applying the prevalence’s correction no other significant differences were observed.

5.5 Comparing HLA allele and haplotype distributions in C282Y chromosomes carrying a “low” or “high CD8+ trait”

We next compared HLA alleles and haplotype distributions in chromosomes grouped according to the “low” or “high” CD8+ trait and no significant differences were observed for either HLA-A or -B alleles between the two groups. The most striking result, however, was the evident greater allelic variability in the group with a “high CD8+ trait” in comparison to the group with

“low CD8+ trait” both for the HLA-A alleles (respectively a total of 12 different alleles in 53 chromosomes *vs* a total of 9 alleles in 79 chromosomes) and the HLA-B alleles (respectively a total of 17 alleles in 53 chromosomes vs a total of 13 alleles in 79 chromosomes). Haplotypic variability was also greater in the group with a “high CD8+ trait” in comparison to the group with “low CD8+ trait” (respectively a total of 36 different haplotypes in 53 chromosomes *vs* a total of 30 haplotypes in 79 chromosomes). Figures 1-3 are illustrative of these results.

Discussion

The present study was designed to explore the question of the association of the MHC-class I region with the genetic regulation of CD8+ T lymphocyte numbers in humans.

Low numbers of CD8+ T cells, both in peripheral blood and in the liver, are known to be negatively correlated with liver iron stores in HH [2, 11, 25]. Therefore, this immunophenotype profile is proposed to be a good clinical marker of the severity of iron overload in HH patients [11, 14].

It is known that CD8+ T lymphocyte numbers are genetically regulated, and partially influenced by genes localized at the MHC region. This was first demonstrated in humans in studies performed in HH patients [12]. Those studies also support the hypothesis that a putative MHC-linked gene(s) controlling CD8+ T cell numbers could be the same gene contributing to the clinical heterogeneity observed in HH [12-13, 15].

Based on the evidence described by Cruz et al (2008) [14], the highly conserved 500 Kb haplotype (around the microsatellite D6S105) defined by the SNP markers PGBD1, ZNF193, ZNF165 seemed to be a very promising region where the putative gene controlling CD8+ T cell numbers could be localized. In the present work, we confirmed a high frequency of the AAT relative to the GGG haplotype and its general association with low or high CD8+ T lymphocytes respectively. It would seem attractive to further narrow the region within this restricted haplotype trying to find a putative gene(s) involved in CD8+ T lymphocyte regulation. However, taking as an example the strategy used by Feder and co-workers when they discovered the HFE gene associated with HH [3] we decided as the next step to enlarge instead of trying to narrow the region

of interest, assuming the extreme linkage disequilibrium in the region [26]. Based on this assumption, we evaluated the MHC region from the *HFE* gene to the HLA-B, a region constituted by about 4Mb (gene markers used are, from centromeric position to telomeric position, HLA-B, HLA-A, PGBD1, ZNF193, ZNF165 and the *HFE* mutation) and correlated that information with the CD8+ T lymphocyte profile. This allowed us to construct a model of conserved extended haplotypes marking the inheritance of the putative genetic trait setting a "low" or a "high" CD8+ T lymphocyte profile.

With this approach, we observed a strong association among the HLA haplotype A3B7, the restricted haplotype AAT and the C282Y mutation, with a strong linkage disequilibrium between the HLA-A*03 and -B*07 alleles as described before [18, 24-25], confirming the remarkable conservation of an ancestral extended haplotype carrying the C282Y mutation [14]. This extended haplotype was the most commonly found in HH patients with both a low and a high CD8+ lymphocyte profile. But curiously, the pattern of linkage disequilibrium was different in chromosomes carrying or not a genetic trait associated with low CD8+ T lymphocytes. This was observed by an analysis of the HLA allele and haplotype distribution in two groups of chromosomes, i.e., those carrying a "low" and those carrying a "high CD8+ trait", here defined by a complex segregation analysis of extended haplotypes (see results, section 4). Although the HLA-A3B7 haplotype was the most commonly found in both groups, the linkage disequilibrium between the HLA-A*03 and -B*07 alleles was only observed in chromosomes carrying the "low CD8+ trait" suggesting that the two groups of chromosomes may have a distinct recombination history. In accordance to that is the fact that HLA diversity is much higher in chromosomes

carrying the “high CD8+ trait”, in spite of the lower number of chromosomes observed. In contrast, the “low CD8+ trait” chromosomes show a higher conservation not only of the most common HLA-A3B7 haplotype, but also other haplotypes (although with much less strong association) suggesting some kind of positive selection.

The fact that the only HLA allele significantly associated in all chromosomes (carrying either the “low” or the “high CD8+ trait”) is the HLA-A*03, in association with the extended haplotype A3B7-AAT, points to the conservation of the region between *HFE* mutation and HLA-A independently of CD8+ T cell numbers. This suggests that the putative CD8+ T lymphocyte trait may be localized centromeric to the HLA-A. Although in this study no direct association was found with the HLA-B locus, this does not rule out the hypothesis that the putative gene may be in this region. Recently, Ferreira et al (2010) demonstrated the existence of an imputed SNP (rs2524054) located in the MHC class I gene cluster (in HLA-C region, next to HLA-B) that influenced the CD4:CD8 ratio and CD8 levels [27]. The authors also show that the A allele of rs2524054 (class I) was associated with decreased CD8 levels. We still cannot rule out, however, the hypothesis that the candidate gene involved in the setting of CD8+ T-lymphocyte numbers may be in a more centromeric region in relation to HLA-B. Supporting that idea is the evidence of a higher allelic and haplotypic variability in the “high” comparatively to the “low CD8+ trait” carrying chromosomes. The best way to clarify the questions raised is to perform a deep sequencing of the whole region including all HLA loci in informative patients regarding their CD8+ T lymphocyte profiles.

The novel finding that the same ancestral haplotype A3B7-AAT is common in both low and high CD8+ T lymphocyte numbers (although much more commonly with low) raises a new question about the evolutionary history of the HH haplotypes in the HH population. It is generally well accepted that the hemochromatosis mutation was a recent event which occurred on a founder chromosome carrying the HLA-A3B7 haplotype [24]. Later studies have shown that the number of copies of the ancestral haplotype was associated with the clinical expression, with a more severe phenotype observed in patients carrying two copies of the ancestral haplotype as compared with those with one or without any copy [28-29]. In 2006 Cruz et al. hypothesised that the most ancestral C282Y carrying HLA-A3-B7 haplotype also carried the genetic trait determining low CD8+ T lymphocytes and that the more diverse haplotypes associated with high CD8+ T lymphocytes should be more recent in the evolutionary history of the HH haplotypes, as expected by recombinational events along generations [15]. The present results could support a new alternative hypothesis where the most ancestral *HFE* mutation could have originally evolved in a high diversity of HLA haplotypes in association with a high CD8+ T lymphocyte profile, and more recently a selective sweep occurred increasing significantly the frequency of the particular haplotype A3B7-AAT associated with low lymphocyte numbers, from which other haplotypes associated with low CD8+s could have been further derived by recombination. This could eventually explain the finding of a general low penetrance of the C282Y mutation (putatively associated with high lymphocytes and mild iron overload) and the remarkable high frequency of the HLA HLA-A3B7 haplotype in patients expressing the disease. It is well known that, despite being a

mutation of high prevalence in Caucasians (1 in 200people) [5] the number of symptomatic patients is not as high as expected.

Conclusions

An important message deriving from this study is that in all studies addressing the question of the penetrance of Hereditary Hemochromatosis, the evaluation of CD8+ T lymphocyte numbers should be included as an important marker. The eventual finding of a putative gene involved in the setting of CD8+ T lymphocyte numbers could be of a great value as a new genetic marker not only for the prognosis and penetrance of Hereditary Hemochromatosis, but also in all clinical situations where CD8+ T lymphocytes are important players. The present study points, for the first time, to a possible localization of that putative gene centromeric to the HLA-A locus.

List of abbreviations

MHC, Major Histocompatibility Complex.

ICBAS, Abel Salazar Institute for the Biomedical Sciences.

IRIS, Iron Genes and the Immune System.

IBMC, Institute for Molecular and Cell Biology.

UMIB, Multidisciplinary Biomedical Research Unit.

CGPP, Center of Predictive and Preventive Genetics.

HH, Hereditary Hemochromatosis.

TfSat, Transferrin Saturation.

Mb, Mega bases pair.

HLA, Human Leukocyte Antigen.

TfR2, Transferrin Receptor 2.

TBIS, Total Body Iron Stores.

Kb, Kilobases.

SNP, Single Nucleotide Polymorphism.

SAH, Santo António Hospital.

PGBD1, PiggyBac Transposable Element Derived 1 gene.

ZNF193, Zinc Finger Protein 193 gene.

ZNF165, Zinc Finger Protein 165 gene.

AS-PCR, Allele-Specific Polymorphism Chain Reaction

PCR, Polymorphism Chain Reaction.

DNA, Deoxyribonucleic acid.

PCR-SSP, Polymerase Chain Reaction - Sequence Specific Primers.

FAD, Allele Frequency in the Diseased population.

FAP, Allele Frequency in the Control population.

SP, Study Population.

CP, Control Population.

TP, Number of true positive results.

FP, Number of false positive results.

TN, Number of true negative results.

FN, Number of false negative results.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SM and GP conceived and designed the study, analyzed and made the interpretation of data and wrote the manuscript. GP, additionally, diagnosed and treated the Portuguese hemochromatosis patients and compiled their clinical data. SM, additionally, performed the HLA class I and SNP genotyping of patients and performed the statistical analysis. MC oversaw the performance and interpretation of most of the laboratory assays for SNP genotyping at the IBMC laboratory and helped in the SNP genotyping of patients. AB oversaw the performance and interpretation of most of the laboratory assays for HLA class I genotyping at the ICBAS laboratory. EC contributed to the selection of study cases, and in the collection, analysis an interpretation of clinical data. SA gathered and performed the DNA extraction from most of all biological samples. BMS is the director of the Immunogenetics Laboratory on ICBAS, and supervised the work on HLA typing.

Acknowledgements

The authors wish to thank to Joana Gomes for help in gathering information from some patients and families, to Luís Correia who was most helpful in collecting the genotypic information of the CGPP patients and the Immunogenetics Laboratory group for their help in the laboratory assays.

This work was partially financed by Roche Farmacêutica (Bolsa de Iniciação à Investigação Clínica – Programa Roche/ICBAS/HSA 2008/2010) and from FCT project PTDC/SAU-GMG/67868/2006.

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Figures

Figure 1

Short Title: HLA-A distribution according to the CD8+ T lymphocytes number trait.

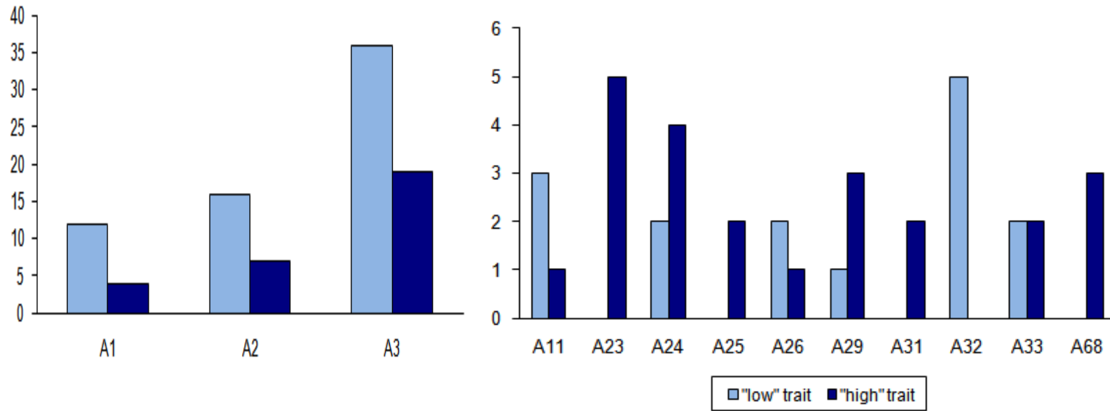


Figure 2

Short Title: HLA-B distribution according to the CD8+ T lymphocytes number trait.

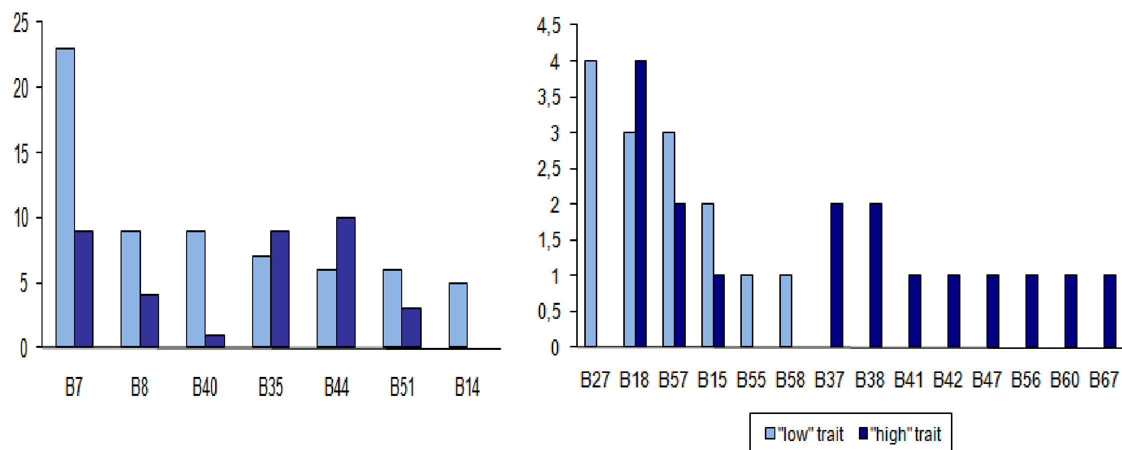
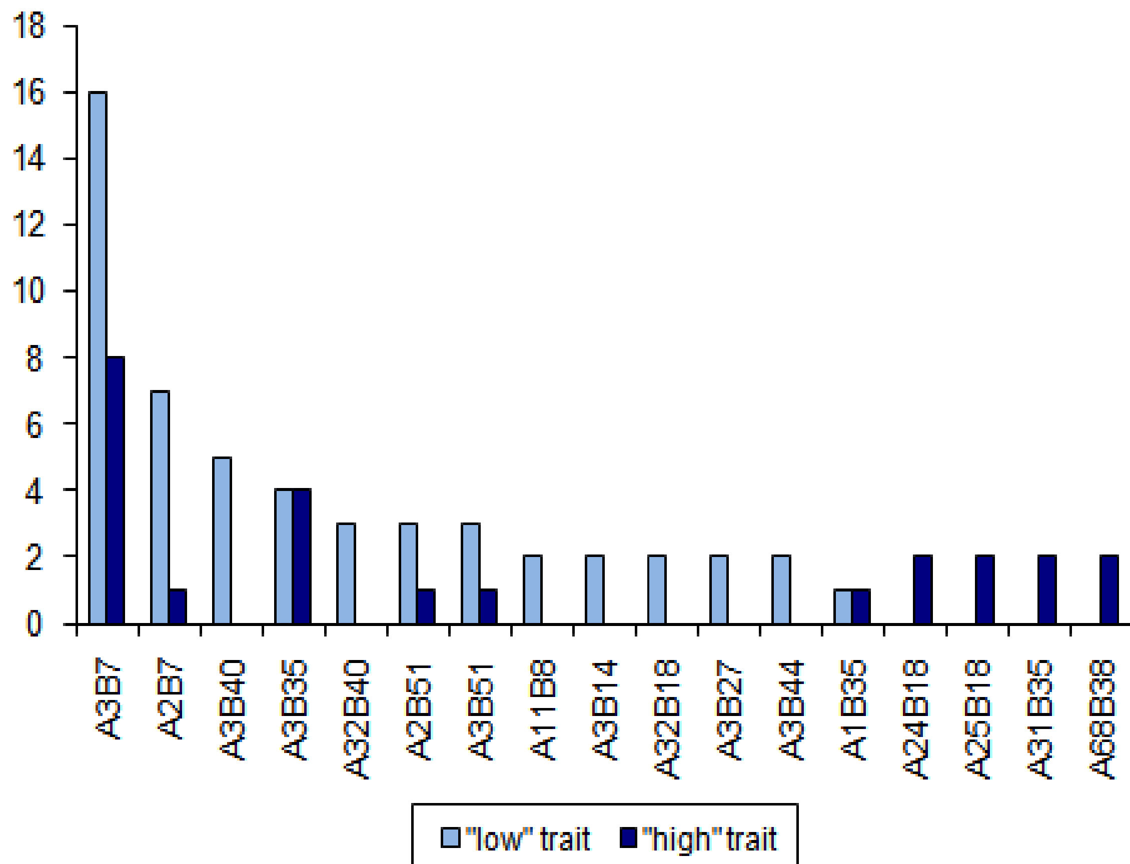


Figure 3

Short Title: HLA haplotype distribution according to the CD8+ T lymphocytes number trait.



Tables and Captions

Table 1 – Haplotype frequencies of SNP markers in unrelated C282Y homozygous subjects.

	Independent CGPP population	**SAH study population (n=71)**			
	Haplotype frequency (n=123)	**Haplotype frequency**	**Total CD8+ T cells (x10^6/ml)**	**TBIS (g)**	**Symptomatic patients**
AAT*	94.3% (232/246)	89.4% (127/142)	**0.37 ± 0.20** (0.08 – 0.77) (n=54)	**8.5 ± 4.66** (1.1 – 17.4) (n=40)	**53.5%** (31/58)
GGG	4.9% (12/246)	6.3% (9/142)	**0.58 ± 0.23** (0.28 – 1.04) (n=8)	**3.7 ± 3.30** (1.4 – 9.9) (n=6)	**12.5%** (1/8)
Others	0.8% (2/246)	4.2% (6/142)	-	14.3 ± 4.90 (8.7 – 18.1) (n=3)	100% (3/3)

* For the purpose of haplotype definition in 106 subjects from the independent CGPP population, where complete information on SNP genotyping was not available, the AAT haplotype was assigned based on the presence of at least the A-allele on PGBD1. The expected error in these cases is not greater than 1.41% (2/142) as estimated by the general frequency of PGBD1-A individuals who are not AAT.

Table 2 – HLA allele frequencies in unrelated C282Y homozygous subjects.

	HLA	N	FreqSP	FreqCP**	Fisher	δ
HLA-A	1	18	0.122	0.111	n.s.	-
	2	26	0.177	0.263	n.s.	-
	3	**60**	**0.408**	**0.110**	**0.0001**	**0.335**
	11	6	0.041	0.069	n.s.	-
	23	5	0.034	0.045	n.s.	-
	24	6	0.041	0.104	n.s.	-
	25	2	0.014	0.015	n.s.	-
	26	3	0.020	0.035	n.s.	-
	29	5	0.034	0.053	n.s.	-
	31	2	0.014	0.025	n.s.	-
	32	6	0.041	0.039	n.s.	-
	33	4	0.027	0.033	n.s.	-
	34	1	0.007	0.004	n.s.	-
	68	3	0.020	0.047	n.s.	-
HLA-B	**7**	**35**	**0.238**	**0.060**	**0.0001**	**0.189**
	8	15	0.102	0.074	n.s.	-
	13	1	0.007	0.001	n.s.	-
	14	6	0.041	0.068	n.s.	-
	15	4	0.027	0.057	n.s.	-
	18	7	0.048	0.049	n.s.	-
	27	5	0.034	0.031	n.s.	-
	35	16	0.109	0.118	n.s.	-
	37	3	0.020	0.015	n.s.	-
	38	2	0.014	0.022	n.s.	-
	40	10	0.068	0.033	n.s.	-
	41	1	0.007	0.009	n.s.	-
	42	1	0.007	0.001	n.s.	-
	44	18	0.122	0.152	n.s.	-
	47	1	0.007	0.002	n.s.	-
	49	1	0.007	0.001	n.s.	-
	51	11	0.075	0.109	n.s.	-
	55	1	0.007	0.014	n.s.	-
	56	1	0.007	0.004	n.s.	-
	57	5	0.034	0.030	n.s.	-
	58	1	0.007	0.018	n.s.	-
	60	2	0.014	0.001	0.0001	0.014
	67	1	0,007	0.001	n.s.	-

** Control population (CP) is composed by a total of 7937 individuals (15874 HLA-A and -B alleles) from the region of Porto, Portugal. Results are published in: "New Allele Frequency Database: http://www.allelefrequencies.net. Middleton D, Menchaca L, Rood H, Komerofsky R. Tissue Antigens 2003, 61, 403-407".

Table 3 – HLA haplotype frequencies in unrelated C282Y homozygous subjects.

HLA	SNP	N	FreqSP	FreqPC***	Fisher	δ
A2B7	AAT	8	0.054	0.0001	0.0001	0.054
A3B7	**AAT**	**26**	**0.177**	**0.0130**	**0.0001**	**0.166**
A34B7	AAT	1	0.007	0.0001	n.s.	-
A1B8	AAT GGT	7 1	0.048 0.007	0.0340	n.s.	-
A2B8	AAT	1	0.007	0.0001	n.s.	-
A3B8	AAT AGG	3 1	0.020 0.007	0.0001	n.s.	-
A11B8	AAT	2	0.014	0.0001	n.s.	-
A1B13	AAT	1	0.007	0.0001	n.s.	-
A2B14	AAT	1	0.007	0.0110	n.s.	-
A3B14	AAT	3	0.020	0.0001	0.0001	0.020
A33B14	GGG	2	0.014	0.0130	n.s.	-
A1B15	AAT	1	0.007	0.0001	n.s.	-
A2B15	AAT	2	0.014	0.0110	n.s.	-
A3B18	AAT	1	0.007	0.0001	n.s.	-
A24B18	AAT	2	0.014	0.0001	n.s.	-
A25B18	AAT	2	0.014	0.0001	n.s.	-
A32B18	AAT	2	0.014	0.0001	n.s.	-
A2B27	AAT	1	0.007	0.0001	n.s.	-
A3B27	AAT	3	0.020	0.0001	0.0001	0.020
A24B27	AAT	1	0.007	0.0001	n.s.	-
A1B35	AAT	2	0.014	0.0001	n.s.	-
A2B35	AAT GGG	1 1	0.007 0.007	0.0100	n.s.	-
A3B35	AAT	8	0.054	0.0120	n.s.	-
A24B35	AAT	1	0.007	0.0110	n.s.	-
A26B35	AAT	1	0.007	0.0001	n.s.	-
A31B35	AAT	2	0.014	0.0001	n.s.	-
A1B37	AAT	1	0.007	0.0001	n.s.	-
A11B37	AAT	1	0.007	0.0001	n.s.	-
A29B37	GGG	1	0.007	0.0001	n.s.	-
A68B38	AAT	2	0.014	0.0001	n.s.	-
A3B40	AAT	5	0.034	0.0001	0.0001	0.034
A26B40	AAT	1	0.007	0.0001	n.s.	-
A32B40	AAT	3	0.020	0.0001	0.0001	0.020
A33B40	AAT	1	0.007	0.0001	n.s.	-
A3B41	AAT	1	0.007	0.0001	n.s.	-
A29B42	AGG	1	0.007	0.0001	n.s.	-
A1B44	AAT	1	0.007	0.0001	n.s.	-
A2B44	AAT	4	0.027	0.0150	n.s.	-
A3B44	AAT	2	0.014	0.0001	n.s.	-
A11B44	GGG	1	0.007	0.0001	n.s.	-
A23B44	AAT AGG GGG	1 1 3	0.007 0.007 0.020	0.0120	n.s.	-
A24B44	AAT	1	0.007	0.0140	n.s.	-
A29B44	GAT	2	0.014	0.0190	n.s.	-
A33B44	AAT	1	0.007	0.0001	n.s.	-
A68B44	GGG	1	0.007	0.0001	n.s.	-
A3B47	AAT	1	0.007	0.0001	n.s.	-
A2B49	AAT	1	0.007	0.0001	n.s.	-
A2B51	AAT	6	0.041	0.0001	0.0001	0.041

A3B51	AAT	4	0.027	0.0001	0.0001	0.027
A11B51	AAT	1	0.007	0.0001	n.s.	-
A11B55	AAT	1	0.007	0.0001	n.s.	-
A26B56	AAT	1	0.007	0.0001	n.s.	-
A1B57	AAT	3	0.020	0.0130	n.s.	-
A3B57	AAT	1	0.007	0.0001	n.s.	-
A24B57	AAT	1	0.007	0.0001	n.s.	-
A29B58	AAT	1	0.007	0.0001	n.s.	-
A1B60	AAT	1	0.007	0.0001	n.s.	-
A32B60	AAT	1	0.007	0.0001	n.s.	-
A3B67	AAT	1	0.007	0.0001	n.s.	-

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Table 4 – HLA allele frequencies in C282Y homozygous subjects related with CD8+ T lymphocytes number trait.

	High CD8+ T-lymphocytes number trait						Low CD8+ T-lymphocytes number trait						
								Uncorrected data			Corrected data		
	HLA	N	FreqSP	Fisher	δ	FreqPC**	N	FreqSP	Fisher	δ	FreqSP	Fisher	δ
HLA-A	1	4	0.075	n.s.	-	0.111	12	0.152	n.s.	-	**0.279**	**0.0020**	**0.189**
	2	7	0.132	n.s.	-	0.263	16	0.203	**0.0001**	-0.082	0.372	n.s.	-
	3	**19**	**0.358**	**0.0001**	**0.279**	0.110	**36**	**0.456**	**0.0001**	**0.388**			
	11	1	0.019	n.s.	-	0.069	3	0.038	n.s.	-	0.070	n.s.	-
	23	5	0.094	n.s.	-	0.045							
	24	4	0.075	n.s.	-	0.104	2	0.025	**0.0001**	-0.088	0.047	n.s.	-
	25	2	0.038	n.s.	-	0.015							
	26	1	0.019	n.s.	-	0.035	2	0.025	n.s.	-	0.047	n.s.	-
	29	3	0.057	n.s.	-	0.053	1	0.013	n.s.	-	0.023	n.s.	-
	31	2	0.038	n.s.	-	0.025							
	32					0.039	5	0.063	n.s.	-	0.116	n.s.	-
	33	2	0.038	n.s.	-	0.033	2	0.025	n.s.	-	0.047	n.s.	-
	68	3	0.057	n.s.	-	0.047							
HLA-B	7	9	0.170	n.s.	-	0.060	23	0.291	**0.0001**	**0.246**			
	8	4	0.075	n.s.	-	0.074	9	0.114	n.s.	-	0.161	n.s.	-
	14					0.068		0.063	n.s.	-	0.089	n.s.	-
	15	1	0.019	n.s.	-	0.057	2	0.025	n.s.	-	0.036	n.s.	-
	18	4	0.075	n.s.	-	0.049	3	0.038	n.s.	-	0.054	n.s.	-
	27					0.031		0.051	n.s.	-	0.071	n.s.	-
	35	9	0.170	n.s.	-	0.118	7	0.089	n.s.	-	0.125	n.s.	-
	37	2	0.038	n.s.	-	0.015							
	38	2	0.038	n.s.	-	0.022							
	40	1	0.019	n.s.	-	0.033	9	0.114	n.s.	-	**0.161**	**0.0001**	**0.132**
	41	1	0.019	n.s.	-	0.009							
	42	1	0.019	n.s.	-	0.001							
	44	10	0.189	n.s.	-	0.152	6	0.076	**0.0001**	-0.090	**0.107**	**n.s.**	**-**
	47	1	0.019	n.s.	-	0.002							
	51	3	0.057	n.s.	-	0.109	6	0.076	n.s.	-	0.107	n.s.	-
	55					0.014	1	0.013	n.s.	-	0.018	n.s.	-
	56	1	0.019	n.s.	-	0.004							
	57	2	0.038	n.s.	-	0.030	3	0.038	n.s.	-	0.054	n.s.	-
	58					0.018	1	0.013	n.s.	-	0.018	n.s.	-
	60	1	0.019	n.s.	-	0.000							
	67	1	0.019	n.s.	-	0.000							

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Table 5 – HLA haplotypes frequencies in C282Y homozygous subjects related with low CD8+ T lymphocytes number trait.

CHROMOSOMES CARRYING THE LOW CD8+ NUMBER TRAIT									
HLA	**SNP**	**Uncorrected data**				**FreqPC****	**Corrected data**		
		N	**FreqSP**	**Fisher**	**δ**		**FreqSP**	**Fisher**	**δ**
A2B7	AAT	7	0.089	0.0001	0.089	0.0001	**0.111**	**0.0001**	**0.111**
A3B7	**AAT**	**16**	**0.203**	**0.0001**	**0.192**	**0.0130**			
A1B8	AAT	6	0.076	n.s.	-	0.0340	0.095	n.s.	-
A3B8	AGG	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A11B8	AAT	2	0.025	0.0001	0.025	0.0001	0.032	**0.0001**	0.032
A2B14	AAT	1	0.013	n.s.	-	0.0110	0.016	n.s.	-
A3B14	AAT	2	0.025	0.0001	0.025	0.0001	0.032	**0.0001**	0.032
A33B14	GGG	2	0.025	n.s.	-	0.0130	0.032	n.s.	-
A1B15	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A2B15	AAT	1	0.013	n.s.	-	0.0110	0.016	n.s.	-
A3B18	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A32B18	AAT	2	0.025	0.0001	0.025	0.0001	0.032	**0.0001**	0.032
A2B27	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A3B27	AAT	2	0.025	0.0001	0.025	0.0001	0.032	**0.0001**	0.032
A24B27	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A1B35	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A3B35	AAT	4	0.051	n.s.	-	0.0120	0.063	n.s.	-
A24B35	AAT	1	0.013	n.s.	-	0.0110	0.016	n.s.	-
A26B35	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A3B40	AAT	5	0.063	n.s.	-	0.0001	0.079	**0.0001**	0.079
A26B40	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A32B40	AAT	3	0.038	0.0001	0.038	0.0001	0.048	**0.0001**	0.048
A1B44	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A2B44	AAT	3	0.038	n.s.	-	0.0150	0.048	n.s.	-
A3B44	AAT	2	0.025	0.0001	0.025	0.0001	0.032	**0.0001**	0.032
A2B51	AAT	3	0.038	0.0001	0.038	0.0001	0.048	**0.0001**	0.048
A3B51	AAT	3	0.038	0.0001	0.038	0.0001	0.048	**0.0001**	0.048
A11B55	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-
A1B57	AAT	3	0.038	n.s.	-	0.0130	0.048	n.s.	-
A29B58	AAT	1	0.013	n.s.	-	0.0001	0.016	n.s.	-

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Table 6 – HLA haplotype frequencies in C282Y homozygous subjects with high CD8+ T lymphocytes number trait.

CHROMOSOMES CARRYING THE HIGH CD8+ NUMBER TRAIT						
HLA	**SNP**	**N**	**FreqSP**	**FreqPC*** **	**Fisher**	**δ**
A2B7	AAT	1	0.019	0.0001	n.s.	-
A3B7	**AAT**	**8**	**0.151**	**0.0130**	**0.0001**	**0.140**
A1B8	AAT	1	0.019	0.0340	n.s.	-
A2B8	AAT	1	0.019	0.0001	n.s.	-
A3B8	AAT	2	0.038	0.0001	0.0001	0.038
A2B15	AAT	1	0.019	0.0110	n.s.	-
A24B18	AAT	2	0.038	0.0001	0.0001	0.038
A25B18	AAT	2	0.038	0.0001	0.0001	0.038
A1B35	AAT	1	0.019	0.0001	n.s.	-
A2B35	AAT	1	0.019	0.0100	n.s.	-
A2B35	GGG	1	0.019			
A3B35	AAT	4	0.075	0.0120	n.s.	-
A31B35	AAT	2	0.038	0.0001	0.0001	0.038
A1B37	AAT	1	0.019	0.0001	n.s.	-
A29B37	GGG	1	0.019	0.0001	n.s.	-
A68B38	AAT	2	0.038	0.0001	0.0001	0.038
A33B40	AAT	1	0.019	0.0001	n.s.	-
A3B41	AAT	1	0.019	0.0001	n.s.	-
A29B42	AGG	1	0.019	0.0001	n.s.	-
A2B44	AAT	1	0.019	0.0150	n.s.	-
A23B44	AAT	1	0.019			
A23B44	AGG	1	0.019	0.0120	n.s.	-
A23B44	GGG	3	0.057			
A24B44	AAT	1	0.019	0.0140	n.s.	-
A29B44	GAT	1	0.019	0.0190	n.s.	-
A33B44	AAT	1	0.019	0.0001	n.s.	-
A68B44	GGG	1	0.019	0.0001	n.s.	-
A3B47	AAT	1	0.019	0.0001	n.s.	-
A2B51	AAT	1	0.019	0.0001	n.s.	-
A3B51	AAT	1	0.019	0.0001	n.s.	-
A11B51	AAT	1	0.019	0.0001	n.s.	-
A26B56	AAT	1	0.019	0.0001	n.s.	-
A3B57	AAT	1	0.019	0.0001	n.s.	-
A24B57	AAT	1	0.019	0.0001	n.s.	-
A1B60	AAT	1	0.019	0.0001	n.s.	-
A3B67	AAT	1	0.019	0.0001	n.s.	-

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