Cell cycle and apoptosis in T cells from Hereditary Hemochromatosis patients

O ciclo celular e a apoptose dos linfócitos T de doentes com Hemocromatose Hereditária

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Junho 2012
This work, presented for the purpose of obtaining the degree of Master of Medicine, includes a proposed research project and the respective implementation report, developed in the context of the Department of "Iniciação à Investigação Clínica" of the Mestrado Integrado em Medicina (MIM) of Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto (ICBAS / UP) and Centro Hospitalar do Porto (CHP) during the academic years 2010/2011 (design and drafting of the proposal) and 2011/2012 (implementing the design, analysis and interpretation of results and preparation of the report).

The project was implemented at the Hemochromatosis Outpatient Clinic (Clinical Hematology), under the guidance of Prof. Dr. Graça Porto (Clinical Hematology Service, Department of Medicine, CHP).
AGRADECIMENTOS

Agradeço à Professora Doutora Graça Porto pela oportunidade, pela imensa disponibilidade e pela transmissão de conhecimentos.

Agradeço à Professora Doutora Margarida Lima pela motivação, pelos ensinamentos e por apoiar esta disciplina que enriqueceu a minha formação como médica e como pessoa.

Agradeço à Drª Sónia Fonseca pela imprescindível ajuda na execução do procedimento laboratorial.

Agradeço à Enfermeira Graça Melo pelo modelo de competência e pela colaboração.

Agradeço ainda a todas as restantes pessoas que contribuíram para a concretização deste projeto.
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ABSTRACT | RESUMO

Abstract: Hereditary Hemochromatosis (HH) is an autosomal recessive metabolic disease that leads to progressive iron loading of parenchymal cells, resulting in tissue damage and organ failure. The great majority of patients are homozygous for the mutation C282Y in the HFE gene, which encodes a non classical MHC-class I protein, whose role in iron homeostasis is not fully explained. The clinical presentation is heterogeneous, varying from a simple biochemical abnormality to a devastating iron overload disease. Immune mechanisms have been implicated in the clinical heterogeneity of HH. Previous studies have shown that patients with abnormally high CD4/CD8 ratios display a faster re-entry of iron into the serum transferrin pool after intensive phlebotomy treatment than patients without these abnormalities. Moreover, low numbers of CD8+ T cells observed in the peripheral blood of patients homozygous for the C282Y mutation correlates with higher hepatic tissue iron. Whether the low CD8+ T cell count directly contributes to the progression of iron accumulation or is simply associated with other genetic factors involved in iron metabolism is still not clarified and the mechanisms that regulate the numbers of CD8+ T lymphocytes need to be investigated. Using flow cytometry, we proposed to investigate if low numbers of CD8+ T lymphocytes observed in the blood of HH patients are related to cell cycle and/or apoptosis abnormalities.

The population analyzed in this study was composed by HH patients homozygous for the C282Y mutation of the HFE gene from the Hemochromatosis Clinic from HSA/CHP and blood donors. For all the individuals, we performed the quantification of total peripheral blood T cells and CD4+ and CD8+ T cell populations, the quantification of the percentage of apoptotic peripheral blood CD8+ T cells and of CD4+ T cells, the quantification of the percentage of peripheral blood CD8+ T cells, of CD4+ T cells at the G0/G1, S and G2/M phases of the cell cycle, and measurement biochemical parameters of iron status.

Although the present work could not provide an answer to the starting question on the possible mechanism underlying the defects in CD8+ T lymphocyte numbers in HH patients, it contributed, however, to better demonstrate the general marked differences in apoptosis and cell cycle markers between the subpopulations of CD4+ and CD8+ T lymphocytes, and permitted also to reveal, for the first time, an impact of systemic iron parameters on T lymphocyte apoptosis, and some differences in cell cycle progression in HH patients.
**Resumo:** A Hemocromatose Hereditária (HH) representa um distúrbio metabólico, transmitido de forma autossômica recessiva, no qual a acumulação progressiva de ferro nas células parenquimatosas leva a lesão tecidual e disfunção orgânica. Na grande maioria, os doentes são homozigóticos para a mutação C282Y no gene HFE, que codifica uma proteína MHC classe I não clássica, e cujo papel na homeostasia do ferro não está bem esclarecido. A apresentação clínica da doença é muito variável, desde a alteração bioquímica isolada, até a disfunção multiorgânica, e o sistema imune está envolvido na modulação desta heterogeneidade. Estudos prévios demonstraram que doentes com razões CD4+/CD8+ mais elevadas mostram uma reacumulação de ferro mais rápida após tratamento intensivo com flebotomias, em comparação com doentes com razões normais. Níveis mais baixos de linfócitos T CD8+ no sangue periférico destes doentes correlacionam-se com níveis mais elevados de ferro no fígado. Não é claro ainda se o baixo número de linfócitos T CD8+ contribui diretamente para a acumulação progressiva de ferro, ou está apenas relacionado com outros factores genéticos envolvidos no seu metabolismo, devendo investigar-se os mecanismos que regulam estas contagens celulares. Recorrendo à citometria de fluxo, propusemo-nos analisar se os baixos níveis de linfócitos T CD8+ observados no sangue periférico de doentes com HH se correlacionam com alterações no ciclo celular ou apoptose destas células.

A população analisada neste estudo foi composta por doentes com HH homozigóticos para a mutação C282Y do gene HFE, provenientes da Consulta de Hemocromatose do HSA / CHP e por donadores de sangue. Para todos os indivíduos, foi realizada a quantificação das células T totais no sangue periférico e das populações de células T CD4 + e CD8 +, a quantificação da percentagem de células CD8 + T e de células T CD4 + apoptóticas do sangue periférico e a quantificação da percentagem de células T CD8 + e de células T CD4 + do sangue periférico nas fases G0/G1, S e G2 /M do ciclo celular. Foram ainda medidos os parâmetros bioquímicos do ferro.

Embora o presente trabalho não possa dar uma resposta à pergunta de partida sobre o possível mecanismo subjacente às variações do número de linfócitos T CD8 + em doentes com HH, contribuiu, no entanto, para demonstrar melhor as diferenças marcadas na apoptose e marcadores do ciclo celular entre as subpopulações de linfócitos T CD4 + e CD8 +, e permitiu também, pela primeira vez, revelar o impacto de parâmetros de ferro sistémico na apoptose de linfócitos T, e algumas diferenças na progressão do ciclo celular em doentes com HH.
Cell cycle and apoptosis in T cells from Hereditary Hemochromatosis patients

TITLE | TÍTULO

Cell cycle and apoptosis in T cells from Hereditary Hemochromatosis patients

INTERVENIENTS | INTERVENIENTES NO PROJETO

Institutions, Departments and Services | Instituições, Departamentos e Serviços
- Centro Hospitalar do Porto (CHP)
  - Hospital de Santo António (HSA)
    - Departamento de Medicina (DM)
    - Serviço de Hematologia Clínica (SHC)

Research team | Equipa de Investigação

Constitution | Constituição

Student | Aluno
- Maria João Silva: Aluna da Disciplina de Iniciação à Investigação Clínica (DIIC) do Curso de Mestrado Integrado em Medicina (MIM) do ICBAS/UP.

Supervisors | Orientadores
- Prof. Doutora Graça Porto: Médica, Imunohemoterapeuta, Chefe de Serviço, Serviço de Hematologia / Consulta de Hemocromatose, Professora do ICBAS/UP, Investigadora do IBMC (Supervisor).
- Doutor Jorge Pinto: Investigador do IBMC (Co-supervisor).

Discipline supervisor | Supervisores
- Prof. Doutora Margarida Lima: Médica, Imunohemoterapeuta, Assistente Hospitalar Graduada do SHC do HSA/CHP; Responsável pelo Laboratório de Citometria (LC) do SHC do HSA/CHP; Professora Auxiliar Convidada do ICBAS/UP; Regente da DIIC.

Other investigators | Outros investigadores
- Dra. Sónia Fonseca, Técnica de Diagnóstico e Terapêutica (TDT) do LC do SHC do HSA/CHP.
- Enf. Graça Melo, Enfermeira da Consulta de Hemocromatose do SHC do HSA/CHP.

Functions and responsibilities | Funções e responsabilidades

The conception and formulation of the proposal, as well as the execution of the project are responsibilities of the student. The supervisor will monitor the formulation of the proposal, the execution of the project and the analysis and interpretation of the results. The discipline supervisor will supervise all the steps of the project, including the conception, the implementation, and the presentation of the results. The follow investigators will collaborate in specific tasks of the project, and described forward.

Time dedicated to the project | Tempo dedicado ao projeto
- Total: 5,05 persons*month
- Student: 10% - 22 months (1 x 0,10 x 22 = 2,2 persons*month)
- Supervisors (x2): 2,5% - 22 months (2 x 0,025 x 22 = 1,1 persons*month)
- Disciple supervisor: 2,5% - 22 months (1 x 0,025 x 22 = 0,55 persons*month)
- Other investigators (x2): 10% - 6 months (2 x 0,10 x 6 = 1,2 persons*month)
Introduction | Introdução

Hereditary Hemochromatosis (HH) is one of the most common hereditary metabolic diseases in Caucasians (Cardoso CS et al, 2001). In this clinical/pathological entity progressive iron loading of parenchymal cells leads to tissue damage and organ failure, in a characteristic pattern that affects liver, pancreas, joints, skin, hypophysis or heart (reviewed by Porto G et al, 2008).

The great majority of patients are homozygous for the mutation C282Y in the HFE gene, assigned to the short arm of chromosome 6, that encodes a non classical MHC-class I protein, whose role in iron homeostasis is not fully explained (Feder JN et al, 1996). This mutation is inherited in an autosomal recessive pattern, but other genetic defects involved in cellular and systemic iron metabolism are expected to interact with HFE. In the north of Portugal the allele frequency of the C282Y mutation is 0.058, corresponding to a frequency of homozygous of 0.8%.

The clinical presentation is very heterogeneous, varying from a simple biochemical abnormality to a full-blown picture of devastating iron overload disease. In order to be considered severe iron overload it is necessary the demonstration of abnormally increased hepatic iron stores (Porto G et al, 2008). It is of great interest to understand what modulates this clinic variability, leading us to the best therapeutic approach.

Immune mechanisms have been implicated in the clinical heterogeneity of HH (Porto G & De Sousa M, 2007). The postulate that immunological system could have a role in monitoring tissue iron toxicity, as part of its surveillance function, was first described by De Sousa in 1978, based on her observations of lymphocyte traffic and positioning (De Sousa M, 1978). Later studies have identified a correlation with other elements of immune system, as monocyte/macrophages and NK cells.

A study performed in 1991 revealed that patients with abnormally high CD4/CD8 ratios (>2.9) displayed a faster re-entry of iron into the serum transferrin pool after intensive phlebotomy treatment than patients without those abnormalities (reviewed by Porto G et al, 2008). In 2001, a study performed specifically in patients homozygous for the C282Y mutation related low percentages of CD8+ T cells in the peripheral blood with higher levels of hepatic tissue iron (Cardoso EM et al, 2001). Several molecular and functional abnormalities, in addition to the counts were also identified in the populations of CD8+ T cells (reviewed by Porto G et al, 2008). These abnormalities are not described in secondary forms of iron
overload. The fact that anomalies are remarkably stable in each individual patient, that they are not corrected by phlebotomy treatment, and that they are observed in asymptomatic patients at young ages, favours the hypothesis that they are intrinsic to the genetic defect and not a consequence of the progressive iron overload (Porto G et al, 2007). The involvement of the MHC region on this regulation was first demonstrated in humans in 2004 (Cruz E et al, 2004), and later results support the notion that gene(s) in this region contribute both to the control of CD8+ T-cell numbers and the clinical heterogeneity observed in HH. Whether the identified phenotype of low CD8+ T cells directly contributes to the progression of iron accumulation or is simply associated with other genetic factors involved in iron metabolism needs to be investigated (Cruz E et al, 2006a).

The described abnormalities in lymphocyte populations were systematically found in the subpopulation of CD8+ T lymphocytes, but the association with total body iron stores is also reflected in the total lymphocyte counts (Porto G et al, 2007). Although no specific abnormalities have so far been found in CD4+ T lymphocytes, the possibility that they also might be present is not fully excluded.

The mechanisms that regulate the numbers of lymphocytes in the peripheral blood are still not clarified. The control system of cell cycle has a main role on the regulation of the numbers of a certain population of cells. No studies have addressed before whether is there any alteration in cell cycle in lymphocytes from HH patients. The only described alteration is an adaptive response to a genotoxic agent (Porto B et al, 2009), reflected in a decrease in induced chromosome breakage. We might speculate if this correlates with cell cycle progression.

Using flow cytometry it is possible to analyze the cell cycle, based on cellular deoxyribonucleic acid (DNA) content. The capacity for multiparametric measurement of large cell populations rapidly and accurately offered by cytometry has made this methodology indispensable in studies of cell proliferation and cell death (Darzynkiewicz Z et al, 2001). A variety of flow cytometric methods to analyze the cell cycle progression, developed over the past three decades, reveals distribution of cells in three major phases of the cycle (G0/G1, S and G2/M) and makes it possible to detect apoptotic cells with fractional DNA content. The discrimination between quiescent (G0) and proliferating cells is achievable, based on the presence of proliferation-associated proteins.

Using flow cytometry it is also possible to analyze apoptosis and a variety of flow cytometric methods to quantify apoptotic cells have been described. Staining with fluorochrome conjugated Annexin V
(due to phosphatidylserine externalization to the outer leaflet of the plasma membrane early during apoptosis) is typically used in conjunction with a fluorescent vital dye such as propidium iodide (PI) or 7-7-Amino-Actinomycin D (7-AAD) to allow the identification and quantification of apoptotic cells (Annexin V positive / PI and 7-AAD negative).

In this study we propose to investigate, by flow cytometry, if low numbers of CD8+ T lymphocytes observed in the blood of HH patients are related to cell cycle and/or apoptosis abnormalities.
Background and rationale of the study | Enquadramento e justificação do estudo

The project supervisor, Prof. Graça Porto, is Consultant at the Clinical Hematology Service in HSA and the Responsible for the Hemochromatosis Clinic in this Service since 1985. This Outpatient Clinic has developed for the last 25 years as a specialized center for the diagnosis, treatment and prevention of Hemochromatosis and obtained its certification according to the ISO 9001:2000 standards in 2005. The regular movement of the Clinic, that is open twice a week, is of about 1200 consultations/year, corresponding to an average number of 260 patients, most of them (40%) being cases of Hereditary Hemochromatosis. This allows an easy and efficient access to biological samples and clinical records from well characterized patients and families. Moreover, as part of the regular clinical work, the project supervisor is also involved in the observation and selection of regular blood donors at the Blood Bank, providing the opportunity of an easy recruitment for the study of healthy subjects from the normal population.

Prof. Graça Porto is also the Director of a new research group at IBMC (BCRIB, Basic and Clinical Research on Iron Biology). The main objective of the group is to understand iron homeostasis through the investigation of the reciprocal functional interactions between Iron Metabolism & The Cells of the Immune System (IS), focusing on the basic questions: how do cells of the IS contribute to the regulation of systemic iron homeostasis and how is iron balance sensed by those cells? A major clinical impact of the present research is expected to be a deeper understanding of the pathophysiology of common or rare disorders of iron homeostasis, most genetically determined, eventually leading to the development of new markers of disease or new forms of treatment and/or prevention. The members of the proposing group have a long historical background of research in the former IBMC group “Iron Genes and the Immune System (IRIS)” headed by Maria de Sousa, having been actively involved in several main achievements, namely:

1. The first demonstration that the clinical severity of iron overload in Hereditary Hemochromatosis (HH), the most common genetic disorder of iron overload, is related to CD8 T lymphocyte numbers in the context of the Human Leukocyte Antigen (HLA) genotypes (Porto et al, Eu J Haematol 52:283-290, 1994; Porto et al, Hepatology 25:397-402, 1997; Cruz et al, Blood Cells Mol Dis; 37:33-39, 2006)

3. The first evidence that lymphocytes express hepcidin and modulate hepcidin and ferroportin expression in response to elemental iron (Pinto et al, *Immunology* 130: 217-230, 2010)

It is now an ambition of the group to move from the established evidence that lymphocytes can contribute to the regulation of iron metabolism, to the clarification of the mechanisms of how it happens. It is also our ambition to elucidate how CD8+ T lymphocyte numbers are regulated and maintained at stable homeostatic levels in the peripheral blood. The specific aims of the present project are therefore part of that larger general aim of the group.

Motivations of the student
To acquire skills in terms of scientific reasoning.

To develop the competence of working with a research team with structured functions and responsibilities.

To contact with laboratory techniques and with the dynamic between clinic and primary sciences in the scope of medicine.

To get personal accomplishment.

**Pitfalls | Problemas**

As mentioned before, numerous studies correlated CD8+ T cells with presenting clinical picture, but it is still unclear what leads to these anomalies. No studies have addressed so far the issue of cell cycle in CD8+ lymphocytes from HH patients.

**Research questions | Questões a investigar**

What are the mechanisms that justify lower numbers of CD8+ T lymphocytes in peripheral blood from HH patients, as compared to healthy individuals?

Are they related to different cell proliferation indexes and/or cell death by apoptosis?

**Working hypothesis | Hipóteses de trabalho**

Our general working hypothesis is that low numbers of CD8+ T lymphocytes, found in peripheral blood from HH patients, may be associated with alterations in cell cycle or progression of CD8+ T cells into apoptosis.
Aims | Objectivos

The general aim of this project is to analyze if there are qualitative alterations on peripheral blood lymphocytes from HH patients, possible related to their numbers.

Specifically, this research aims to analyze T lymphocytes from HH patients, homozygous for the mutation C282Y, and normal controls (all recruited at the Hematology Service of HSA), using cell cycle and apoptosis target components.

Goals | Metas

It is expected that the study of cell cycle and apoptosis in T cells contribute to the knowledge about variability in these population numbers, among HH patients.

Implication | Implicações

Considering the fact that lymphocyte counts have a consistent pattern of correlation with clinic of HH, the explanation of what is underlying abnormalities may lead us to a better comprehension of this heterogeneity. If anomalies are found, they might be implicated in the expression of the disease.

Study design | Desenho do estudo

Classification | Classificação

Local: National and institutional study.

Type: Analytic, observational, cross-sectional and case-control study.

Nature: Clinical and laboratorial study.

Universe, population and sample | Universo, população e amostra

Universe | Universo

Patients: HH patients homozygous for the C282Y mutation of the HFE gene.

Controls: Healthy adult individuals.

Population | População

Patients: The population analyzed in this study will be composed by HH patients homozygous for the C282Y mutation of the HFE gene from the Hemochromatosis Clinic from HSA/CHP. A total of about 500 patients are registered and are regularly followed-up at the clinic, and 90 of these are genetically characterized as HH homozygous for the C282Y mutation, i.e., the population of interest for the present study.
Immunological characterization: The immunological characterization of patients included the number of peripheral blood CD8+ T lymphocytes. For the purpose of phenotypic characterization of patients, CD8+ T lymphocyte numbers were classified as “low” when they were $\leq 0.41 \times 10^6$/ml and “high” when they were $>0.41 \times 10^6$/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).

Controls: The control population for this project will be composed by the regular blood donors attending the HSA Blood Bank.

Sample | Amostra

Patients: The sample population will be composed by two groups of patients: a group of 15 subjects with low numbers of CD8 + T lymphocytes, and another group of 15 subjects with high numbers of CD8 + T lymphocytes.

Controls: The sample population for this project will be composed by a group of 15 subjects, selected among the population of regular blood donors attending the HSA Blood Bank.

Participant selection | Selecção dos participantes

Participants will be selected in a non-probabilistic consecutive way (by convenience).

Patients and blood donors will be consecutively recruited at the time of their consultation and volunteer blood donation, respectively.

Eligibility criteria | Critérios de elegibilidade

Inclusion criteria:

- To be followed at the Hemochromatosis Clinic, as a HH patient homozygous for the C282Y mutation of the HFE gene.
- Have a scheduled consultation during the period of recruitment
- Agree to participate in the project
Exclusion criteria:

- Patients with clinical conditions known to influence total lymphocytes numbers (such as autoimmune or viral diseases) will be excluded.
- Controls whose donation is the first will be excluded.

**Working plan | Plano de trabalho**

**Tasks | Tarefas**

**TASK 1:** Inform about the study and ask to participate

**TASK 2:** Data collection from clinical process

**TASK 3:** Blood sample collection

**TASK 4:** Analytical procedures

**TASK 5:** Analysis and interpretation of results

**TASK 1 – Inform about the study and ask to participate**

- **Duration:**
  3 months
- **Starting and ending dates:**
  01-10-2011 a 31-12-2011
- **Institutions, Departments and Services:**
  HSA/CHP – DM – SHC – Consulta de Hemocromatose e Sector de Dadores de Sangue
- **Investigators:**
  Maria João Silva; Graça Porto.
- **Aims:**
  Inform the patients and blood donors about the goals and features of the study. Request their participation in the study, and signature of informed consent.
- **Description:**
  Patients: During the period of consultation, the student (Maria João Silva) will be presented to the patients by the supervisor and responsible for the Hemochromatosis Clinic (Graça Porto), will inform them about the study, requesting their participation. If they agree to participate, they will have to sign the informed consent.
  Controls: On specific days in which the supervisor is responsible for the observation and selection of regular blood donors at the Blood Bank, the student will go to the department; the supervisor will present the student to the blood donors, and she will inform them about the study, requesting their participation. If they agree to participate, they will have to sign the informed consent.
- Functions and responsibilities:

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<td>Graça Porto</td>
<td>Present the student to the patients/blood donors.</td>
</tr>
<tr>
<td>Maria João Silva</td>
<td>Inform the patients/blood donors about the study and request their participation.</td>
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**TASK 2 – Data collection from clinical files**

- Duration:
  
  3 months

- Starting and ending dates:
  
  01-10-2011 a 31-12-2011

- Institutions, Departments and Services:
  
  HSA/CHP – DM – SHC – Consulta de Hemocromatose

- Investigators:
  
  Maria João Silva; Graça Porto.

- Aims:
  
  Collect clinical data from patients and controls clinical files.

- Description:
  
  During the period of consultation, according to the participant recruitment, it will be collected the corresponding analytic data registered at the moment of diagnosis in their clinical files; in the next week, will be collected the results from the CBC and biochemical study, which is normally made after regular phlebotomies.

  The collection of controls’ data will be held under the same rules.

  The list of analytic parameters is described in the form attached. The supervisor will overlook the process of data collection from clinical files.

- Functions and responsibilities:

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<tr>
<td>Maria João Silva</td>
<td>Data collection and registering</td>
</tr>
<tr>
<td>Graça Porto</td>
<td>Supervision of data collection and registering</td>
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**TASK 3 – Blood sample collection**

- Duration:
  
  3 months

- Starting and ending dates:
  
  01-10-2011 a 31-12-2011

- Institutions, Departments and Services:
  
  HSA/CHP – DM – SHC – Consulta de Hemocromatose; Sector de Dadores de Sangue

- Investigators:
  
  Nurse (Consulta de Hemocromatose) – Enf. Graça Melo.

- Other collaborators:
Nurses (Dadores de Sangue, SHC, HSA/CHP).

- Aims:
  Collect blood samples.

- Description:
  It will be carried out the collection of 2 blood samples (1 tube with EDTA-K3, with 4.5ml of blood; 1 tube with sodic heparin with 7.5 ml of blood);
  The procedure does not involve risks, besides the implicated in any venous puncture;
  It will be collected at most 2 samples from patients and 2 samples from blood donors per week (1 sample of each, at each period of consultation);
  The blood samples will be sent to the LC (Laboratório de Citometria) SHC-HSA-CHP , addressed to Técn. Sónia Fonseca.

- Functions and responsibilities:

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<tr>
<td>Graça Melo</td>
<td>Collect blood samples from patients</td>
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<td>Deliver the samples in LC</td>
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<tr>
<td>Other nurses (Dadores de Sangue)</td>
<td>Collect blood samples from blood donors</td>
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<td>Deliver the samples in LC</td>
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**TASK 4 – Analytical procedures**

- Duration:
  3 months

- Starting and ending dates:
  01-10-2011 a 31-12-2012

- Institutions, Departments and Services:
  HSA/CHP – DM – SHC – LC

- Investigadores:
  Maria João Silva; Sónia Fonseca; Margarida Lima.

- Aims:
  To quantify total of peripheral blood T-cells and CD4+ and CD8+ T cell populations;
  To quantify the percentage of peripheral blood CD8+ T cells, and of CD4+ T cells at the S and G2/M phases of the cell cycle;
  To quantify the percentage of apoptotic peripheral blood CD8+ T cells and of CD4+ T cells.

- Description:
  The quantification of the CD4+ and CD8+ T cells populations, the CD8+ and CD4+ T lymphocytes cell cycle study, and the quantification of apoptotic CD8+ and CD4+ T lymphocytes will be performed by flow cytometry.
- Functions and responsibilities:

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<tbody>
<tr>
<td>Sónia Fonseca</td>
<td>Performance of the technique and analysis of results</td>
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<tr>
<td>Maria João Silva</td>
<td>Cooperation with the performance of the technique and analysis of results</td>
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<tr>
<td>Margarida Lima</td>
<td>Supervising of the study and analysis of results</td>
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**TASK 5 – Analysis and interpretation of results**

- Duration:
  2 months

- Starting and ending dates:
  02-01-2012 a 30-03-2012

- Institutions, Departments and Services:
  HSA/CHP – DM – SHC

- Investigators:
  Maria João Silva; Graça Porto.

- Aims:
  To study the relationship between CD8+ and CD4+ T cell numbers and their phenotypic profile (%S and G2/M cells and % of apoptotic cells).

- Description:
  Data will be analyzed with the support of statistic software (SPSS or Statgraphics);
  Descriptive statistics: The mean, median and standard deviation of the percentage and absolute numbers of total T-cells, CD4+ T-cells and CD8+ T-cells and CD4/CD8 ratios;
  Inferential statistics: Association studies between CD8+ and CD4+ numbers and the corresponding phenotypic profile (%S and G2/M cells and % of apoptotic cells) will be performed.

- Functions and responsibilities:

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<tr>
<td>Graça Porto</td>
<td>Supervision of analysis and interpretation of results</td>
</tr>
<tr>
<td>Maria João Silva</td>
<td>Analysis and interpretation of results</td>
</tr>
</tbody>
</table>

**Material and Methods | Material e Métodos**

**Sample collection | Colheita das amostras**

Blood samples will be obtained at the time of the patients and controls regular visits to the clinic and blood bank respectively. It is expected that at the time of the visit routine measurements of biochemical parameters of iron metabolism (serum iron, serum transferrin, transferrin saturation and serum ferritin) and CBC will be obtained. At this moment, the participants will be informed about the study and asked to give their Informed Consent.
Samples for T-cell immunophenotyping and cell cycle studies will be collected into K3-ethylene-diamine-tetracetic acid (EDTA-K3)-containing tubes, whereas samples to be used in apoptosis studies will be collected into tubes containing sodium heparin. Overall, about 10 ml of blood will be collected.

**Sample processing | Processamento das amostras**

All technical procedures will be executed at the Flow Cytometry Lab (CHP – HSA – SHC)

**T cell immunophenotyping**

**Rationale:** Flow cytometry has become a standard laboratory tool in the identification of lymphocyte populations and subpopulations, a method referred to as immunophenotyping. The clinical application of this technology has been facilitated by the development of instruments and data analysis systems suitable for routine use in diagnostic laboratories. In addition, the expanded range of monoclonal antibodies (Mab) specific for lymphocyte surface antigens directly conjugated to a number of different fluorochromes are now available. By staining cells with Mab with different specificities conjugated with fluorochromes that emit light of different wavelengths it is possible to differentiate between different lymphocyte populations by staining cells in a single tube. For instance, anti-CD45 Mab stains all leukocytes with different intensity (lymphocytes have a high CD45 expression), anti-CD3 stains T cells and anti-CD4 and anti-CD8 Mab can be used to distinguish CD4+ and CD8+ T-cell populations. Some of the fluorochromes that are currently used in combination are the Fluorescein isothiocyanate (FITC, maximum emission ~520 nm, green fluorescence), Phycoerythrin (PE, maximum emission ~576 nm, orange fluorescence), the Phycoerythrin-Texas Red-X (ECD, maximum emission ~620 nm, red fluorescence) and the Phycoerythrin-Cy5 (PC5, maximum emission ~670 nm, deep red fluorescence), all of each are excitable with the argon ion laser (488 nm light length emission) used in most benchtop cytometers.

**Flow cytometry protocol:** The blood sample will be submitted to cell surface immunophenotyping with FITC-conjugated mouse anti-human CD45 (pan leukocyte antigen), RD-conjugated mouse anti-human CD4 (CD4-RD1) and ECD-conjugated mouse anti-human CD8 (CD8-ECD) and PC5-conjugated mouse anti-human CD3 (CD3-PC5) Mab (IgG) (CD45-FITC / CD4-RD1 / CD8-ECD / CD3-PC5), using a non-wash whole blood direct immunofluorescence staining method. Erythrocyte lysis and leukocyte fixation will be performed with the Immunoprep Reagent System™ (Beckman Coulter, BC), which contains reagents to lyse the red blood cells (Reagent A), to stop the lysing process (Reagent B) and to fix the cells (Reagent C),
using an automated sample preparation station (TQ-prep™, BC), following the manufactures’ instructions. Stained samples will be acquired in a flow cytometer (EPICS-XL-MCL™, BC) using the System II™ software (BC).

**Flow cytometry data analysis:** Stored listmode data will be analyzed using the System II™ Software (BC). The percentage of total T cells (low SSC signal, CD45+high, CD3+ cells) among lymphocytes and the percentage of CD4+ (low SSC signal, CD45+high, CD3+CD4+ cells) and CD8+ (low SSC signal, CD45+high, CD3+CD8+ cells) T cells among total T cells and among lymphocytes will be calculated.

**Distribution of CD8+ and CD4+ T cells throughout the cell cycle phases**

Rationale: The DNA measurement and cell cycle studies will be performed by flow cytometry, based upon the ability of propidium iodide (PI) to bind stoichiometrically to double strand DNA under appropriate staining conditions. Cells stained in this manner will emit red fluorescence in direct proportion to their DNA content and based on this parameter the percentage of cells in G0+G1 (DNA content = n), S and G2+M (DNA content = 2n) phases of the cell cycle will be evaluated. In order to determine the DNA content in specific lymphocyte populations, blood cells will be stained for cell surface molecules prior to DNA staining with PI. This will be done by staining cells with a FITC-conjugated mouse anti-human CD8 or CD4 Mab (green fluorescence), following by staining with a FITC-conjugated rabbit anti-mouse IgG polyclonal antibody (Ab).

**Flow cytometry protocol:** The blood sample will be submitted to cell surface immunophenotyping with FITC-conjugated mouse anti-CD4 or anti-CD8 Mab (IgG) followed by staining with FITC conjugated rabbit anti-mouse IgG polyclonal Ab and basic cellular DNA measurement based on flow cytometry techniques, using the COULTER DNA PREP Reagents Kit™, according to a protocol that was described in detail (Lima M et al, 2000). This reagent kit contains non-ionic detergents to permeabilizing cells (DNA PREP LPR reagent), PI (to stain double strain nucleic acids, DNA and dsRNA) and RNAses (to destroy RNA) (DNA PREP Stain reagent). After staining, samples will be acquired in a flow cytometer (EPICS-XL-MCL, BC) using the System II™ software (BC).

**Flow cytometry data analysis:** Stored listmode data will be analyzed using specific software for DNA analysis “Multicycle for Windows™” (Phoenix Flow System, PFS). Using this software, the percentage of CD8+ T-cells and CD4+ T-cells in each phase of the cell cycle (G0+G1, S and G2M) will be calculated.
Detection of apoptotic of T cells

Rationale: Annexin V is a protein that binds phospholipids in the presence of calcium, which has a high affinity for phosphatidylserine (PS), a negatively charged phospholipids located in the inner leaflet of the plasma membrane of the viable cells. PS is exposed at the cell surface early during the apoptotic process and externalization of PS on the cell surface allows cells to bind Annexin V. The loss of membrane integrity accompanies the latest stages of cell death resulting from either apoptosis or necrosis and this phenomenon brings cells permeable to some dyes such as PI and 7-Amino-Actinomycin D (7-AAD). Thus, the intact membranes viable cells do not stain with Annexin V and exclude PI and 7-AAD, whereas the membranes of dead and damaged cells stain with Annexin V and are permeable to PI and 7-AAD. In addition, apoptotic cells stains with Annexin V and exclude PI and 7-AAD. Therefore, staining with FITC or PE conjugated Annexin V is typically used in conjunction with PI or 7-AAD, respectively, to allow the identification and quantification of viable (Annexin V negative and PI / 7-AAD negative), early apoptotic (Annexin V positive and PI / 7-AAD negative) and dead (Annexin V positive and PI / 7-AAD positive). This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because in both cases, the dead cells will stain with Annexin and PI / 7-AAD.

Flow cytometry protocol: Apoptotic T cells will be assessed through flow cytometry, using the Annexin V-FITC/7-AAD Kit © (BC), containing FITC conjugated Annexin V, 7-AAD staining solution and Annexin V binding buffer. Samples will be acquired in a flow cytometer (EPICS-XL-MCL™, BC) using the System II™ software (BC).

Flow cytometry data analysis: Stored listmode data will be analyzed using the System II™ software (BC). The percentage of viable (Annexin V negative / 7-AAD negative), early apoptotic (Annexin V positive / 7-AAD negative) and late apoptotic or already dead (Annexin V positive / 7-AAD positive) CD8+ T-cells will be calculated.

Data analysis | Análise dos dados

Association studies between CD8+ and CD4+ T lymphocytes numbers and the corresponding phenotypic profile (%S and G2/M cells and % of apoptotic cells) will be performed. Data will be further correlated with the other clinical parameters (CBC and biochemical).

Data will be analyzed with the support of statistic software (SPSS or Statgraphics).
Equipment, material e reagents | Equipamento, material e reagentes

Equipment | Equipamento

It will be used the equipment available at the LC of SHC-HSA-CHP.

<table>
<thead>
<tr>
<th>Laboratory Equipment</th>
<th>Automated hematologic counters: Coulter LH 780 (BC)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Flow cytometers: EPICS-XL-MCL&lt;sup&gt;Tm&lt;/sup&gt; (BC)</td>
</tr>
<tr>
<td></td>
<td>Sample preparation stations: TQ-PreP&lt;sup&gt;Tm&lt;/sup&gt; workstation (BC)</td>
</tr>
<tr>
<td></td>
<td>Refrigerators</td>
</tr>
<tr>
<td></td>
<td>Centrifuges</td>
</tr>
<tr>
<td>Sample preparation stations: TQ-PreP&lt;sup&gt;Tm&lt;/sup&gt; workstation (BC)</td>
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</tr>
<tr>
<td></td>
<td>Automated Micropipettes</td>
</tr>
<tr>
<td>Hardware</td>
<td>Computers and Printers</td>
</tr>
<tr>
<td>Software</td>
<td>System II&lt;sup&gt;Tm&lt;/sup&gt; (BC); Multicycle for Windows&lt;sup&gt;Tm&lt;/sup&gt; (PFS)</td>
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</table>

BC, Beckman Coulter; PFS (Phoenix Flow Systems)
### Reagents | Reagentes

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<tr>
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<th>Description</th>
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<td>Enzifarma</td>
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<td></td>
<td>Heparin (sodium)</td>
<td>BDB</td>
<td>-</td>
<td>Enzifarma</td>
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<td>Cell preparation (washing and suspension)</td>
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</table>

BD, Becton Dickinson; BC, Beckman Coulter; DK, Dako; IOT, Immunotech

**Material consumível | Consumable material**

- 5 ml polypropylene tubes
- Pasteur pipettes
- Pipette tips
Cell cycle and apoptosis in T cells from Hereditary Hemochromatosis patients

Calendarization | Calendarização

Duration | Duração
Global: 22 months Execution: 6 months

Start and end dates | Datos de início e conclusão

Chronogram | Cronograma

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<th>2011</th>
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<tr>
<td>Preparation of project proposal</td>
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<td>Thesis defense</td>
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</table>

Output indicators | Indicadores de produção

Oral communications and posters | Comunicações orais e posters
- Oral presentation of the proposal at the 3<sup>th</sup> JIIC (Jornadas de Iniciação á Investigação Clínica (June / July 2011)
- Oral presentation of the results at the 4<sup>th</sup> JIIC (June / July 2012)
- Presentation of the results in poster in scientific meeting of the specialty (ex. Sociedade Portuguesa de Imunologia) (2012)

Manuscripts | Trabalhos escritos
- Research Project proposal (2011)
- Thesis defense (2012)
- Article for publication in national or international medical journal with referees (2012)
Bibliographic references | Referências bibliográficas


FINANTIAL PLAN | PLANO FINANCEIRO

Budget | Orçamento

All the analysis will be executed at LC (SHC-HSA-CHP), with reagents purchased using funds made available by ICBAS, and the analysis will be carried out after working hours.

<table>
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<td>Reagents and other consumables (*)</td>
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<td>Poster (impression)</td>
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<td>Congress (inscription)</td>
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(*) Described in detail in the next page

Funding | Financiamento

This study will be financed by ICBAS/UP.
### Cell cycle and apoptosis in T cells from Hereditary Hemochromatosis patients

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<th>Reagents and other consumables</th>
<th>Description</th>
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<th>Price / Units (€)</th>
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**TOTAL** 7117,52

BD, Becton Dickinson; BC, Beckman Coulter; DK, Dako; IOT, Immunotech
Polypropylene tubes, Pasteur pipettes, and pipette tips will be provided by the Lab.
GLOSSARY | GLOSSÁRIO

Abreviations and acronims | Abreviaturas e acrónimos

7-AAD, 7-Amino-Actinomycin D
BC, Beckman Coulter
BD, Becton Dickinson
BCRIB, Basic and Clinical Research on Iron Biology
CHP, Centro Hospitalar do Porto
CBC, Complete Blood Count
DIIC, Disciplina de Iniciação à Investigação Clínica
DK, Dako
DM, Departamento de Medicina do HSA/CHP
DNA, Deoxyribonucleic acid
ECD, Phycoerythrin-Texas Red-X (maximum emission ~620 nm, red fluorescence)
EDTA, Ethylene-diamine-tetracetic acid
FITC, Fluorescein isothiocyanate (maximum emission ~520 nm, green fluorescence)
HH, Hemocromatose Hereditária
HSA, Hospital de Santo António
IBMC, Instituto de Biologia Molecular e Celular
ICBAS, Instituto de Ciências Biomédicas Abel Salazar
IOT, Immunotech
IS, Immune system
JIIC, Jornadas de Iniciação à Investigação Clínica
LC, Laboratório de Citometria do HSA/CHP
MIM, Mestrado Integrado em Medicina
PBS, Phosphate buffered saline
PC5, Phycoerythrin-Cy5 (maximum emission ~670 nm, deep red fluorescence),
PE, Phycoerythrin (maximum emission ~576 nm, orange fluorescence)
PI, Propidium iodide (Iodeto de propideo)
PS, Phosphatidylserine
SHC, Serviço de Hematologia Clínica do HSA/CHP
UP, Universidade do Porto

Technical terms | Termos técnicos
ANNEXES | ANEXOS

- Formulários de recolha de dados clínicos e laboratoriais
- Termos de consentimento informado (doentes e dadores de sangue)
- Folhetos informativos (doentes e dadores de sangue)
- Folha de rosto de estudo de investigação
- Pedidos de autorização
- Termos de responsabilidade
- Autorizações locais
**Formulário de recolha de dados**

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**Avaliação hematológica**

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<td>Neutrófilos</td>
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<td>Linfócitos</td>
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<td>Monócitos</td>
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**Avaliação bioquímica**

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<tr>
<td>Transferrina</td>
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<td>Saturação de Transferrina</td>
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<td>Ferritina Sérica</td>
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**Avaliação imunológica**

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<tr>
<td>Linfócitos T CD4 (%linfo)</td>
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<td></td>
</tr>
<tr>
<td>Linfócitos T CD8 (%linfo)</td>
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</table>
Termo de consentimento informado (Doentes)

O ciclo celular e a apoptose de linfócitos T de doentes com hemocromatose hereditária

Eu, abaixo-assinado: __________________________: 

Fui informado de que o estudo de investigação acima mencionado se destina a estudar a Hemocromatose Hereditária, uma doença que se caracteriza pela acumulação de ferro no organismo. Os doentes com Hemocromatose Hereditária têm com frequência uma diminuição dos linfócitos T CD8 no sangue e essa diminuição parece ter relação com a acumulação de ferro. Neste estudo pretendemos estudar os mecanismos que conduzem à diminuição dos linfócitos T CD8 e para isso é necessário analisar os dados clínicos e fazer algumas análises ao sangue.

Sei que neste estudo está prevista a colheita de dados do processo clínico e a colheita de uma amostra de sangue vai ser utilizada para fazer análises.

Foi-me garantido que todos os dados relativos à identificação dos Participantes neste estudo são confidenciais.

Foi-me garantido que o sangue colhido não será utilizado para a realização estudos genéticos.

Sei que posso recusar-me a participar ou interromper a qualquer momento a participação no estudo, sem nenhum tipo de penalização por este facto.

Compreendi a informação que me foi dada, tive oportunidade de fazer perguntas e as minhas dúvidas foram esclarecidas.

Aceito participar de livre vontade no estudo acima mencionado.

Concordo que seja efetuada a colheita de duas amostras de sangue para realizar as análises que fazem parte deste estudo.

Também autorizo a divulgação dos resultados obtidos no meio científico, garantindo o anonimato.

Nome do Participante no estudo.

Data: ___/___/______ Assinatura

Nome da Aluna:

Data: ___/___/______ Assinatura

__________________________________________

Maria João Silva, MIM ICBAS/UP – 2011/2012

30
Termo de consentimento informado (Dadores de sangue)

O ciclo celular e a apoptose de linfócitos T de doentes com hemocromatose hereditária

Eu, abaixo-assinado_______________________________________________

Fui informado de que o estudo de investigação acima mencionado se destina a estudar a Hemocromatose Hereditária, uma doença que se caracteriza pela acumulação de ferro no organismo e em que há com frequência uma diminuição dos linfócitos T CD8 no sangue, que parece ter relação com a acumulação de ferro. Neste estudo pretende-se estudar os mecanismos que conduzem à diminuição dos linfócitos T CD8 através de algumas análises ao sangue e para isso são necessárias amostras de sangue de pessoas saudáveis.

Foi-me garantido que todos os dados relativos à identificação dos Participantes neste estudo são confidenciais.

Foi-me garantido que o sangue colhido não será utilizado para a realização estudos genéticos.

Sei que posso recusar-me a participar ou interromper a qualquer momento a participação no estudo, sem nenhum tipo de penalização por este facto.

Compreendi a informação que me foi dada, tive oportunidade de fazer perguntas e as minhas dúvidas foram esclarecidas.

Aceito participar de livre vontade no estudo acima mencionado.

Concordo que seja efetuada a colheita de duas amostras de sangue para realizar as análises que fazem parte deste estudo.

Também autorizo a divulgação dos resultados obtidos no meio científico, garantindo o anonimato.

Nome do Participante no estudo.

Data ___/___/_____ Assinatura

Nome da Aluna.

Data ___/___/_____ Assinatura
O ciclo celular e a apoptose de linfócitos T de doentes com hemocromatose hereditária

**Folheto informativo (Doentes)**

A Hemocromatose Hereditária é uma doença que se caracteriza pela acumulação de ferro nos órgãos.

Os doentes com Hemocromatose Hereditária tem uma diminuição dos linfócitos T CD8+ no sangue e essa diminuição parece ter relação com a acumulação de ferro.

Neste estudo vamos investigar os mecanismos que conduzem a uma diminuição dos linfócitos T CD8 no sangue.

Para isso, precisamos de estudar doentes com Hemocromatose e por isso estamos a pedir a sua colaboração.

Se concordar em participar, será consultado o seu processo clínico e serão colhidas duas amostras de sangue para fazer algumas análises.

O sangue colhido não será utilizado para estudos genéticos.

Contamos com a sua participação.

Muito obrigada!

Maria João Silva

DIIC, Mestrado Integrado em Medicina – ICBAS/UP

**Folheto informativo (Dadores de Sangue)**

A Hemocromatose Hereditária é uma doença que se caracteriza pela acumulação de ferro nos órgãos.

Os doentes com Hemocromatose Hereditária tem uma diminuição dos linfócitos T CD8+ no sangue e essa diminuição parece ter relação com a acumulação de ferro.

Neste estudo vamos investigar os mecanismos que conduzem a uma diminuição dos linfócitos T CD8 no sangue.

Para isso, precisamos de amostras de sangue de pessoas normais e por isso estamos a pedir a sua colaboração.

Se concordar em participar, serão colhidas duas amostras de sangue para fazer as análises que fazem parte deste estudo.

O sangue colhido não será utilizado para estudos genéticos.

Contamos com a sua participação.

Muito obrigada!

Maria João Silva

DIIC, Mestrado Integrado em Medicina – ICBAS/UP
Folha de rosto do estudo de investigação

TÍTULO
O ciclo celular e a apoptose de linfócitos T de doentes com Hemocromatose Hereditária

CLASSIFICAÇÃO
Trabalho Académico de Investigação X Conferidor de grau X (Mestrado Integrado X)

VERSÃO
Novo X

CALENDARIZAÇÃO
Data início: Outubro 2010 Data conclusão: Julho 2012
Prazo a cumprir: Julho 2011

INVESTIGADORES

ALUNOS E ORIENTADORES

Aluno
MARIA JOÃO SILVA; ICBAS – UP; MIM; 5ºANO; mjoaoslv@gmail.com; 933320155.

Orientador / Supervisor da Instituição de Ensino
PROFª. DOUTORA MARGARIDA LIMA; MÉDICA, IMUNOHEMOTERAPEUTA; ASSISTENTE HOSPITALAR GRADUADA, SHC DO HSA/CHP; PROFESSORA CONVIDADA, ICBAS/UP; mnc.lima@clix.pt; 966 327 115

Orientador / Supervisor no CHP
PROFª. DOUTORA GRAÇA PORTO; MÉDICA, IMUNOHEMOTERAPEUTA; CHEFE DE SERVIÇO, SHC DO HSA/CHP; PROFESSORA CATEDRÁTICA, ICBAS/UP; gporto@ibmc.up.pt; 968345652.

PROMOTOR O próprio X
MARIA JOÃO SILVA; ICBAS – UP; MIM; 5ºANO; mjoaoslv@gmail.com; 933320155.

INSTITUIÇÕES E SERVIÇOS

Unidades, Departamentos e Serviço do CHP
HSA/CHP – DM – SHC

Outras Instituições intervenientes
ICBAS/UP
CARACTERÍSTICAS do estudo

**Alvo do estudo**

- Humanos: X

**Natureza do estudo**

- Clínico: X
- Laboratorial: X
- Analítico: X
- Observacional: X
- Transversal: X

**Participantes**

- Existência de grupo controlo: Sim: X
- Seleção dos Participantes: Não aleatória: X

**Estudos observacionais**

- Tipo: Caso-controlo: X

**Estudos experimentais**

- Não se aplica

**Outros aspectos relevantes para a apreciação do estudo**

- Participação de grupos vulneráveis: Não: X
- Convocação de doentes / participantes: Não: X
- Consentimento informado: Sim: X
- Realização de inquéritos / questionários: Não: X
- Realização de entrevistas: Não: X
- Colheita de produtos biológicos: Sim: X (Não CHP: X) (Não anonimizados: X)
- Armazenamento de produtos biológicos: Não: X
- Criação de bancos de produtos biológicos: Não: X
- Realização de exames / análises: Sim: X (Não CHP: X)
- Realização de estudos genéticos: Não: X
- Recolha de dados: Sim: X (Dados clínicos laboratoriais: analíticos: X / imagem: X)
- Criação de bases de dados: Não: X
- Saída para outras instituições: Não: X

**ORÇAMENTO E FINANCIAMENTO**

- Orçamento total: 7417,52 Euros
- Contrato financeiro em anexo: Não: X
- Financiamento: Interno (CHP): 0,00 Euros
- Externo (Outros): 7417,52 Euros
- Entidade(s) financiadora(s): Bolsa Iniciação à Investigação – ICBAS

**INDICADORES**

- Dissertação Mestrado Integrado em Medicina

Data: 

Assinatura do proponente (Aluno):
**Pedido de autorização institucional**
Trabalho académico de investigação: **O CICLO CELULAR E A APOPTOSE DE LINFÓCITOS T DE DOENTES COM HEMOCROMATOSE HEREDITÁRIA**

**Presidente do Conselho de Administração do CHP**

Exmo. Senhor Presidente do Conselho de Administração do CHP

Maria João Silva, na qualidade de Aluna da Disciplina de Iniciação à Investigação Clínica do curso de Mestrado Integrado em Medicina do ICBAS/UP e do CHP, vem por este meio, solicitar a Vossa Exa. autorização para realizar no Centro Hospitalar do Porto o Estudo de Investigação acima mencionado, de acordo com o programa de trabalhos e os meios apresentados.

Data ___/___/____ Assinatura

**Presidente da Comissão de Ética para a Saúde do CHP**

Exma. Senhora Presidente da Comissão de Ética para a Saúde do CHP

Maria João Silva, na qualidade de Aluna da Disciplina de Iniciação à Investigação Clínica do curso de Mestrado Integrado em Medicina do ICBAS/UP e do CHP, vem por este meio, solicitar a Vossa Exa. autorização para realizar no Centro Hospitalar do Porto o Estudo de Investigação acima mencionado, de acordo com o programa de trabalhos e os meios apresentados.

Data ___/___/____ Assinatura

**Diretora do Departamento de Ensino, Formação e Investigação do CHP**

Exma. Senhora Diretora do Departamento de Ensino, Formação e Investigação do CHP

Maria João Silva, na qualidade de Aluna da Disciplina de Iniciação à Investigação Clínica do curso de Mestrado Integrado em Medicina do ICBAS/UP e do CHP, vem por este meio, solicitar a Vossa Exa. autorização para realizar no Centro Hospitalar do Porto o Estudo de Investigação acima mencionado, de acordo com o programa de trabalhos e os meios apresentados.

Data ___/___/____ Assinatura
**Pedido de financiamento**

Trabalho académico de investigação: **O CICLO CELULAR E A APOPTOSE DE LINFÓCITOS T DE DOENTES COM HEMOCROMATOSE HEREDITÁRIA**

**Bolsa de iniciação à investigação clínica**

Carta ao Presidente do Conselho Diretivo

Exmo. Senhor Presidente do Conselho Diretivo do ICBAS/UP

Prof. Doutor António Sousa Pereira

MARIA JOÃO SILVA, 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. a atribuição de 7417,52 € da Bolsa de Iniciação à Investigação Clínica, para financiamento do Trabalho Académico acima mencionado, de acordo com orçamento apresentado.

Data ___/___/_____

Assinatura
Termos de Responsabilidade

Trabalho académico de investigação: O CICLO CELULAR E A APOPTOSE DE LINFÓCITOS T DE DOENTES COM HEMOCROMATOSE HEREDITÁRIA

Aluno

Eu, abaixo assinado, Maria João Silva, na qualidade de Aluna da Disciplina de Iniciação à Investigação Clínica do curso de Mestrado Integrado em Medicina do ICBAS/UP e do CHP, comprometo-me a executar o Trabalho Académico de Investigação acima mencionado, de acordo com o programa de trabalhos e os meios apresentados, respeitando os princípios éticos e deontológicos e as normas internas da instituição.

Data ___/___/_____
Assinatura

Orientador

Eu, abaixo assinado, Graça Porto, médica, na qualidade de Orientador, solicito autorização do Conselho de Administração para que o Aluno acima referido possa desenvolver no CHP o seu Trabalho de Investigação. Informo que me comprometo a prestar a orientação necessária para uma boa execução do mesmo e a acompanhar o Aluno nas diferentes fases da sua realização, de acordo com o programa de trabalhos e meios apresentados, bem como por zelar pelo respeito dos princípios éticos e deontológicos e pelo cumprimento das normas internas da instituição.

Data ___/___/_____
Assinatura

Supervisor do CHP

Eu, abaixo assinado, Margarida Lima, médica, na qualidade de Professora Responsável pela Disciplina de Iniciação à Investigação Clínica do curso de Mestrado Integrado em Medicina do ICBAS/UP e do CHP, comprometo-me a prestar a orientação necessária para uma boa execução do Trabalho de Investigaçã0, de acordo com o programa de trabalhos e meios apresentados. Mais declaro que acompanharei o Aluno, responsabilizando-me por supervisionar a execução do trabalho no CHP, bem como por zelar pelo respeito dos princípios éticos e deontológicos e pelo cumprimento das normas internas da instituição.

Data ___/___/_____
Assinatura
Termos de autorização local

Trabalho académico de investigação: O CICLO CELULAR E A APOPTOSE DE LINFÓCITOS T DE DOENTES COM HEMOCROMATOSE HEREDITÁRIA

Diretores de Serviço

Na qualidade de Director de Serviço, declaro que autorizo a execução do Estudo de Investigação 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.

<table>
<thead>
<tr>
<th>Serviço</th>
<th>Nome do Diretor</th>
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<th>Assinatura</th>
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<tbody>
<tr>
<td>Hematologia Clínica</td>
<td>Dr. Jorge Coutinho</td>
<td><em><strong>/</strong></em>/___</td>
<td>__________</td>
</tr>
</tbody>
</table>

Diretores / Conselhos de Gestão de Departamento

Na qualidade de Diretor do Departamento, declaro que autorizo a execução do Estudo de Investigação 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.

<table>
<thead>
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<tr>
<td>Medicina</td>
<td>Prof. Doutor Lopes Gomes</td>
<td><em><strong>/</strong></em>/___</td>
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Cell cycle and apoptosis in T cells from Hereditary Hemochromatosis patients

EXECUTION REPORT | RELATÓRIO DE EXECUÇÃO
The project here reported, previously approved by Centro Hospitalar do Porto, was executed between 7th November of 2011 and 30th April of 2012. It was held in Clinical Hematology Service (Hemochromatosis Clinic and Cytometry Laboratory), and involved the cooperation of all the collaborators previously mentioned. The general aim of the project was to analyse T lymphocytes from Hereditary Hemochromatosis (HH) patients, homozygous for the mutation C282Y, and normal controls, using cell cycle and apoptosis target components, and verify if there are qualitative alterations on peripheral blood lymphocytes from HH patients, possibly related to their numbers.

Following the project proposal, where the background and objectives of the work were explained in more detail, this report describes the population studied, the procedures executed, the results obtained and respective discussion.
DESIGN AND METHODS

Patients

The study population consisted of 20 patients with HH (13 males and 7 females, with a median age of 52 years, ranging from 23 to 74 years). These included 10 individuals classified with the immunological phenotype of “low CD8+ T cells” (50%) and 10 individuals classified with the immunological phenotype of “high CD8+ T cells” (50%).

All patients were diagnosed and are regularly followed-up at the Hemochromatosis Outpatient Clinic of Hospital de Santo António, Porto, where all the clinical information is recorded. Only patients genetically characterized as homozygous for the C282Y mutation of HFE were included. Clinical and genetic data from these patients have been published previously (Porto G et al, 1997; Porto G et al, 2001; Cardoso C et al, 2001).

During the study period, patients were evaluated at different stages of their treatment course. Four patients were under intensive phlebotomy treatment, and the remaining 16 were receiving maintenance therapy. The inclusion of patients at different stages of iron load was important in order to allow an analysis of cell cycle parameters in relation to a wide range of TfSat values.

All clinical material was collected and stored with the informed consent of the patients according to the approval of the Ethical Committee of Hospital Santo António, Porto.

Controls

The control population was randomly recruited from the population of regular blood donors of the Hospital de Santo António, Porto and consisted of 13 individuals (9 males and 4 females with a median age of 47 years, ranging from 26 to 64 years). From the initial sample collected, one individual was excluded because of an atypical pattern of entrainment on flow cytometry analysis. Thus, the final sample consisted of 12 individuals, 8 males and 4 females, with a median age of 45 years, ranging from 26 to 64 years. homozygosity for the C282Y allele was assumed to be negative through the biochemical analysis of iron status. Informed consent for the study was also obtained from all recruited subjects according to the approval of the Ethical Committee of Hospital Santo António, Porto.
Sample collection

Blood samples were obtained at the time of the patients and controls regular visits to the clinic and blood bank respectively. Samples for T-cell immunophenotyping and cell cycle studies were collected into K3-ethylene-diamine-tetracetic acid (EDTA-K3)-containing tubes, whereas samples to be used in apoptosis studies were collected into tubes containing sodium heparin.

Experimental Procedure

All technical procedures were executed at the Flow Cytometry Lab (Department of Haematology, Hospital de Santo António Porto).

T cell immunophenotyping

Flow cytometry protocol: The blood sample was submitted to cell surface immunophenotyping with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD45pan leukocyte antigen, R-phycoerythrin (R-PE, also known as RD1) -conjugated mouse anti-human CD4 and phycoerythrin-Texas Red (PE-Texas Red, also known as ECD)-conjugated mouse anti-human CD8 and phycoerythrocyanin5(PC5)-conjugated mouse anti-human CD3 monoclonal antibodies (mAb) (CD45-FITC / CD4-RD1 / CD8-ECD / CD3-PC5) (Beckman Coulter, BC), using a non-wash whole blood direct immunofluorescence staining method. Erythrocyte lysis and leukocyte fixation was performed in the TQ-prep™ automated sample preparation station (BC), using the Immunoprep Reagent System™ (BC), which contains reagents to lyse the red blood cells (Reagent A), to stop the lysing process (Reagent B) and to fix leukocytes (Reagent C), following the manufactures’ instructions. Stained samples were acquired in an EPICS-XL-MCL™ flow cytometer (BC), using the System II™ software (BC).

Flow cytometry data analysis: Stored listmode data were analyzed using the System II™ Software (BC). The percentage of total T cells (low SSC signal, CD45+high, CD3+ cells) among lymphocytes and the percentage of CD4+ (low SSC signal, CD45+high, CD3+CD4+ cells) and CD8+ (low SSC signal, CD45+high, CD3+CD8+ cells) T cells among total T-cells and among lymphocytes was calculated.

Detection of apoptotic of T cells

Flow cytometry protocol: Apoptotic T cells were assessed through flow cytometry, using the Annexin V-FITC/7-AAD Kit © (BC), containing FITC conjugated Annexin V, 7-AAD staining solution and...
Annexin V binding buffer, following the instructions of the manufacture. Apoptosis studies were performed on mononuclear peripheral blood cells separated by centrifugation on the Lymphoprep™1.077 g/ml density gradient (NycomedPharma AS, Oslo, Norway), washed and suspended in RMPI culture medium, and kept overnight in the refrigerator before further processing. Samples were acquired in a flow cytometer (EPICS-XL-MCL™, BC) using the System II™ software (BC).

**Flow cytometry data analysis:** Stored listmode data was analyzed using the System II™ software (BC). The percentage of viable (Annexin V negative / 7-AAD negative), early apoptotic (Annexin V positive / 7-AAD negative) and late apoptotic or already dead (Annexin V positive / 7-AAD positive) CD8+ and CD4+ T-cells was calculated.

**Distribution of CD8+ and CD4+ T cells throughout the cell cycle phases**

**Flow cytometry protocol:** The blood sample was submitted to cell surface immunophenotyping with FITC-conjugated mouse anti-CD4 or anti-CD8 IgGmAb followed by staining with FITC conjugated rabbit anti-mouse IgG polyclonal Ab and basic cellular DNA measurement based on flow cytometry techniques, using the DNA PREP Reagents Kit™ (BC) according to a protocol that was described in detail (Lima M et al, 2000). This reagent kit contains non-ionic detergents to permeabilizing cells (DNA PREP LPR reagent), PI (to stain double strain nucleic acids, DNA and dsRNA) and RNAse (to destroy RNA) (DNA PREP Stain reagent). After staining, samples were acquired in an EPICS-XL-MCL flow cytometer (BC) using the System II™ software (BC).

**Flow cytometry data analysis:** Stored listmode data were analyzed using specific software for DNA analysis “Multicyle for Windows™” (Phoenix Flow System, PFS). Using this software, the percentages of CD8+ and CD4+ T-cells in each phase of the cell cycle (G0/G1, S and G2/M) were calculated.

**Other laboratory studies**

Other laboratory studies for all subjects included a complete blood cell count and biochemical parameters of iron status, including the measurement of serum iron (Fe), ferritin, transferrin (Tf) and transferrin saturation (TfSat).
**Statistical analysis**

Correlations between variables were analysed by simple linear regression. Differences in group means or sample distributions were tested respectively by the Student’s t-test and the Kolmogorov-Smirnov (KS) two sample test. For the purpose of analysis, logarithmic transformation was applied to the values of serum ferritin, since these have a logarithmic distribution. The p value of 0.05 was taken as the level of statistical significance.

Data were analyzed by StatGraphics software (Statgraphics Statistical Graphics System, version 16.0).
RESULTS

Analysis of Apoptosis

*Overall comparisons between CD4+ and CD8+ T Cells (Figure 1)*

In general, a highly significant difference was found in the percentage of apoptotic cells between the CD4+ and CD8+ T lymphocyte subpopulations (T Test \(p=9.4\times10^{-8}\), KS Test \(p=0.000002\)), the average apoptosis percentage being 21.8% ± 9.9% in CD4+ T cells, and 47.3% ± 21.8% in CD8+ T cells.

Apoptosis in CD4+ T cells and CD8+ T cells was not influenced by gender but it was influenced by age in CD8+ T cells (\(R^2=13.5\%\), \(r=0.37\), \(p=0.0380\)), not in CD4+ T cells.

*HH patients vs. controls*

In general, apoptosis in CD4+ or CD8+ T cells was not statistically different between the groups of controls and HH patients and, in these, it was not influenced by the therapy stage (intensive vs. maintenance). In terms of association with T lymphocyte numbers, also no significant correlation was found between apoptosis and either CD4+ T or CD8+ cell counts (cells/mm3) in both HH patients and controls.

*Correlation with iron parameters*

Regarding the iron metabolism parameters, an overall significant correlation was found between the percentage of apoptosis in both CD4+ and CD8+ T cells and serum iron (respectively \(p=0.0359\) and \(p=0.0353\)) and also transferrin saturation (respectively \(p=0.0316\) and \(p=0.0205\)). Results of the respective correlation coefficients and Rsquare values are summarized in Table 1. No significant correlation was found, in general, with serum ferritin or transferrin.
When considering the HH patients population alone, the correlations between, apoptosis and serum iron and transferrin saturation was even more striking in both CD4+ and CD8+ T cells. These results are illustrated in Figure 2. Moreover, in the case of HH patients, a significant correlation was also found between the log of ferritin and the percentage of apoptosis in both CD4+ and CD8+ T cells (respectively $R^2=20.5\%$, $r=0.452$, $p=0.0452$ and $R^2=22.7\%$, $r=0.476$, $p=0.0339$).

<table>
<thead>
<tr>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T Cells</td>
</tr>
<tr>
<td>S.L.R. Iron Parameters</td>
</tr>
<tr>
<td>Fe ($\mu g/dL$)</td>
</tr>
<tr>
<td>$R^2=13.9%$</td>
</tr>
<tr>
<td>$p=0.0359$</td>
</tr>
<tr>
<td>SatTf (%)</td>
</tr>
<tr>
<td>$R^2=14.5%$</td>
</tr>
<tr>
<td>$p=0.0316$</td>
</tr>
<tr>
<td>Log Ferritin</td>
</tr>
<tr>
<td>(ng/mL)</td>
</tr>
</tbody>
</table>

Table1. Overall correlations between apoptosis in CD4+ and CD8+ T cells populations and iron parameters. N.S.- Not significant. S.L.R. – Simple linear regression.

In face of the observation that iron parameters influence the percentage of apoptotic cells, we further analyzed the HH patients population in comparison to controls according to TfSat values, i.e., after dividing
HH patients in two groups with TfSat above or below 50%. The only significant difference found was in the average apoptosis in CD8+ T cells between HH patients with TfSat<50% or ≥50% (p=0.028), but none of these groups was statistically different from controls who showed average values at an intermediate value between patients with low or high TfSat (respectively 49.8%±26.4%, 35.8%±8.6% and 56.3%±23.0% in controls, HH patients with TfSat< 50% and HH patients with TfSat≥50%).

Distribution of CD8+ and CD4+ T cells throughout the cell cycle phases

*Overall comparison of CD4+ vs. CD8+ T Cells (Figure 3)*

The overall proportions of cells along the 3 phases of cells cycle was also highly significantly different between the populations of CD4+ and CD8+ T cells. On average, 99.5% ±0.30% of CD4+ T cells vs. 98.4% ±1.56% of CD8+ T cells (T test p=0.00023, KS p=0.00067) were in phase G0/G1, while 0.431% ±0.219% of CD4+ T cells and 1.218% ±1.063% of CD8+ T cells (T test p=0.0001, KS p=0.00067) were in phase S of the cell cycle, and 0.068% ±0.1186% of CD4+ T cells and 0.381% ±0.630% of CD8+ T cells (T test p=0.0079, KS p=0.000002) were in phase G2/M phase of the cell cycle.

The percentages of both CD4+ and CD8+ T cells in each phase of cell cycle were not related to age or sex except for the percentage of CD4+ T cells in G2/M phase which was found higher in females (p=0.0159). There was no correlation between percentage of CD4+ T cells in each cell cycle phase and CD4+ T cells counts (cells/mm3), nor between the percentages of CD8+ T cells in each cell cycle phase and

![Figure 3. Comparison of percentage of cells in G0/G1, G2/M and S phases of cell cycle in CD4+ and CD8+ T cells population.](image)
the CD8+ T cells counts (cells/mm3). There was also no correlation between any of the iron parameters and the percentages of CD4+ or CD8+ T cells in different phases of cell cycle.

**HH patients vs. Control population**

In contrast to the results observed for apoptosis, in the case of cell cycle analyses, some differences could be observed between HH patients and controls. Average values of the percentages of CD4+ and CD8+ T lymphocytes in the different cell cycle phases are summarized in Table 2 where results are compared between patients and controls. In general, there were more lymphocytes (both CD4+ and CD8+) in S and G2/M, and less in G0/G1 phase in HH patients than in controls. These differences, however, reached only a statistical significance in G2/M (for both CD4+ and CD8+) and in G0/G1 for CD4+ cells only.

<table>
<thead>
<tr>
<th>Cell Cycle Phases</th>
<th>Controls</th>
<th>HH Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>% G0G1 CD4+ T Cells</td>
<td>99.7% ±0.16%</td>
<td>99.4% ±0.35%</td>
</tr>
<tr>
<td>% G0G1 CD8+ T Cells</td>
<td>98.9% ±1.25%</td>
<td>98.1% ±1.69%</td>
</tr>
<tr>
<td>% G2M CD4+ T Cells</td>
<td>0.008% ±0.029%</td>
<td>0.105% ±0.136%</td>
</tr>
<tr>
<td>% G2M CD8+ T Cells</td>
<td>0.2% ±0.357%</td>
<td>0.49% ±0.735%</td>
</tr>
<tr>
<td>% S CD4+ T Cells</td>
<td>0.342% ±0.162%</td>
<td>0.485% ±0.235%</td>
</tr>
<tr>
<td>% S CD8+ T Cells</td>
<td>0.942% ±1.00%</td>
<td>1.385% ±1.089%</td>
</tr>
</tbody>
</table>

Table 2. Comparison between % of CD4+ and CD8+ T cells in each cell cycle phases between controls and HH patients.

A significant correlation between percentages of cells in each cell cycle phases and apoptosis was not observed in this study.
DISCUSSION

The present study was motivated by the search of a putative mechanism to explain the systematic finding of abnormally low CD8+ T lymphocytes in HH patients homozygous for the C282Y mutation in HFE (Porto G et al, 2007). To the best of our knowledge, this is the first study reporting the profile of peripheral blood lymphocytes in HH patients in terms of their percentages of apoptotic cells and their distribution in the different cell cycle phases. Although the results did not show any strong evidence that abnormalities in apoptosis or cell cycle progression could explain the abnormalities in CD8+ T cell numbers in these patients, they brought into light some new interesting aspects of lymphocyte biology and iron overload with potential fundamental and clinical relevance.

The first result to highlight in this study is the clear demonstration of marked differences in apoptosis and cell cycle progression between the subpopulations of CD4+ and CD8+ T lymphocytes. Although a higher percentage of apoptotic cells among the CD8+ subpopulation has been already observed in previously published studies of apoptosis in humans in the context of sepsis and pulmonary disease, this result has never been a focus of discussion or properly addressed in any of those studies (J. S. Nielsen et al, 2011; Sung Chul Lim, et al 2011). In our view, this is a relevant finding that deserves fully consideration in future studies of lymphocyte activation and proliferation in the clinical setting.

The second interesting finding in this study was the novel observation of a significant correlation between apoptosis and systemic iron parameters. Iron is essential for proper cell function, synthesis and division, but an excess of Fe^{2+} can cause the generation of reactive oxygen species which will provoke oxidative cell damage (N. Bresgen et al. 2010). Iron induced apoptosis is a reported phenomenon in human hepatocytes, rat neurons, but has never been described in human lymphocytes (N. Bresgen et al. 2007; Saadeldien HM et al. 2012). In this study, we found an interesting relationship between iron parameters and apoptosis in lymphocytes from the HH patient’s population, this association being more striking between TfSat values, and the CD8+ T cells. It should be stressed here that all previously described abnormalities in lymphocyte populations in HH were systematically found in the sub-population of CD8+T lymphocytes (Porto G et al, 2007). It could therefore be tempting to speculate that iron overload would contribute to decreased numbers of those cells in HH patients. However, a direct relationship between apoptosis and cell numbers was not found in the present study. A possible explanation for the lack of correlation of apoptosis
with the cell numbers could be the occurrence of compensatory mechanisms of cell proliferation in the context of increased apoptosis. The results on cell cycle markers may support that notion. The fact that HH patients had less resting cells and more cells in G2/M and S could be consistent with the hypothesis of a “more dynamic” pool of lymphocytes. To illustrate this notion we present in Figure 4 a summary of the comparison between patients and controls in terms of the apoptosis and cell cycle markers in CD8+ T lymphocytes. Although a higher percentage of apoptotic cells as well as cells in S or G2/M phases is apparent, this result reaches statistical significance only for cells in G2/M.

![Figure 4. Summary of the comparison between HH patients and controls in the percentages of CD8+ and CD4+ T lymphocytes in apoptosis (left graph) and in cycle phases S and G2M (right graph). * Significantly different (p=0.0025).](image)

The increased number of cells in G2/M, however, does not necessarily mean that there are more cells in mitosis, but could also mean that there are more cells arrested in G2 for DNA repair. If this is the case, it suggests that patients may be somehow responding to oxidative injury, such as the case iron induced cell death. A similar model was addressed by Porto B. et al in a study about the genotoxic effect of diepoxynbutane (DEB) where HH patients` lymphocytes were shown to have a lower DEB-induced chromosome instability possibly due to an adaptive response of the HH lymphocytes with increased DNA repair (Porto, B et al. 2007). Further studies of lymphocyte cell division in HH patients should be designed in order to clarify if indeed, HH patients have more proliferating cells to compensate for an increased iron induced apoptosis or if they simply accumulate more cells arrested in G2 for DNA repair and, as a consequence, have a decreased total number of functional lymphocytes.
CONCLUSION

The present research, although it could not clarify the mechanism underlying the abnormalities in T lymphocyte counts of HH patients, reinforced the notion that there are phenotypic changes in this cell population, possibly reflecting a response to iron induced cell death.

Considering the fact that lymphocyte counts have a consistent pattern of correlation with clinic of HH, this might be considered as a step into a better comprehension of this heterogeneity.
BIBLIOGRAPHIC REFERENCES


