Universidade de Aveiro

Departamento de Biologia

2009

Mónica Isabel Encarnação Costa

MHC-class I markers in non-alcoholic steatohepatitis

Marcadores genéticos do MHC-Classe I na esteatohepatite não alcoólica

Universidade de Aveiro

Departamento de Biologia

2009

Mónica Isabel Encarnação Costa

MHC-class I markers in non-alcoholic steatohepatitis

Marcadores genéticos do MHC-Classe I na esteatohepatite não alcoólica

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Graça Porto, Professora Catedrática Convidada do Instituto de Ciências Abel Salazar, Investigadora no Instituto de Biologia Molecular e Celular do Porto na patologia da Hemocromatose Hereditária e Médica no Serviço de Hematologia Clínica do Hospital de Santo António. Co-orientação realizada pelo Professora Doutora Maria da Conceição Lopes Vieira dos Santos do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente

Professora Doutora Maria de Lourdes Pereira

Professora associada com agregação ao Departamento de Biologia da Universidade de Aveiro

Professora Doutora Maria da Graça Beça Gonçalves Porto

Professora catedrática convidada do Instituto de Ciências Biomédicas Abel Salazar

Professora Doutora Maria da Conceição Lopes Vieira dos Santos

Professora associada com agregação ao Departamento de Biologia da Universidade de Aveiro

Professora Doutora Maria de Fátima Matos Macedo

Professora auxiliar convidada da Escola Ciências da Saúde de Aveiro

agradecimentos

Gostaria de expressar os meus mais sinceros agradecimentos a todos os que contribuíram para a realização deste estudo.

À minha orientadora Professora Doutora Graça Porto, gostaria de manifestar a minha admiração por toda a motivação, energia e entrega que tem na sua pesquisa científica, sem dúvida um exemplo inspirador de liderança. Elaborar esta tese sob a sua orientação foi muito gratificante em momentos de profunda aprendizagem. Muito obrigada por todo o seu apoio.

À Doutora Eugénia Cruz, as palavras não são suficientes para agradecer tamanha dedicação a este projecto, a minha dívida de gratidão é enorme, resta-me somente a esperança de algum dia poder retribuir. Muito obrigada pelas longas horas passadas em frente ao computador, pela paciência e por tudo o que me ensinou e incentivou a aprender.

Ao Doutor Jorge Vieira e Doutora Cristina Vieira gostaria de agradecer toda a disponibilidade, conselhos e sugestões. Pela partilha de ideias no debate deste estudo, por me terem recebido no seu grupo de investigação e me terem proporcionado um crescimento muito importante a nível científico. Muito obrigada por todo o apoio.

À Doutora Conceição Santos por ter aceitado ser minha co-orientadora, muito obrigada pelas sugestões e pela sua disponibilidade.

A todos os meus colegas do grupo IRIS por toda a amizade, especialmente à Vera Dias por todo o apoio. Aos meus colegas do grupo de Evolução Molecular: Ramiro, Micael e Bruno obrigado por me terem recebido, ensinado e por toda a amizade.

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

À Antonieta e Carla, amigas sempre presentes que me apoiam incondicionalmente, obrigada por toda a motivação.

Aos meus pais, dirijo o meu mais sincero obrigada por serem as pessoas que mais acreditam em mim e nos quais tenho o maior orgulho e extremo carinho.

palavras-chave

Doença Hepática, Ferro, Esteatohepatite Não alcoólica (NASH), Hemocromatose Hereditária (HH), Complexo maior de Histocompatibilidade (MHC), Antigénio leucocitário Humano (HLA), gene HFE, Linfócitos T CD8+

resumo

O Fígado é o principal órgão regulador do metabolismo do ferro. Distúrbios relacionados com a sobrecarga de ferro podem ser devido a factores genéticos, como na HH com mutações conhecidas para o gene HFE, mas também associados a doença hepática crónica, como a NASH. Vários estudos relatam uma elevada frequência das mutações do gene HFE em pacientes com NASH. Do modelo genético e imunológico da HH, sabe-se que estes doentes têm um haplótipo conservado na região MHC que está associado com o número de células CD8 + T. Os indivíduos que têm baixos números de linfócitos T CD8 +, acumulam mais ferro no tecido hepático do que os têm elevado número de linfócitos CD8+ T. A partir destes pressupostos, questionamos se este efeito é também observado em pacientes com NASH e se a região MHC, utilizando os marcadores genéticos do modelo da HH, teria qualquer impacto na gravidade da doença.

Foram analisados dados clínicos, imunológicos e histopatológicos de 59 pacientes com NASH. Pacientes com NASH foram genotipados para oito marcadores genéticos na região MHC correlacionados com o número de células CD8 + T (HLA-A, B e C; PGBD1; ZNF193 e HFE) e dois perto do HLA-A: RS7240078 e RS4713207. Controlos (264 indivíduos) foram utilizados para a comparação das frequências de todos os alelos, excepto para PGBD1 e ZNF193 onde um subgrupo (n = 56) foi usado.

Não foram observadas diferenças estatisticamente significativas entre NASH e controlos para os marcadores genéticos e dados imunológicos. Foi encontrada uma associação entre números de linfócitos CD8 + T com fibrose e inflamação, sendo estas variáveis inversamente correlacionados. Os alelos HLA-A33 e HLA-A29 foram encontrados em dois haplótipos conservados na região definida pelos marcadores. O haplótipo contendo o HLA-A 33 encontra-se associado à presença de fibrose simultaneamente com uma baixa média de células CD8 + enquanto os indivíduos com o haplótipo do HLA-A 29 não desenvolviam fibrose e tinham linfócitos CD8+T mais elevados.

Os resultados demonstram que o perfil imunológico dos NASH não reflecte o mesmo padrão da HH. Este facto pode ser devido à condição inflamatória dos NASH, porque as células CD8+ T à periferia não reflectem o que ocorre nos tecidos como é observado no modelo da HH.

keywords

Liver disease, Iron, Non-alcoholic steatohepatitis (NASH), Hereditary Hemochromatosis (HH), Human Leucocyte Antigen (HLA), HFE, CD8+ T lymphocytes, Major Histocompatibility Complex (MHC) Class I.

abstract

Liver is the main organ in regulation of iron metabolism. Disorders of iron overload can be due to genetic factors, as in HH associated with the HFE gene mutations, and also associated with chronic liver disease, as in NASH. Several studies report a high frequency of HFE mutations in NASH patients. From HH genetic and immunological model it is known that a conserved haplotype in the MHC region is associated with CD8+T cell numbers. Individuals that have low CD8+T cell numbers have more iron accumulated in liver tissue, than individuals with high CD8+T cells. From these assumptions, we questioned if this effect is also seen in NASH patients and if the MHC region, using the genetic markers of HH, would have any impact in disease severity.

Clinical, immunological and histopathological data from 59 NASH patients were analyzed. NASH patients were genotyped for eight genetic markers of the MHC region correlated with CD8+T cell numbers (HLA –A, B and C; PGBD1; ZNF193; HFE; RS7240078 and RS4713207. Controls (264 individuals) were used for comparing frequencies of all genes except for PGBD1 and ZNF193 where a subgroup (n=56) was used.

No statistically significant differences were observed between NASH and controls for the genetic markers and immunological data. A negative association was found between CD8+T cell numbers and both fibrosis and inflammation. Two conserved haplotype were found with HLA-A33 and HLA-A29. HLA-A33 haplotype was found associated with the presence of fibrosis and low CD8+T cells average while haplotype carrying HLA-A*29 had less fibrosis and high CD8+T cells.

In conclusion, the results show that immunological profile in NASH patients do not reflect the same picture as in HH. This may be due to a different distribution of lymphocytes cells between blood and tissues given the inflammatory condition in NASH.

Index

Index of figures

Index of tables

1-INTRODUCTION

1.1 IRON IN LIVER DISEASE

The liver is the largest parenchymal organ in the body with multiple metabolic functions. Its normal function is vital for the intermediary metabolism of carbohydrates, fats and proteins. It is the organ that synthesizes all major plasma proteins, except immunoglobulins. The most important one is albumin. Others are coagulation proteins, proteins of transport of hormones, vitamins and many other biologically active substances [1]. By secreting the hormone peptide hepcidin, the liver is now recognized to play a central regulatory role in iron homeostasis [2]. In humans, there is no known excretory pathway for iron, so severe iron overload may result from excessive iron absorption, caused by a genetic defect known as hereditary Hemochromatosis (HH), or as a consequence of parenteral administration of iron, mainly in the form of blood transfusions. In addition, mild or moderate forms of iron overload are commonly observed associated with several forms of chronic liver disease, either with chronic alcohol consumption, viral hepatitis, or dysmetabolic disorders as non-alcoholic fatty liver disease (NAFLD).

1.2 HEREDITARY HEMOCHROMATOSIS

Hereditary Hemochromatosis (HH) is a homozygous-recessive inherited disorder that results in iron overload in the parenchymal cells of the liver and other organs due to excessive intestinal absorption. Tissue iron overload causes damage and dysfunction of the target organs. Total body iron pool ranges from 2 to 6 g in normal adults, of which 0.5g is stored in the liver (98% in hepatocytes). In HH, the net iron accumulation ranges from 0.5 to 1.0 g/year and total iron stored may exceed 50g, one third will be in the liver [1]. Manifestations of the disease appear after 20g of iron accumulation. The disease is characterized by **clinical**, **genetic** and **immunological** features.

The most severe clinical consequences are: hepatic cirrhosis, diabetes, arthritis, sexual dysfunction due to hypogonadotropic hypogonadism, cardiomyopathy, hiperpigmentation, and hepatocellular carcinoma [3]. Treatment of HH consists in regular phlebotomies for depletion of iron stores, establishing the serum ferritin level less than 50ng/ml [4].

Genetically, the most common form of HH is associated with the *HFE* gene. This gene was discovered by Feder *et al.* in the short arm of chromosome 6 at 6p21.3, near the HLA gene locus [5]. The search for this gene started long ago (1975), by Simon and collaborators that described for the first time an association between HH and HLA antigens: HLA-A and HLA-B in the major histocompatibility complex region (MHC). After several family studies, it was confirmed the autossomic recessive transmission of HH in *linkage* with the HLA-A*03-B*07 haplotype. The *HFE* gene, discovered later in 1996 by Feder *et al.*, encodes an HLA class I-like molecule. The protein structure consists in a transmembrane region and a small cytoplasmatic portion (Fig.1). The structural organization of HFE molecule includes a signal sequence, alpha-1 and alpha-2 domains that recognizes the peptide, and an immunoglobulin domain alpha-3. For protein surface expression, the correct conformation of immunoglobulin domain alpha-3 is needed with a non-covalent interaction with β2 microglobulin molecule (β2-m) and presentation of peptide binding site formed by α1 and α2. The peptide binding site interacts with transferrin receptor (TfR1) that binds to circulating transferrin and regulate iron homeostasis [1, 4].

Initially, two mutations were described in the HFE gene: H63D and C282Y. The C282Y mutation is more frequent among Caucasians (incident between 1.3- 14%), and is a cysteine-to-tyrosine replacement at amino acid 282 that inactivates this 343 amino-acid protein. This is due to a single G--»A transition at nucleotide 845 (G845A). The result is the alteration of the α3 domain and consequently elimination of the heterodimer HFE-β2m eradicating the cell surface expression of the molecule [4, 6]. This mutation is present in more than 83% in homozygosity of patients with HH. It is estimated that, for the Caucasian population, this homozigoty is approximately 0.3-1.0% [5, 7].

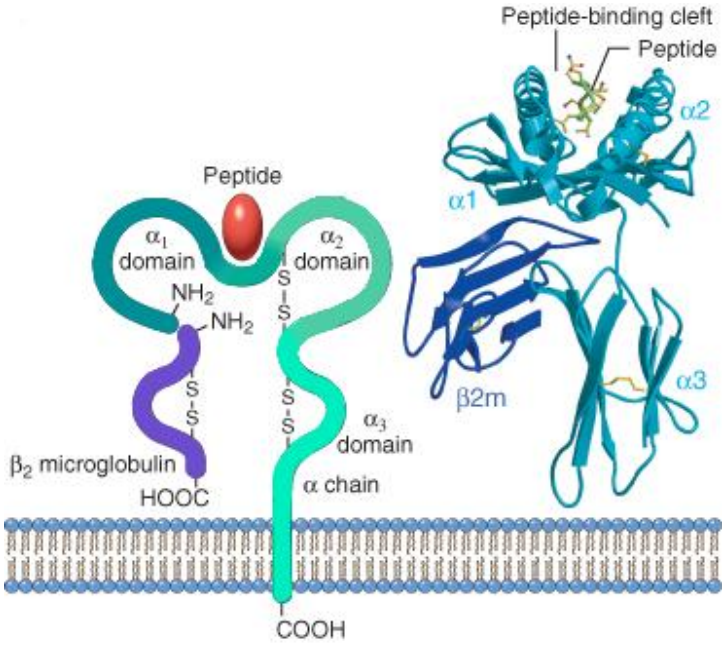

Figure 1 Schematic diagram of a MHC class I molecule (adapted from [1]).

The H63D mutation has a high frequency in Europe, above 15% [4]. It is associated to a mild phenotype of HH, when in heterozigoty with C282Y. It consists in a substitution of a cytosine to a guanine in the nucleotide 187, so it would appear a histidine instead of an aspartate at amino-acid 63. It does not affect the cell surface expression but alters the α1 domain modifying the affinity of transferrin to its receptor [4].

1.3 IRON AND IMMUNITY

The *HFE* gene belongs to the MHC class I genes family. The MHC is an extremely important group of genes involved in the immunological system. The postulate that the immunological system participates in the monitorization of iron toxicity was described by De Sousa in 1978 [8], based on her observations on lymphocytes traffic and positioning.

It was postulated that these cells were involved in a protector mechanism against metal toxicity through recognition and binding of metals [9]. This fact motivated

immunological studies in HH patients. Iron is an important nutrient for cell division in general. In the case of lymphocytes, it is known that activation and expansion depend on the expression of transferrin receptors needed for DNA synthesis and cellular division. It is also known that both T cells (inactivated and activated) synthesize ferritin [9].

In HH patients, it was observed abnormally high CD4/CD8 lymphocytes T ratios displaying a faster re-entry of iron in circulating transferrin after heavy phlebotomies, comparing with patients with normal CD4/CD8 ratios [10, 11].

The high CD4/CD8 ratios were due to low numbers of CD8+ T cells, which are inversely correlated with the amount of iron accumulated during time, reflecting a more severe disease [5, 12, 13]. Low numbers of CD8+T cells in the peripheral blood are associated with low numbers of these cells in the liver [14]. Patients with "low CD8+ T cells profile" have a more severe accumulation of iron with age, in contrast with patients with "high CD8+ T cells profile" [12]. Therefore, the CD8+T cells are used as prognostic marker for the accumulation of iron with age.

The most common form of HH is associated with C282Y mutation of the *HFE* gene. Although there is a great homogeneity in the genotype, the clinical phenotype is variable. Heterogeneity observed in the amount of iron accumulated in the patients' tissues may be explained by the existence of modifying factors as: sex and age of the individuals but also by environmental factors and the involvement of others genes that may interact together.

The existence of theses modifying factors may explain the low clinical penetrance of the disease. One of the modifiers that have been referred is the CD8+T cell profile. Recently, these cells were shown to be genetically regulated by gene/s at the MHC region. Studies in families with HH, allowed the observation of LD between particular HLA-A types and CD8+T cell counts. The inheritance of two copies of haplotypes carrying the HLA-A alleles: A*03, A*02 and A*01 was associated with low lymphocytes (0.30±0.14 $x10^6$/ml). Patients carrying one copy of that haplotype had an intermediate number of CD8+T cells (0.46±0.19 $x10^6$/ml) and patients without any of those haplotypes had the highest CD8+T cells numbers (0.79±0.19 $x10^6$/ml) [7].

Later, Vieira and collaborators identified a region in a normal population next to D6S105-150 as being the most probable localization of the gene that influences the levels of CD8+T cells [15]. This same region had been proposed some years ago, as a candidate region to look for modifiers of HH [16]. Pratiwi and collaborators conducted an extensive genetic analysis of genes in HFE region in a Australian population of HH. Two distinct peaks of association were identified, separated by 2 Megabases, which were not compatible with a single gene disorder [16]. One of the peaks found was at the region of the D6S2239, at 14 Kilobases (Kb) from HFE. The other corresponded to D6S105, located about 200 Kb from D6S2222 (Fig.2). This observation is compatible with the presence of a genetic modifier of the disease near this region [16].

Figure 2 Physical map of genetic markers used by Cruz et al. (2008) study and their relative location at scale.

Haplotypes that contains the HLA-A*1 allele are not the same in normal population and in C282Y homozygous patients, what explains why the allele A1 is associated with high numbers of CD8+T lymphocytes in normal population and low in HH patients [15].

The frequent presence of haplotype C282Y in linkage *desiquilibrium* with the allele HLA-A*03, and less relevant with HLA-B*07 associated to lower lymphocytes, led to infer the ancestral haplotype where the mutation C282Y occurred. Recombination in the region in between HLA-A and D6S2222 could have driven the C282Y mutation to others haplotypes found in control population (A*11, A*23, A*24, A*26, A*29, A*31, A*32, A*33

and A*68), leading to extinction of the linkage with the responsible “low CD8*T cells” profile gene [7].

Toomajian et al. (2003) found one triallelic polymorphism (A/C/G) at site 7633 located in first intron in HFE gene [17]. They found that 7633-A allele, was under positive selection in normal population, suggesting that it has hitchhiked on a particular large region. Vieira and collaborators showed an association between HFE-7633-A and microssatellite D6S105-150 predicting high CD8+T cell numbers [15].

In a recent publication in HH patients, Cruz et al. (2008) conducted a more extensive haplotype study and covered 1 megabase with 7 different markers surrounding the D6S105 microssatelite. The markers used included 5 SNPs in the genes: ZSCAN12 (Zinc finger and Scan Domain12), PGBD1 (piggyback transposable element derived 1), ZNF193 (Zinc finger protein 193), ZNF165 (Zinc finger protein 165), ZNF184 (Zinc finger protein 184); and two microsatellites D6S2222 and D6S105 [18](Fig.2).

The SNPs were selected based on their location and frequencies in the population. In a sample of 56 C282Y homozygous patients, a total of 112 haplotypes were obtained. The most common haplotype observed, with a frequency of 73%, was: ZSCAN12-A, PGBD1-A, ZNF193-A, ZNF165-T, D6S2222-247, D6S105-150. ZNF184-G. The high frequency of this haplotype suggests that it might be the ancestral one where C282Y mutation appeared. The results also revealed that 3 SNPs tested: PGBD1 (A/G), ZNF193 (A/G) and ZNF165 (T/G) each one were statistically significant associated with CD8+ T cell numbers. Those polymorphisms define a conserved haplotype with 500kb predicting CD8+T cells count, higher than each SNP separately. The haplotype constituted by the alleles PGBD1-A, ZNF193-A and ZNF165-T, designated A-A-T, was associated with low CD8+T numbers (0.37±0.17 x 10^6/ml) in opposite with the haplotype PGBD1-G, ZNF193-G and ZNF165-G, designated G-G-G, reflecting an high number of those cells (0.55±0.14 x10^6/ml) [18]. Moreover, the A-A-T haplotype when present in homozygosity (A-A-T/A-A-T) was associated with higher hepatic iron accumulation than in heterozygous patients for this haplotype (A-A-T/G-G-G) that exhibit a less deposition of iron in tissues. The G-G-G haplotype was also found in 10 Canadian patients [18]. This leads to the hypothesis that

the G-G-G haplotype could have a protective role in patients` clinical features and the same associations with CD8+Tcells and iron stores were observed. One limitation of this study is the little representation of G-G-G haplotype in these patients [18].

Clinical implications of these findings may be extremely important, because the genotype of the 3 SNPs can distinguish two groups of patients according to different prognostics. Patients with G-G-G haplotype may have a different surveillance in relation to therapeutic strategies as they may accumulate less iron and may need fewer phlebotomies.

1.4 NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of disorders of the liver due to causes other than alcohol that ranges from simple accumulation of fat exceeding 5-10% by weight (steatosis), to non-alcoholic steatohepatitis (NASH) with advanced fibrosis which can lead to cirrhosis and hepatocellular carcinoma. It is possible to distinguish a condition of simple fatty liver, characterized by the histologic finding of steatosis, from a state of NASH characterized by hepatocellular injury/inflammation with or without fibrosis. Even though, the frequency of NASH disorder is increasing [19-21].

NAFLD is considered to be the hepatic manifestation of insulin resistance (IR) [22]. The prevalence of NAFLD is around 20-30% in the general population. Only 5-17% of NAFLD patients progress to NASH. It is unclear why some patients who develop NAFLD go on to develop NASH while others do not. The occurrence of NASH is clinically important because approximately 15-25% of these patients will progress to cirrhosis [19]. From this cirrhotic stage, 30-40% of patients will develop a liver-related death over a 10-year period [19].

Although the initial stage of NASH is usually asymptomatic, it may be associated with non-specific symptoms such as upper abdominal pain, malaise or fatigue. In terms of histology NASH can progress from steato-inflammation to bridging fibrosis, cirrhosis and hepatic failure [20].

The laboratory features of the disease traduce abnormally high levels of serum concentrations of aspartate transaminase (AST) and alanine transaminase (ALT). This pattern of high AST and ALT concentrations helps to distinguish NAFLD from alcoholic hepatitis where AST values are elevated in relation to ALT concentration. In NAFLD it is common to observe elevations in serum ferritin with normal transferrin saturation and mild hepatic iron staining in liver biopsies [20]. However, the serum ferritin may also be increased in other inflammatory conditions and it is not clear what event comes first.

The physiopathology of NASH remains a two-hits hypothesis, the first hit consists of a metabolic syndrome often associated to obesity or overweight patients, but also including insulin resistance and diabetes. For NAFLD progression to NASH, a second hit is needed with the occurrence of ROS (Fig.3) [19].

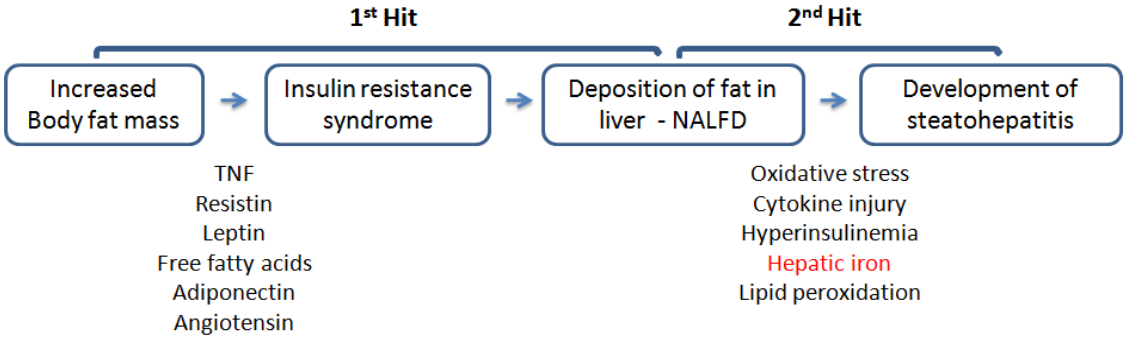

Figure 3 Scheme of suggested pathogenesis of NASH as a two hits process (Adapted from [19]).

Bonkovsky et al. studied the association of *HFE* mutations with NASH in a group of 57 patients in USA. The results showed an increased prevalence of the *HFE* mutations in homozygosity or heterozygosity combined in NASH patients (69.4%) relative to a control population with 348 subjects (40.5%, p=0.001). Patients with mutations were the ones with higher levels of serum ALT [20].

While in NASH cell injury begins with fat deposition in liver (NAFLD) as a result of insulin resistance and increased fat mass, in HH cell injury results directly from iron deposition consequence of an imbalance of iron absorption due a genetic cause.

In both situations, oxidative stress is involved. In NASH patients iron may be an additional factor for disease progression. Oxidative stress reactions (ROS) in the liver can be provoked by alcohol, hepatitis C virus or insulin resistance associated to obesity. Iron overload may exacerbate the ROS and promote liver injury and fibrosis. In some cases, ROS may directly promote carcinogenesis. Oxidative stress can also depress hepcidin expression in hepatocytes by reduced activity of C/EBPα. The presence of HFE C282Y mutation, can depress hepcidin even further and increase iron absorption and accumulation in the liver, generating more ROS (Fig.4).

The degree of hepatic fibrosis shows a positive association between hepatic iron concentration and duration of exposure to excess iron [2]. In HH it has been demonstrated that excess hepatic iron promotes the activation of hepatic stellate cells, reversible by iron removal [23].

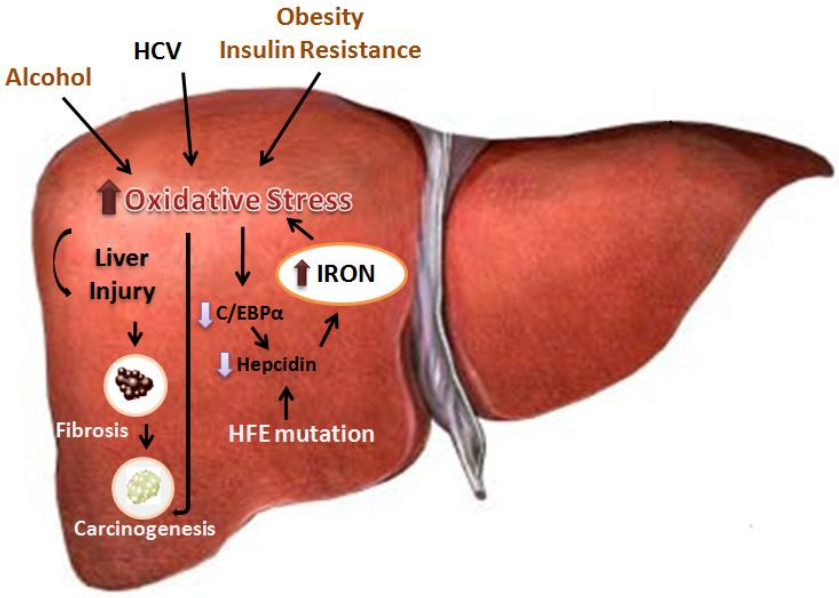

Figure 4 The role of Iron in liver disease. (adapted from [2]).

1.5 *OBJECTIVES*

It is known that NASH is associated with increased oxidative stress (ROS) and mitochondrial dysfunction. Iron overload is a producer of oxidative stress, therefore it appears that iron may exacerbate NASH pathology. The prevalence of the *HFE* mutations in NASH is reported to be higher than in controls, suggesting that iron may play a role is this pathogenesis. The risk of developing fibrosis is an interplay between genetic influences (as the HFE mutations) and environmental factors [24]. The predictive haplotype G-G-G, localized at MHC region described recently in HH, might also be involved in the progression of NASH, a mechanism that could be through the effect of CD8+T lymphocytes.

In the present thesis a NASH population is analyzed in relation to several genetic markers at MHC Class I region, the same markers that in HH are correlated with CD8+T cells and iron stores. We test the hypothesis if, similarly to HH, there are a relationship between the MHC region including HLA genes, haplotype PGBD1, ZNF193, ZNF165 and the HFE mutations, and the severity of NASH measured by the degree of steatosis, fibrosis, and inflammation. This hypothesis that the same modifiers of expression observed in HH could also modify the clinical course in NASH is very attractive.

2-MATERIAL AND METHODS

2.1 POPULATION STUDIED

2.1.1 NON-ALCOHOLIC STEATOHEPATITIS PATIENTS

A total of fifty-nine NASH patients were included in this study. They were diagnosed and followed up at Gastroenterology Clinics at Eduardo Santos Silva Hospital, Gaia. Forty-three were males with mean age 45 ± 12 years (range 26-78), and sixteen were females with mean age 45 ± 12 years (range 31-68). The diagnosis of NASH was established by a combination of clinical and histopathological features: consumption of alcohol below 20g/day and liver biopsy with steatosis and lobular inflammation (with absence of features suggestive of other diagnosis such as autoimmune, granulomatous or viral hepatitis). Clinical and laboratory data included in this work were partially reviewed from clinical files of the subjects or were determined for this study.

2.1.2 CONTROLS

A total of 264 apparently healthy unrelated subjects previously analyzed (Cruz et al, 2004) [25] were included in this study. They were blood donors recruited from the Blood Bank of Santo António Hospital, Porto. One hundred and sixteen were males with mean age 43 ± 13 years (range 20-76), and 148 were females with mean age 45 ± 13 years (range 18-88). These subjects were used as controls for immunological parameters (total lymphocytes, CD4+ and CD8+T cell numbers), for estimating HLA-A, -B, –C and HFE mutations normal frequencies. From this starting sample, a subgroup of 56 subjects was randomly selected and genotyped for four SNP markers (see Genetic characterization of subjects). Twenty-seven were males with mean age 40 ± 11 years (range 23-64), and twenty-six females with mean age 39 ± 12 years (range 23-62).

2.1.3 INFORMED CONSENT AND APPROVAL OF THE STUDY

Informed consent from patients and controls was obtained according to the Helsinki declaration. The study was approved by the ethical committee of Eduardo Santos Silva Hospital and Santo António Hospital.

2.1.4 GENERAL CHARACTERIZATION OF PATIENTS

Clinical data from patients were carefully reviewed from their clinical files by one dedicated physician. A liver biopsy was performed in all patients and all have steatosis and inflammation, as part of diagnosis criteria. In addition, fibrosis and siderosis was also evaluated. Scoring of these parameters was performed always by the same physician. Steatosis was graded 1-3: 22 patients with grade I, 19 with grade II and 14 with grade III (in four patients grade was not available); inflammation was graded 1-3: 46 with grade I, eight with grade II and two grade III (in three patients grade was not available); fibrosis was graded 0-4: 33 patients without fibrosis, 15 with grade I, five with grade II, two with grade III and one with grade IV (in three patients grade was not available); siderosis was graded 0-3: 27 without siderosis, 18 with grade I, eight with grade II and one with grade III (in five patients grade was not available).

Parameters included in the study were: body mass index (BMI), biochemical parameters of liver disease (alanine aminotransferase-ALT, aspartate aminotransferase-AST, gamma-glutamyl transferase-GGT) and biochemical parameters of iron metabolism (serum iron, transferrin saturation [TfSat] and serum ferritin).

Immunological parameters analyzed included peripheral blood total lymphocyte counts and CD4+ and CD8+ T-lymphocyte counts. Total lymphocyte counts were determined in an automatic blood cell counter (Advia 120. Hematology Systems, Bayer®). CD4+ and CD8+ T lymphocytes were determined by flow cytometry as described in detail elsewhere [25].

2.1.5 GENETIC CHARACTERIZATION OF SUBJECTS

Several genetic markers localized at the MHC class I region in the short arm of chromosome 6 were previously genotyped or genotyped for the purpose of this study in patients and controls (Fig.5).

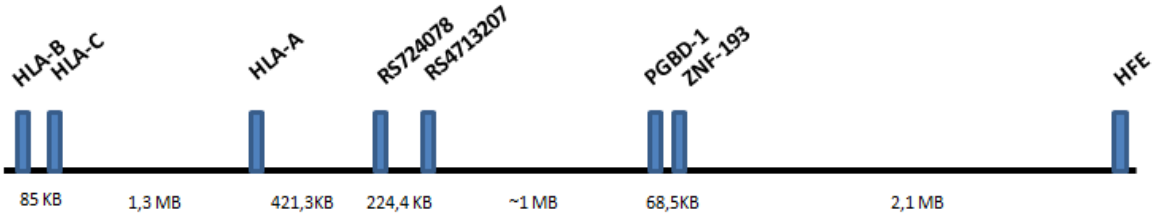

Figure 5 Physical map of genetic markers used in this study and their relative distance between consecutive marker.

These markers include: genotype for *HFE* gene mutations (C282Y and H63D) and for *HLA-A*, *-B*, *-C*. In controls, all subjects had genotype information for HLA-A alleles, 242 individuals gather information for HLA-B and 207 had genotype for HLA-C. In one patient there was no information of HLA genotype. Detailed description of genotype techniques was previously published elsewhere [25]. In addition patients and the subgroup of 56 controls were genotyped for four SNPs, two were localized in the genes: *PGBD1* (rs1997660 at position 28377642bp) and *ZNF193* (rs7206 at position 28309117), that were previously genotyped in HH patients (Cruz et al 2008)[26]. The other two were localized near HLA-A gene: RS724078 at position 1835888 and RS4713207 at position 2060281 (Fig.5).

2.2 METHODS

Genomic DNA was extracted using the blood extraction Kit from Promega: Maxwell®16 DNA Purification. For genotyping these SNPs, we first performed the approach of Allele-Specific PCR (AS-PCR), which consists in designing specific primers that will match each SNP polymorphism at the 3'-end of the prime.

For assessing the AS-PCR protocol, DNA samples were quantified using the Thermo Scientific NanoDrop spectrophotometer and normalized at the concentration of

70ng/µl. Using Oligo 4.0 software, primers to amplify each SNP allele were designed respecting the best compatibility parameters between oligonucleotides probes.

Only three genetic markers gathered these optimal conditions and were genotyped by AS-PCR: *PGBD1*, *ZNF193* and *RS724078*. The PCR annealing temperatures were optimized for each allele amplification (Table 1).

Table 1 Primers used for each SNP allele amplification, with specific annealing temperature, by AS-PCR.

SNP	Primers	Tª annealing		PCR Product
	FW: 5` TGT TTG GTG TCT TAC TTT	A	G	
PGBD1	RV(A):5` CTC ATT GTT TTC TTC TAT	53ºC	54ºC	671bp
	RV(G):5` CTC ATT GTT TTC TTC TAC			
	FW: 5`GAA AAG CCG TAT CAG TGC3`	A	G	
ZNF193	RV(A):5`GTA AGA AAG AGG TAA AAT 3`	52ªC	54ºC	382bp
	RV(G): 5`GTA AGA AAG AGG TAA AAC 3`			
	FW(A): 5`CTA CTG AGG AAA CAA GCA 3`	A	G	
RS724078	FW(G):5`CTA CTG AGG AAA CAA GCG 3`	49ºC	51ºC	764bp
	RV: 5` TGA CCA ATG ACC AAT GAT 3`			

Homozygous controls (positive and negative for each allele) were used in all PCR reactions. The thermocycler program had the following conditions: initial denaturation step at 95ºC (2`), 35 cycles (denaturation step at 95ºC (30``); annealing at specific temperature (45``) and extension at 72ºC (2`30) with a final extension at 72ºC (5`). The PCR products were stored at 4-8ºC. After the PCR reaction, the products were electrophoresed in a 1.5% agarose gel and samples amplification were compared with the specific controls.

AS-PCR protocol could not be established for the other SNP (RS4713207), since oligonucleotide probes in that region couldn´t assure a PCR genotyping. This difficulty was overcome by direct sequencing. Furthermore, when results of AS-PCR genotyping for *PGBD1* and *ZNF193* were not in agreement with the expected result, according to the most common haplotypes in the sample, those individuals were also sequenced. The sequencing protocol begins with a standard PCR with a volume of 25 µl using specific primers to frame the SNP genome region. The sequencing primers used for each gene

were those described in table 2 with specific annealing temperature and PCR product length.

Table 2 Primers used in sequencing PCR for each SNP gene region and specific annealing temperature.

SNP	Primers	Tª annealing	PCR Product
PGBD1	FW:5´AAA ATG TCC CTT TAT GAA T 3` RV:5´CAA ATC TCC GAA AAT CCA A 3`	50ºC	419bp
ZNF193	FW:5´TCT TTC TTC CTT TCT CCA T 3` RV:5´TCT GTT ATC CTT TGT AGT T 3`	46ºC	275bp
RS724078	FW: 5`AGC AGC CAG CAT CAC AGA 3` RV:5`ATC ATT GGT CAT TGG TCA 3`	52ºC	277bp
RS4713207	FW: 5`ATT TTC CTT TAC CTA CCC 3` RV: 5` TAT TAT TTT CTG GCT TCT 3`	48º C	706bp

Amplicons were then electrophoresed and extracted from the agarose gel with the QIAquick Gel Extraction Kit (Quiagen, Izasa Portugal, Lda). Sequencing mixs were prepared with Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California USA) according to manufacturer instructions and one of the sequencing primers. Finally samples were loaded in an ABI Prism 310 Genetic Analyzer Sequencer (Applied Biosystems, California USA).

2.3 HAPLOTYPE INFERENCE

Extended haplotypes were defined using the genotype information of the 8 genetic markers (Fig.5), using the program PHASE v 2.1 (http://www.stat. washington.edu/stephens/phase/) as described previously in Vieira et al. 2007 [15]. In some patients the genotype information of the markers rs4713207 (n=4) and rs724078 (n=16) was missing. In those cases, missing alleles were inferred using PHASE.

2.4 STATISTICAL ANALYSIS

Group means were compared by a parametric (Student's T test, ANOVA) or non-parametric (Mann-Whitney and Kruskal-Wallis) tests as appropriate according to variable distribution. The Shapiro-Wilk test was used to test the fitness of data to the normal distribution, for $p>0.05$. A normal distribution could be assumed for CD8+T cell numbers ($p=0.107$), TfSat ($p=0.128$) and serum iron ($p=0.068$). Independence between categorical data and differences between allele frequencies of the genetic markers, between NASH patients and controls were tested using the Fisher exact test. When large numbers of statistical tests are performed, correction for multiple comparisons is required. This is the case of comparisons of frequencies of HLA alleles that are extremely polymorphic. In order to overcome this problem the Bonferroni`s correction was applied.

Severity of NASH was evaluated in liver biopsies by the degree of steatosis, inflammation, siderosis and fibrosis. In order to have sufficient number of patients for statistical analyses involving these parameters, patients were grouped in two classes for each of those parameters: 1 and >1 for inflammation and steatosis and 0 and ≥1 for fibrosis and siderosis. Statistical analysis involving these parameters were performed using parametric or non-parametric tests, identified above, as appropriate.

All statistical tests were performed at 0.05 level of significance and all *p* values are two-sided. Data were analyzed by SPSS (Statistical Package for Social Sciences, version 17.0) software.

3-RESULTS

3.1 GENERAL CHARACTERIZATION OF NASH PATIENTS

General characterization of patients is shown in Table 3. No statistically significant differences were found in any of the parameters analyzed according to gender therefore, analyses were performed in males and females together. All subjects had average body mass index above the normal ranges, indicating an overweight condition. In the group of 59 NASH patients, all had average normal values of TfSat and serum iron according to reference ranges. Nevertheless, average values of serum ferritin were elevated in both males and females in relation to reference ranges. Average values of liver enzymes, ALT, AST and GGT, were increased, which is in accordance with NASH physiopathology. Considering immunological data, no significant differences were observed between NASH and controls in average values of total lymphocytes numbers, CD4+ T cells and CD8+ T cells counts (Table 3).

In all patients a liver biopsy was performed and all had steatosis and inflammation (as part of the diagnostic criteria). Fibrosis and siderosis was also evaluated in the liver biopsy. These four parameters were scored as described in Material and Methods (2.1.4). These parameters were analyzed between them and studied in relation to liver enzyme levels (AST, ALT and GGT) and iron parameters (TfSat, ferritin and serum iron). The grade of inflammation was correlated with fibrosis ($p<0.0001$, $r=0.58$, $R^2=0.32$) but not with steatosis and siderosis. The grade of siderosis was associated with ferritin levels ($p=0.035$, Kruskal-Wallis test), TfSat and serum iron ($p=0.0003$ and $p=0.0002$ One-way ANOVA, respectively), but not with liver enzymes. Severity of inflammation was associated with ALT levels ($p=0.051$, Kruskal-Wallis test) and was not associated with serum ferritin. Severity of fibrosis was associated with mean values of AST ($p=0.016$, Kruskal-Wallis test) and was not associated with serum ferritin levels.

Table 3 General characterization of Non-Alcoholic Steatohepatitis patients.

	All Patients (n=59)	**Males (n=53)**	**Females (n=16)**	**Reference values***
Age	45±12 (26-78)	45±12 (26-78)	45±12 (33-68)	-
Body Mass Index (Kg/m^2)	29.8±5.0 (21-48)	28.8±3.6 (21-35)	32.1±7.1 (21-48)	18.5-25
Transferrin saturation (%)	36±12 (16-74)	37±13 (16-74)	33±11 (15-52)	15-45
Serum ferritin (ng/ml)	282±255 (17-949)	323±254 (17-949)	307±248 (12-492)	11-150 (F) 11-282 (M)
Serum Iron (μg/dl)	112±33 (49-225)	112±36 (49-225)	112±26 (63-147)	53-167
Hepatic Iron Concentration (μg/g)	53.9±75.2 (7-434)	62.1±82.6 (7-434)	31.6±44.6 (1.3-183)	<50
Serum ALT (IU/l)	73±41 (26-206)	70±40 (31-206)	79±45 (26-176)	10-44
Serum AST (IU/l)	48±26 (18-141)	45±26 (26-141)	54±26 (21-107)	10-34
Serum GGT (IU/l)	109±106 (25-664)	111±116 (16-301)	103±80 (25-340)	10-66
Total lymphocytes (x10^6/ml)	2.04±0.52 (1.20-3.80)	1.99±0.43 (1.20-2.96)	2.18±0.71 (0.99-3.80)	2.14±0.53 (0.89-3.75)
Total CD4+ T cells (x10^6/ml)	0.85±0.26 (0.45-1.72)	0.77±0.18 (0.46-1.17)	1.01±0.32 (0.68-1.35)	0.92±0.28 (0.32-1.77)
Total CD8+ T cells (x10^6/ml)	0.44±0.17 (0.27-0.91)	0.44±0.18 (0.07-0.91)	0.43±0.17 (0.21-0.70)	0.43±0.17 (0.025-0.98)

*Reference values of the clinical biochemistry laboratory of the SAH, except for immunological data which reference values were determined from 264 unrelated healthy subjects used in a previous study.

3.2 GENETIC CHARACTERIZATION OF NASH PATIENTS

3.2.1 ALLELE FREQUENCIES OF HFE MUTATIONS, HLA-A, -B AND –C ALLELES AND SNP MARKERS

All patients were genotyped for HFE mutation and HLA-A, -B and –C alleles and frequencies were compared with 264 controls previously studied (Cruz et al. 2004, [25]), using Fisher exact test. The allele frequencies of *HFE* mutations in this group of NASH patients were not statistically different than controls (Table 4). The C282Y mutation had an allele frequency of 0.017 (0.025 in controls, n.s.) and the H63D mutation had a frequency of 0.241, that was slightly higher than in controls (0.191, n.s.) (Table 4).

Table 4 Comparison of allele frequencies of *HFE* mutations between NASH patients and controls.

HFE Allele Frequency	NASH patients (n=58)	Controls (n=264)*	p value
C282Y	0.017 (n=2)	0.025 (n=13)	0.75 n.s
H63D	0.241 (n=28)	0.191 (n=100)	0.25 n.s

* A previously studied population of 264 unrelated healthy subjects was used as controls. Allele frequencies were compared using the Fisher exact test.

No statistically significant differences were found between frequencies of HLA-A alleles in NASH patients and controls. In HLA-B alleles, only with HLA-B*49 was found a significant difference (p=0.022) between these groups, with a higher frequency in NASH patients (9/116=0.078) than in controls (13/484=0.027). In HLA-C alleles, HLA-C*4 had a statistically significant (p=0.032) lower frequency among NASH patients (11/116=0.095) comparing with controls (73/414=0.176). After Bonferroni` correction these associations were no longer statistically significant (Table 5).

Table 5 Comparison of allele frequencies of HLA-A, -B and -C between NASH patients and controls.

HLA-A	NASH (n=116 alleles)	Controls * (n=528 alleles)	p value
HLA-A*1	0.112 (n=13)	0.123 (n=65)	n.s
HLA-A*2	0.276 (n=32)	0.280 (n=148)	n.s
HLA-A*3	0.086 (n=10)	0.095 (n=50)	n.s
HLA-A*11	0.034 (n=4)	0.051 (n=27)	n.s
HLA-A*23	0.043 (n=5)	0.045 (n=24)	n.s
HLA-A*24	0.181 (n=21)	0.116 (n=61)	n.s
HLA-A*25	0.017 (n=2)	0.009 (n=5)	n.s
HLA-A*26	0.026 (n=3)	0.027 (n=14)	n.s
HLA-A*29	0.069 (n=8)	0.049 (n=26)	n.s
HLA-A*30	0.009 (n=1)	0.015(n=8)	n.s
HLA-A*31	0.017 (n=2)	0.019 (n=10)	n.s
HLA-A*32	0.026 (n=3)	0.038 (n=20)	n.s
HLA-A*33	0.060 (n=7)	0.036 (n=19)	n.s
HLA-A*66	0.009 (n=1)	0.008 (n=4)	n.s
HLA-A*68	0.035 (n=4)	0.036 (n=19)	n.s

Table 5 Comparison of allele frequencies of HLA-A, -B and -C between NASH patients and controls (Cont.).

HLA-B	NASH (n=116 alleles)	Controls * (n=484 alleles)	p value
HLA-B*7	0.026 (n=3)	0.056 (n=27)	n.s
HLA-B*8	0.034 (n=4)	0.064 (n=31)	n.s
HLA-B*13	0.017 (n=2)	0.010 (n=5)	n.s
HLA-B*14	0.095 (n=11)	0.081 (n=39)	n.s
HLA-B*15	0.060 (n=7)	0.064 (n=29)	n.s
HLA-B*18	0.069 (n=8)	0.046 (n=22)	n.s
HLA-B*27	0.052 (n=6)	0.027 (n=13)	n.s
HLA-B*35	0.076 (n=9)	0.097 (n=47)	n.s
HLA-B*37	0.009 (n=1)	0.014 (n=7)	n.s
HLA-B*38	0.017 (n=2)	0.021 (n=10)	n.s
HLA-B*39	0.026 (n=3)	0.010 (n=5)	n.s
HLA-B*40	0.026 (n=3)	0.019 (n=9)	n.s
HLA-B*41	0.009 (n=1)	0.010 (n=5)	n.s
HLA-B*44	0.181 (n=21)	0.174 (n=83)	n.s
HLA-B*45	0.009 (n=1)	0.012 (n=6)	n.s
HLA-B*49	0.078 (n=9)	0.027 (n=13)	0.022 (n.s after BC)
HLA-B*50	0.043 (n=5)	0.048 (n=23)	n.s
HLA-B*51	0.086 (n=10)	0.106 (n=51)	n.s
HLA-B*57	0.052 (n=6)	0.029 (n=14)	n.s
HLA-B*58	0.026 (n=3)	0.012 (n=6)	n.s
HLA-C	**NASH (n=116 alleles)**	**Controls * (n=414 alleles)**	**p value**
HLA-C*1	0.017 (n=2)	0.012 (n=5)	n.s
HLA-C*2	0.069 (n=8)	0.065 (n=27)	n.s
HLA-C*3	0.026 (n=3)	0.056 (n=23)	n.s
HLA-C*4	0.095 (n=11)	0.176 (n=73)	0.032 (n.s after BC)
HLA-C*5	0.095 (n=11)	0.063 (n=26)	n.s
HLA-C*6	0.095 (n=11)	0.099 (n=41)	n.s
HLA-C*7	0.250 (n=29)	0.229 (n=95)	n.s
HLA-C*8	0.086 (n=10)	0.077 (n=32)	n.s
HLA-C*12	0.069 (n=8)	0.053 (n=22)	n.s
HLA-C*14	0.043 (n=5)	0.034 (n=14)	n.s
HLA-C*15	0.034 (n=4)	0.065 (n=27)	n.s
HLA-C*16	0.103 (n=12)	0.060 (n=25)	n.s
HLA-C*17	0.009 (n=1)	0.010 (n=4)	n.s
HLA-C*18	0.009 (n=1)	0	n.s

* A previously studied population of 264 unrelated healthy subjects was used as controls. Allele frequencies were compared using the Fisher exact test. Bonferroni` s correction was applied (BC).

The SNPs in the genes PGBD1 and ZNF193, and SNPs RS724078 and RS4713207 were genotyped in the 59 patients and in a subgroup of controls of 56 subjects. No statistically significant differences were observed in the allele frequencies of these markers between NASH patients and controls (Table 6).

Table 6 Comparison of allele frequencies of SNP markers between NASH patients and controls.

Class2SNP		**NASH**	**Controls * n=112**	**p value**
RS724078 (n=118)	A	0.356 (n=42)	0.384 (n=43)	n.s
	G	0.644 (n=76)	0.616 (n=69)	
RS4713207 (n=118)	A	0.542 (n=64)	0.423 (n=48)	n.s
	G	0.466 (n=54)	0.571 (n=64)	
PGBD1 (n=118)	A	0.644 (n=76)	0.643 (n=72)	n.s
	G	0.362 (n=42)	0.357 (n=40)	
ZNF193 (n=118)	A	0.593 (n=70)	0.554 (n=62)	n.s
	G	0.407 (n=48)	0.446 (n=50)	

* Allele frequencies of the genetic markers were determined in the subgroup of 56 unrelated healthy subjects. Allele frequencies were compared using the Fisher exact test.

3.2.2 HAPLOTYPE FREQUENCIES

Gathering all sequencing information obtained in these 8 SNP for NASH patients and controls, extended haplotypes were inferred using the software PHASE. In Fig.6, haplotypes were aligned taking in to account the HLA–A alleles and then arbitrarily according to the similarity of the alleles from the several genetic markers. The extended haplotypes that were found with frequencies above 10% were those carrying the HLA-A*2, HLA-A*24 and HLA-A*1. Although there are some conserved regions in those haplotypes, they are extremely diverse as illustrated in Figure 6 by use of different colors. Interestingly, the extended haplotypes carrying HLA-A*29 and HLA-A*33 showed a high conservation. 50% (4/8) of HLA-A29 and 57% (4/7) of HLA-A33 haplotype carry the same alleles in all 8 markers tested (Fig. 6 and Table 7).

HLA-B	HLA-C	HLA-A	RS078	RS207	PGBD	ZNF193	HFE
8	7	1	G	A	G	G	wt
58	7	1	G	A	G	G	wt
18	7	1	G	A	G	G	wt
37	6	1	G	A	G	G	H63D
57	6	1	G	A	G	G	H63D
13	6	1	G	A	A	A	H63D
27	2	1	G	A	A	A	wt
27	2	1	G	A	A	A	wt
44	16	1	G	A	A	A	wt
35	4	1	G	G	A	A	wt
58	7	1	G	G	G	G	wt
40	15	1	A	A	G	G	H63D
8	7	1	A	A	A	A	H63D
51	14	2	A	A	A	A	wt
51	14	2	A	A	A	A	wt
51	14	2	A	A	A	A	wt
51	14	2	G	A	A	A	wt
51	14	2	G	A	A	A	wt
44	5	2	A	A	A	A	wt
44	5	2	A	G	A	A	wt
13	6	2	A	A	A	A	wt
50	6	2	A	A	A	A	wt
7	7	2	G	A	A	A	wt
51	12	2	A	A	A	A	wt
39	12	2	A	G	A	A	wt
41	17	2	A	A	A	A	wt
44	2	2	A	A	A	A	wt
35	4	2	A	A	A	A	wt
35	4	2	A	A	A	A	wt
35	4	2	A	A	A	A	wt
35	4	2	A	G	A	A	wt
44	16	2	G	G	A	A	wt
44	16	2	G	G	A	A	wt
44	16	2	G	G	A	A	wt
45	16	2	G	A	A	A	wt
15	1	2	G	A	G	G	wt
35	4	2	G	A	G	G	wt
50	6	2	G	A	G	G	H63D
50	6	2	G	A	A	G	H63D
39	12	2	A	G	G	G	H63D
27	1	2	G	A	A	G	wt
44	5	2	G	A	A	G	wt
44	5	2	G	A	A	A	wt
50	6	2	G	A	A	A	wt
38	12	2	G	G	G	G	C282Y
7	7	3	A	G	A	A	wt
7	7	3	A	G	A	A	H63D
14	8	3	A	G	A	A	wt
27	7	3	A	G	G	G	wt
49	7	3	A	G	G	G	H63D
35	4	3	A	G	G	A	wt
44	4	3	A	G	G	G	H63D
57	18	3	A	A	G	G	wt
51	2	3	A	A	A	A	H63D
18	3	3	G	G	A	A	wt
15	7	11	G	A	A	A	wt
18	12	11	A	G	A	A	wt
18	5	11	G	A	A	A	H63D
15	7	11	G	G	G	G	wt

HLA-B	HLA-C	HLA-A	RS078	RS207	PGBD	ZNF193	HFE
49	7	23	G	G	A	A	wt
49	7	23	G	G	A	A	wt
49	7	23	G	A	A	A	H63D
49	7	23	G	A	G	A	wt
50	6	23	A	G	A	G	wt
44	5	24	G	G	A	A	wt
44	5	24	G	G	A	A	wt
44	5	24	G	G	A	A	wt
44	5	24	G	G	A	A	wt
44	5	24	G	G	A	A	wt
27	2	24	G	G	A	A	wt
15	2	24	G	G	A	A	wt
57	6	24	G	G	A	A	wt
18	7	24	G	G	A	A	wt
8	7	24	A	G	A	A	wt
14	8	24	A	G	A	A	wt
51	15	24	A	G	A	A	wt
57	7	24	G	A	A	A	wt
57	7	24	A	A	A	A	wt
8	16	24	A	A	A	A	wt
14	2	24	G	A	A	G	wt
57	7	24	G	A	G	G	wt
27	2	24	G	A	G	G	H63D
35	8	24	G	A	G	G	H63D
40	3	24	A	A	A	G	H63D
40	3	24	A	G	G	G	wt
18	12	25	G	G	A	A	H63D
18	12	25	G	G	A	A	H63D
38	12	26	G	A	A	A	H63D
49	7	26	G	G	A	A	wt
58	7	26	A	G	A	A	wt
44	16	29	G	A	G	G	H63D
44	16	29	G	A	G	G	H63D
44	16	29	G	A	G	G	H63D
44	16	29	G	A	G	G	H63D
44	16	29	G	G	G	G	H63D
44	16	29	G	A	G	G	wt
14	8	29	G	G	G	G	H63D
35	4	29	A	A	A	A	wt
15	4	30	A	A	G	G	H63D
51	15	31	G	G	A	A	wt
51	15	31	G	G	A	A	wt
15	7	32	A	G	A	A	wt
39	7	32	G	A	G	G	wt
49	7	32	G	G	G	G	wt
14	8	33	G	G	G	G	wt
14	8	33	G	G	G	G	wt
14	8	33	G	G	G	G	wt
14	8	33	G	G	G	G	wt
14	8	33	G	G	G	G	C282Y
14	8	33	G	G	A	A	wt
14	6	33	G	G	G	G	H63D
15	7	66	G	A	A	A	wt
49	7	68	G	A	G	G	wt
49	7	68	A	A	G	G	wt
18	5	68	G	A	G	G	wt
53	4	68	G	G	A	A	wt

Figure 6 Extended haplotypes using 8 genetic markers of the MHC region, in NASH patients grouped according HLA-A types. Color boxes represent conserved haplotypes areas.

3.3 ANALYSES OF THE IMPACT OF GENETIC MARKERS OF THE MHC REGION IN RELATION TO SEVERITY OF DISEASE

For these analyses allele frequencies of each genetic marker were determined in relation to severity of NASH. Severity of disease was evaluated in liver biopsies scored for levels of steatosis, inflammation, fibrosis and siderosis. In order to have sufficient number of patients for statistical analyses, patients were grouped in two classes: 1 and >1 for steatosis and inflammation and 0 and ≥1 for fibrosis and siderosis. For steatosis, inflammation and siderosis, no statistically significant effect was found for any of the genetic markers tested (Fisher exact test, $p>0.05$). When fibrosis was analyzed, a high frequency of the HLA-A*33 was found in patients with fibrosis ≥1 (6/46=0.130) in contrast with patients without fibrosis (1/66=0.015, $p=0.018$, Fisher exact test). A low frequency of HLA-A*29 was found in patients with fibrosis ≥1 (1/46=0.022) in contrast with patients without fibrosis (7/66=0.106), although this result does not reach statistical significance ($p=0.137$).

3.4 ANALYSES OF CD8+T CELLS NUMBERS IN RELATION TO SEVERITY OF DISEASE

The numbers of CD8+T lymphocytes were analyzed in relation to severity of NASH using the same grouping criteria of biopsy parameters in two classes as above.

A statistically significant association was found between CD8+T cell numbers and fibrosis. Patients without fibrosis showed average CD8+T cell numbers higher (0.49±0.17 $x10^6$/ml) than patients with fibrosis ≥1 (0.37±0.16$x10^6$/ml, $p=0.044$ Student-T test) (Fig.7). A negative association was also found between CD8+T cell numbers and inflammation (0.46±0.17 for inflammation=1 and 0.29±0.15 $x10^6$/ml for inflammation >1, $p=0.045$, Student T-test) (Fig.8).

No statistically significant associations were found for total lymphocytes and CD4+ T cells counts and any of the liver histological parameters analyzed.

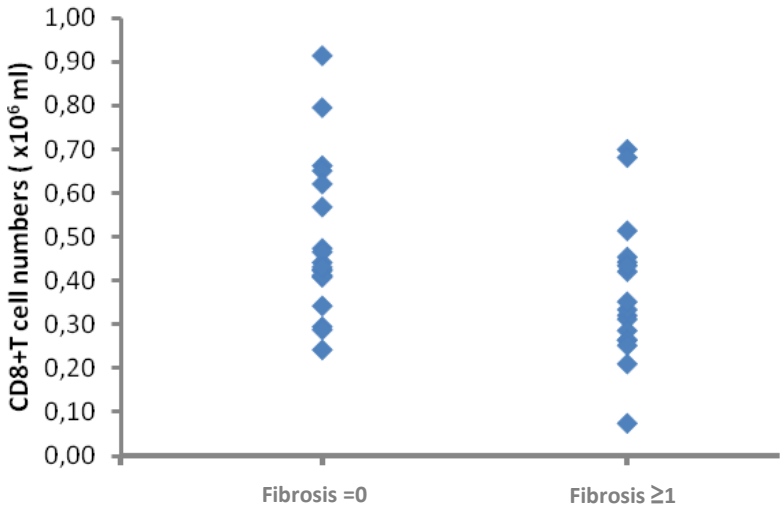

Figure 7 Distribution of CD8+ T cell numbers according to fibrosis score (p=0,044).

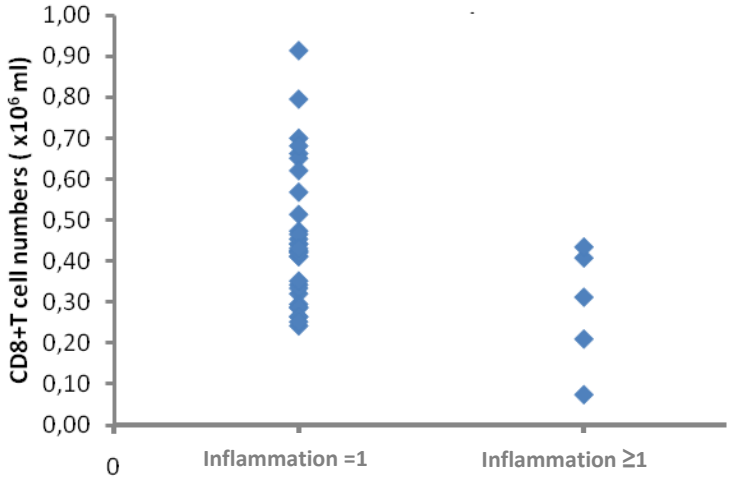

Figure 8 Distribution of CD8+T cell numbers according to inflammation score (p=0,045).

3.5 IMPACT OF EXTENDED HAPLOTYPE AND CD8+ T CELLS NUMBERS IN HEPATIC FIBROSIS IN NASH PATIENTS

According to previously published results in HH subjects [7], genetic markers of the MHC Class I region were shown to be predictors of CD8+T cell numbers. In the context of C282Y mutation, the inheritance of two haplotypes carrying the HLA-A*01, -A*02 or -A*03 were associated with a low CD8+T cell profile in opposite to haplotypes carrying all

other HLA-A alleles. Results in NASH patients showed an impact of certain HLA-A types on fibrosis and negative associations of CD8+ T cells with fibrosis and inflammation. In face of the results previously described in HH patients and the results described in the present study, we went further to test the hypothesis if in this disease the impact of HLA-A region on severity of NASH is mediated by CD8+T cell numbers. For this, we analyzed extended haplotypes carrying HLA-A*33 and HLA-A*29 (the most conserved haplotypes among this group of NASH patients) in relation to CD8+T cell numbers and degree of fibrosis (Table 7). Indeed, in the subgroup of patients carrying the allele HLA-A*33, where all patients had fibrosis except one, the average of CD8+T cell number was low (0.31±0.19 x10^6/ml). In contrast, the subgroup of patients carrying the HLA-A*29, where there is only one patient with fibrosis, had high average of CD8+T cell numbers (0.51±0.16 x10^6/ml).

Table 7 Genetic characterization of NASH patients carrying HLA-A*29 and HLA*A33 haplotype with correspondent CD8+T cell counts and Fibrosis score of liver biopsy.

ID	Gender	Age (years)	Fibrose Score	CD8 (x10^6 ml)	Haplotype 1								Haplotype 2							
					HLA-B	HLA-C	HLA-A	RS078	RS207	PGBD	ZNF193	HFE	HLA-B	HLA-C	HLA-A	RS078	RS207	PGBD	ZNF193	HFE
#1166	M	47	0	n.a	44	16	29	G	A	G	G	H63D	44	16	29	G	A	G	G	H63D
#1849	F	33	0	0.57	44	16	29	G	A	G	G	H63D	49	7	26	G	G	A	A	wt
#993	F	68	0	0.66	44	16	29	G	A	G	G	H63D	18	7	24	G	G	A	A	wt
#1291	F	34	0	n.a	44	16	29	G	G	G	G	H63D	27	2	24	G	G	A	A	wt
#1611	M	45	0	0.62	44	16	29	G	A	G	G	wt	49	7	68	G	A	G	G	wt
#930	F	31	1	0.27	14	8	29	G	G	G	G	H63D	58	7	26	A	G	A	A	wt
#1416	M	26	0	0.41	35	4	29	A	A	A	A	wt	15	2	24	G	G	A	A	wt
#1036	M	66	2	0.07	14	8	33	G	G	G	G	wt	49	7	3	A	G	G	G	H63D
#827	F	33	1	0.31	14	8	33	G	G	G	G	wt	35	4	2	A	G	A	A	wt
#986	M	39	1	0.51	14	8	33	G	G	G	G	wt	15	7	66	G	A	A	A	wt
#997	M	36	1	0.35	14	8	33	G	G	G	G	wt	8	7	1	A	A	A	A	H63D
#685	M	62	1	0.33	14	6	33	G	G	G	G	H63D	13	6	1	G	A	A	A	H63D
#1216	M	43	0	0.43	14	8	33	G	G	G	G	C282Y	14	8	3	A	G	A	A	wt
#1345	F	45	2	0.70	14	8	33	G	G	A	A	wt	51	2	3	A	A	A	A	H63D

* n.a: not available

4-DISCUSSION

Low numbers of CD8+T cells have been described for a long time in HH and have been associated with the severity of disease in terms of iron stores, HH related clinical symptoms and progression of liver pathology [27]. In addition, genes localized at the MHC Class I region were shown to be involved in the regulation of CD8+T cell numbers and therefore associated with the severity of disease. In the present work, we studied the involvement of the same modifiers of the severity of HH (CD8+T cells and genetic markers of MHC Class I region) in the phenotypic expression of NASH in 59 patients.

From general characterization of subjects, the results showed that in this group of NASH patients, serum AST and ALT are increased. Moreover, we also found positive associations of the degree of inflammation and fibrosis with ALT levels. These results are expected since these enzymes are markers of liver injury. In fact it is known that long-term observation of these enzymes may provide an index of individuals at risk of developing metabolic syndrome and diabetes [28]. Siderosis was found to be associated with serum ferritin, showing that the levels of ferritin are indicating liver iron stores.

The involvement of MHC Class I genetic markers in NASH is different from HH

NASH patients were genotyped for 8 markers of the MHC class I region, including HLA-A, -B and-C, *HFE* mutations (C282Y and H63D) and four SNPs in between (Fig. 5). In general, no differences were found in the frequencies of those markers between NASH patients and controls. Allele frequencies of *HFE* mutations (C282Y and H63D) were not statistically significant different between patients and controls, although we found a slightly increase of the H63D (0.024 versus 0.191). The frequencies of these mutations have been previously reported and some authors found increased frequencies mainly of the H63D [20, 29-31]. Nevertheless, this was not confirmed by other studies [32-34]. This may be due to selection criteria of patients in those studies or to geographical population

genetic differences. No significant differences were found between NASH patients and controls for the frequencies of the HLA-A, -B and –C. Although statistical results were found for HLA-B*49 and –C*04 these differences do not have statistical power considering that we made multiple comparisons.

Extended haplotypes showed a high diversity of the genetic markers studied when they are analyzed according to the HLA-A alleles (Fig. 6). The most conserved extended haplotypes are the ones carrying the A*29 and A*33 where they have the same alleles in the 8 markers in 50% of the A*29 haplotypes and in 57% of the A*33 haplotypes. These findings are considerable different from genetics of the same region in HH where haplotypes carring the C282Y mutation are extremely conserved as shown before [7].

CD8+T lymphocytes are related with liver severity in NASH

In NASH patients, CD8+T cell numbers were found associated with severity of this disease in terms of degree of fibrosis. Patients with low CD8+T cell numbers had high degree of fibrosis in contrast with patients with high CD8+T cells that had less fibrosis. This result does not seem to be due to the liver iron content as previously described in HH [12, 13], because siderosis or HIC did not correlate with CD8+T cells. There are two possible explanations for these results. One is related with the iron accumulation in the liver that is not as severe as in HH that may prevent the observation of the referred correlation. The other is related with the presence of inflammation in NASH, where CD8+T cell numbers are inversely associated with liver inflammation. This is clearly in contrast with what is described in HH [14]. These results suggest distinct pathogeneses in these two diseases.

Is there any combined effect of MHC Class I genes and CD8+T cells in liver severity in NASH?

Interestingly, we found an association of the two most conserved haplotypes (A*33 and A*29) with fibrosis and CD8+T cell numbers. While patients carrying HLA-A*33

haplotype had more fibrosis and less CD8+ T cells, patients carrying HLA-A*29 haplotype had less fibrosis and high CD8+T cells. It is possible that in this region there is one or more genes correlated with CD8+T cells and with fibrosis. In this case “protective” gene/s would be transmitted in the haplotype carrying HLA-A*29 (associated with high CD8+T cells and low fibrosis) and “susceptibility” gene/s would be transmitted in the haplotype carrying the HLA-A*33 (associated with low CD8+T cells and high fibrosis). These results may be observed only in the context of these two haplotypes because there is a great conservation of the analyzed region that could allow the transmission of long blocks carrying the same genes through generations. It should be noticed however, that the variability of CD8+T cell is high in each subgroup and the number of patients with those HLA-A alleles is small probably due to their normal low frequencies (less than 5%) (Table 5).

Considering genes located at the MHC region, near the HLA-B gene there is the TNF(α) that is recognized as an important intervenient in the activation of the stellate cells which drives fibrosis [35]. Other genes in the region are transcription factors with unknown functions that could directly or indirectly regulate expression of distant genes.

Therefore we should not exclude an important role of other non-MHC Class I linked cytokines. It has been described that toll-like receptor (TLR)-9 and MCP-1 cytokine are expressed by activated stellate cells and are responsible for lymphocyte T cells placement in injury tissues [24, 36]. Stellate cells are antigen-presenting cells and can regulate, through chemokines, the infiltration of inflammatory cells. Another toll-like receptor, TLR-4, produced by Kupffer and stellate cells, is also able to interact with endotoxins, resulting in the release of pro-inflammatory mediators that can induce hepatic injury and fibrosis [22, 36]. Moreover a polymorphism in TRL-4 gene has been reported to contribute to fibrosis progression in HCV infection [28].

It is evident that the mechanism of fibrogenesis is complex and any step towards its clarification is of great clinical relevance.

5-CONCLUSION

Motivated by the known importance of the MHC region in liver pathology, as demonstrated by the well studied model of HH, this study aimed to search its contribution in NASH metabolic syndrome. In a group of 59 NASH patients with available biopsy evaluation, we found an association between the criteria of fibrosis and CD8+ T cell numbers, being these variables inversely associated. The inflammatory condition is the main physiological difference between NASH and HH pathology. In NASH, peripheral low numbers of CD8+ T cells reflect tissue liver inflammation, while in HH low CD8+ T cells reflect a genetic predisposition. Furthermore, the MHC region in this NASH population is highly variable. Despite that, two conserved haplotypes carrying HLA*A33 and A*29 appeared and were related to a fibrosis predisposition state. This genetic region is very complex and contains many genes, some being transcription factors that could regulate the expression of distant genes. With the present study we gained in terms of increasing our knowledge in NASH pathology and the role of CD8+T cell lymphocytes in the fibrogenic process. The results presented here, however, were obtained with the peripheral blood cells, it would be important in future to confirm the role of CD8+T cells in liver tissue.

6-BIBLIOGRAPHY

1. Kumar VA, Abul K. ;Fausto, Nelson: Robbins & Cotran Pathologic Basis of Disease, 7th Edition edn: Saunders; 2004.

2. Wallace DF, Subramaniam VN: Co-factors in liver disease: The role of HFE-related hereditary hemochromatosis and iron. *Biochim Biophys Acta* 2009, 1790(7):663-670.

3. Brissot P, Troadec MB, Bardou-Jacquet E, Le Lan C, Jouanolle AM, Deugnier Y, Loreal O: Current approach to hemochromatosis. *Blood Rev* 2008, 22(4):195-210.

4. Porto G. CC, Macedo MF, Cruz E.: Hereditary hemochromatosis type I: Genetic, clinical and immunological aspects. In: *Iron Metabolism and Disease.* Edited by Fuchs H, 1 edn; 2008: 435-460.

5. de Sousa M, Porto G: The immunological system in hemochromatosis. *J Hepatol* 1998, 28 Suppl 1:1-7.

6. Cardoso C, Porto G, Lacerda R, Resende D, Rodrigues P, Bravo F, Oliveira JC, Justica B, de Sousa M: T-cell receptor repertoire in hereditary hemochromatosis: a study of 32 hemochromatosis patients and 274 healthy subjects. *Hum Immunol* 2001, 62(5):488-499.

7. Cruz E, Vieira J, Almeida S, Lacerda R, Gartner A, Cardoso CS, Alves H, 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* 2006, 7:16.

8. De Sousa M: Lymphoid cell positioning: a new proposal for the mechanism of control of lymphoid cell migration. *Symp Soc Exp Biol* 1978, 32:393-410.

9. Porto G, De Sousa M: Iron overload and immunity. *World J Gastroenterol* 2007, 13(35):4707-4715.

10. Porto G, Cardoso CS, Gordeuk V, Cruz E, Fraga J, Areias J, Oliveira JC, Bravo F, Gangaidzo IT, MacPhail AP *et al*: Clinical and genetic heterogeneity in hereditary haemochromatosis: association between lymphocyte counts and expression of iron overload. *Eur J Haematol* 2001, 67(2):110-118.

11. Porto G, Reimao R, Goncalves C, Vicente C, Justica B, de Sousa M: Haemochromatosis as a window into the study of the immunological system: a novel correlation between CD8+ lymphocytes and iron overload. *Eur J Haematol* 1994, 52(5):283-290.

12. 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* 2006, 37(1):33-39.

13. Porto G, Vicente C, Teixeira MA, Martins O, Cabeda JM, Lacerda R, Goncalves C, Fraga J, Macedo G, Silva BM *et al*: Relative impact of HLA phenotype and CD4-CD8 ratios on the clinical expression of hemochromatosis. *Hepatology* 1997, 25(2):397-402.

14. Cardoso EM, Hagen K, de Sousa M, Hultcrantz R: Hepatic damage in C282Y homozygotes relates to low numbers of CD8+ cells in the liver lobuli. *Eur J Clin Invest* 2001, 31(1):45-53.

15. Vieira J, Cardoso CS, Pinto J, Patil K, Brazdil P, Cruz E, Mascarenhas C, Lacerda R, Gartner A, Almeida S *et al*: A putative gene located at the MHC class I region around the D6S105 marker contributes to the setting of CD8+ T-lymphocyte numbers in humans. *Int J Immunogenet* 2007, 34(5):359-367.

16. Pratiwi R, Fletcher LM, Pyper WR, Do KA, Crawford DH, Powell LW, Jazwinska EC: Linkage disequilibrium analysis in Australian haemochromatosis patients indicates bipartite association with clinical expression. *J Hepatol* 1999, 31(1):39-46.

17. Toomajian C, Ajioka RS, Jorde LB, Kushner JP, Kreitman M: A method for detecting recent selection in the human genome from allele age estimates. *Genetics* 2003, 165(1):287-297.

18. Cruz E, Whittington C, Krikler SH, Mascarenhas C, Lacerda R, Vieira J, Porto G: A new 500kb haplotype associated with high CD8+ T-lymphocyte numbers predicts a less severe expression of hereditary hemochromatosis. *BMC Med Genet* 2008, 9(1):97.

19. Shifflet A, Wu GY: Non-alcoholic steatohepatitis: an overview. *J Formos Med Assoc* 2009, 108(1):4-12.

20. Bonkovsky HL, Jawaid Q, Tortorelli K, LeClair P, Cobb J, Lambrecht RW, Banner BF: Non-alcoholic steatohepatitis and iron: increased prevalence of mutations of the HFE gene in non-alcoholic steatohepatitis. *J Hepatol* 1999, 31(3):421-429.

21. Varela-Rey M, Embade N, Ariz U, Lu SC, Mato JM, Martinez-Chantar ML: Non-alcoholic steatohepatitis and animal models: understanding the human disease. *Int J Biochem Cell Biol* 2009, 41(5):969-976.

22. Petta S, Muratore C, Craxi A: Non-alcoholic fatty liver disease pathogenesis: the present and the future. *Dig Liver Dis* 2009, 41(9):615-625.

23. Ramm GA, Crawford DH, Powell LW, Walker NI, Fletcher LM, Halliday JW: Hepatic stellate cell activation in genetic haemochromatosis. Lobular distribution, effect of increasing hepatic iron and response to phlebotomy. *J Hepatol* 1997, 26(3):584-592.

24. Wood MJ, Powell LW, Ramm GA: Environmental and genetic modifiers of the progression to fibrosis and cirrhosis in hemochromatosis. *Blood* 2008, 111(9):4456-4462.

25. Cruz E, Vieira J, Goncalves R, Alves H, Almeida S, Rodrigues P, Lacerda R, Porto G: Involvement of the major histocompatibility complex region in the genetic regulation of circulating CD8 T-cell numbers in humans. *Tissue Antigens* 2004, 64(1):25-34.

26. 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.

27. Macedo MF, Cruz E, Lacerda R, Porto G, de Sousa M: Low serum transferrin levels in HFE C282Y homozygous subjects are associated with low CD8(+) T lymphocyte numbers. *Blood Cells Mol Dis* 2005, 35(3):319-325.

28. Lefkowitch JH: Recent developments in liver pathology. *Hum Pathol* 2009, 40(4):445-455.

29. Fargion S, Mattioli M, Fracanzani AL, Sampietro M, Tavazzi D, Fociani P, Taioli E, Valenti L, Fiorelli G: Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. *Am J Gastroenterol* 2001, 96(8):2448-2455.

30. George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, Jazwinska EC, Powell LW: Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. *Gastroenterology* 1998, 114(2):311-318.

31. Nelson JE, Bhattacharya R, Lindor KD, Chalasani N, Raaka S, Heathcote EJ, Miskovsky E, Shaffer E, Rulyak SJ, Kowdley KV: HFE C282Y mutations are associated with advanced hepatic fibrosis in Caucasians with nonalcoholic steatohepatitis. *Hepatology* 2007, 46(3):723-729.

32. Bugianesi E, Manzini P, D'Antico S, Vanni E, Longo F, Leone N, Massarenti P, Piga A, Marchesini G, Rizzetto M: Relative contribution of iron burden, HFE mutations, and insulin resistance to fibrosis in nonalcoholic fatty liver. *Hepatology* 2004, 39(1):179-187.

33. Chitturi S, Weltman M, Farrell GC, McDonald D, Kench J, Liddle C, Samarasinghe D, Lin R, Abeygunasekera S, George J: HFE mutations, hepatic iron, and fibrosis: ethnic-specific association of NASH with C282Y but not with fibrotic severity. *Hepatology* 2002, 36(1):142-149.

34. Deguti MM, Sipahi AM, Gayotto LC, Palacios SA, Bittencourt PL, Goldberg AC, Laudanna AA, Carrilho FJ, Cancado EL: Lack of evidence for the pathogenic role of iron and HFE gene mutations in Brazilian patients with nonalcoholic steatohepatitis. *Braz J Med Biol Res* 2003, 36(6):739-745.

35. Novo E, Parola M: Redox mechanisms in hepatic chronic wound healing and fibrogenesis. *Fibrogenesis Tissue Repair* 2008, 1(1):5.

36. Friedman SL: Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008, 134(6):1655-1669.