

MITOCHONDRIAL HAPLOGROUPS ASSOCIATED WITH END-STAGE HEART FAILURE AND CORONARY ALLOGRAFT VASCULOPATHY IN HEART TRANSPLANT PATIENTS

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Abstract

Aims Mitochondrial haplogroups are known to influence individual predisposition to a wide spectrum of metabolic and degenerative diseases, including ischemic cardiovascular diseases. We have examined the influence of the mitochondrial DNA (mtDNA) background on the development of human end-stage heart failure (HF) in patients undergoing heart transplantation. The influence of mtDNA haplogroups on the incidence of transplant-related complications, mainly cardiac allograft vasculopathy (CAV), and on post-transplant survival was also studied.

Methods and Results The most common mitochondrial haplogroups in European populations were genotyped in 450 heart transplant recipients, 248 heart transplant donors, and 206 healthy controls. Mitochondrial haplogroups were determined by PCR amplification of short mtDNA fragments, followed by restriction fragment length polymorphism analysis. After adjustment for age and sex the frequency of haplogroup H was significantly higher in heart transplant recipients than in controls (OR 1.86 [95% CI 1.27-2.74], $P=0.014$), and in heart donors (OR 1.47 [95% CI 0.99-2.19], $P=0.032$). Likewise, haplogroup Uk was found significantly more frequently among CAV patients than in non-CAV heart allograft recipients (OR 4.1 [95% CI 1.51-11.42], $P=0.042$). Finally, heart donor haplogroups had no influence on the morbidity or mortality after heart transplantation.

Conclusions Mitochondrial haplogroups behave like risk factors for the progress to end-stage HF in a Spanish cardiac transplant population. Mitochondrial DNA variants may have some influence on the appearance of cardiac transplant complications.

Keywords heart failure, coronary allograft vasculopathy, heart transplantation; mitochondria; mitochondrial haplogroups

1. Introduction

Heart failure (HF), the end-stage syndrome of many cardiovascular diseases, is a common, severe condition that places a great demand on health care resources. In Spain, several studies have reported a prevalence of HF of 6.8% in the Spanish population aged over 45 years and of 16% when only taking into account the population aged over 75 years.^{1,2} Because of its high incidence and poor prognosis, HF has been considered the genuine worldwide 21st century epidemic and new therapeutic approaches are encouraged to treat these patients.² In this sense, cardiac transplantation is currently the only satisfactory long-term therapy available to treat refractory HF.¹

In spite of the advances in HF therapy over the last decade, HF remains a relentlessly progressive disease and the reason why heart failure progresses in patients receiving current optimal therapy remains unknown.² There is increasing evidence that defective mitochondrial energetic and abnormal substrate metabolism are key characteristics of the failing heart,⁴ suggesting that benefit may be derived from therapies designed to preserve and optimize cardiac mitochondrial function.⁴

Mitochondria are the site of pyruvate oxidation and the citric acid cycle, and they generate ATP by means of the electron transport chain and oxidative phosphorylation. Of the proteins directly needed for this activity, only 13 are encoded by the mtDNA itself, whereas about 80 proteins are coded by the nucleus and subsequently imported from the cytoplasm into the mitochondria.⁵ Human mitochondrial DNA is a 16,569 base pair double-stranded circular molecule containing 37 genes, and is transmitted by the mother so that it undergoes negligible intermolecular recombination.⁵ In addition, the evolutionary rate of mtDNA is reported to be much higher than that of nuclear DNA. Consequently, polymorphisms in mtDNA are expected to contribute more extensively to the functional differences among individuals than those in nuclear DNA.⁵ Among the

numerous mutations that have accumulated in mtDNA during evolution there are several ethnic specific single nucleotide polymorphisms that enable the definition of discrete and region specific subdivisions of the human population called mitochondrial haplogroups. Mitochondrial energetic function is influenced by functional mtDNA variants that alter mitochondrial coupling efficiency and allow individuals to adapt to different energetic environments.⁵ Besides, these genetic variants have controversially been described to be associated to the individual predisposition to a wide spectrum of metabolic and degenerative diseases, including ischemic cardiovascular disease, as well as to cancer and longevity.⁵⁻⁷

The relationship between mtDNA haplogroups and the development and progression of HF is unknown. For that reason, the primary aim of this study was to analyze the influence of mtDNA haplogroups on the evolutive course to refractory HF. The results reported here clearly show that mitochondrial haplogroups are associated with the progress of end-stage HF. Our analyses, although preliminary, also suggest an influence of the mitochondrial genetic background on coronary allograft vasculopathy in a Spanish cohort of heart transplanted patients.

2. Methods

2.1. Study Subjects

This study was approved by the ethics committee of the Puerta de Hierro University Hospital and complies with the principles of the Declaration of Helsinki.

The studied cohort comprised 450 unrelated patients (mean age, 49.5 ± 13.1 years; range 10-68; 355 men) undergoing heart transplantation at our institution between 1984 and 2008, and 248 donors (mean age, 30.6 ± 11.8 ; range 10-56; 185 men) from the same time period. All patients and donors were Caucasians. Underlying cardiac disease in the cohort was ischemic heart disease in 174 (38.7%) patients, idiopathic dilated cardiomyopathy in 148 (32.9%), other causes of dilated cardiomyopathy in 20 (4.4%), valvular disease in 38 (8.4%), restrictive cardiomyopathy in 19 (4.2%), hypertrophic cardiomyopathy in 20 (4.4%), congenital heart disease in 14 (3.1%), and others in 17 (3.8%). All patients presented end-stage chronic HF and were in the New York Heart Association (NYHA) functional class III (41%) or IV (59%) at the time of cardiac transplantation. Pre-transplant, transplant, and post-transplant data were extracted from our centre's prospective Heart Transplant database. Patients followed our centre's standard post-transplant clinical follow-up program. Endomyocardial biopsy (EMB) and invasive coronariography were used for the diagnosis of rejection and cardiac allograft vasculopathy (CAV), respectively, according to the International Society of Lung and Heart Transplantation criteria.⁸ Our follow-up protocol includes 10 EMBs during the first post-transplant year and afterwards only when clinically indicated. CAV is systematically investigated per protocol at 5 and 10 years post-transplant and in the presence of symptoms or signs of cardiac dysfunction (even subtle ones, such as appearance of premature beats or repolarization abnormalities on EKG). Intravascular ultrasound (IVUS) was performed routinely at the time of coronary studies since 1994,

but did not influence medical management of heart transplant recipients, since changes were introduced only after diagnosis of significant CAV, defined by the presence of angiographic stenoses >50% in main coronary vessels. In the absence of recent coronariography and IVUS, sudden death was always attributed to CAV unless necropsy evidenced an alternative cause.

Patients received triple immunosuppressive therapy with calcineurin inhibitors (Ciclosporine A in most patients), an anti-metabolite drug (azathioprine until 1999 and mycophenolate mofetil thereafter) and prednisone. Steroid withdrawal was attempted only in a minority of patients who developed severe side-effects. All of them received statins for CAV prevention irrespective of their lipid levels. No other drugs were used routinely. Treatment of hypertension was usually started with ACE inhibitors or angiotensin receptor blockers (ARB).

2.2. Healthy controls

The control group comprised 206 Caucasian blood donors from our institution (mean age, 37.7±11.5 years; range, 18-63; 110 men) who were receiving no pharmacologic treatment and showed no evidence of heart disease according to their medical history.

2.3. Mitochondrial haplogroup genotyping

Our study was designed and performed in accordance with the recommendations for the human genotype-phenotype associations.⁹ Genomic DNA was extracted from blood following standard procedures.¹⁰ The samples were haplogrouped by PCR amplification of short mtDNA fragments, followed by restriction fragment length polymorphism (RFLP) analysis to assess the mtDNA haplogroups, as previously described¹¹ but with minor modifications (Table 1).¹² Individuals with haplogroups L and M (pointing to

some non-Caucasian ancestry), with Caucasian haplogroups with low representation (I, W, X), or with haplogroups not ascribed to any of the known Caucasian haplogroups were grouped in haplogroup O. In individuals belonging to haplogroup H we also genotyped polymorphisms that corresponded to subhaplogroups H1 (m.3010G>A), H3 (m.6776T>C) and H* (the rest of H subhaplogroups pooled together). To ensure proper internal control, for each genotype analysis, positive and negative controls from different mtDNA aliquots which were previously genotyped by the same method have been used. Besides, external control samples from standard accepted sets have been haplogrouped. For all the haplogroup markers, the RFLP analyses were carried out by two experienced researchers who were blind to subject data. Primers, PCR and RFLP conditions are available upon request.

2.4. Statistical analysis

Statistical analyses were performed using SPSS software for Windows version 14.0 (Chicago, IL, USA). The number of patients and controls fulfilled sample size estimations based on Fleiss,¹³ which setting a statistical power of 80%, a significance level of 0.05, and a 2:1 patient-control ratio, indicated a minimum of 297 heart transplant recipients and 148 controls for detecting an OR of 1.8 for an association of end-stage heart failure with the most common haplogroup in Spanish Caucasoids (haplogroup H: 39%⁶). Results were expressed as mean \pm standard deviation (SD) for numerical variables and as proportions for categorical variables. One-way analysis of variance (ANOVA) was used to assess the independence of the haplogroup distribution regarding age, body mass index (BMI), ischemia time, and left ventricular ejection fraction (LVEF). The differences in haplogroup frequency distribution between patients and controls were assessed by the χ^2 -independence test for the other cardiovascular risk

factors, such as hypertension and diabetes. Haplogroup frequencies in heart allograft recipients and donors were compared in the same way, but we compared each heart donor haplogroup with all other haplogroups pooled into a single group, as previously described⁷. The odds ratio (OR) and 95% confidence intervals (CI) were calculated to estimate the strength of association between the mitochondrial haplogroups and end-stage HF. To adjust for demographic variables such as age and sex a multivariate logistic regression model has been used. Contingency tables and χ^2 test were also used to predict possible interactions between clinical post-transplant variables and mitochondrial haplogroups. The Kaplan-Meier method and log-rank tests were used to compare the distributions of post-transplant survival according to the different haplogroups. A two-sided *p* value less than of 0.05 was considered statistically significant. The *p*-values of statistically significant differences were then corrected by the Bonferroni method (Corrected P-value= *p*-value*n [number of independent test]). The etiologic fraction (proportion of the genetic risk to a disease contributed by an analysed factor) was calculated by the formula: $f(OR-1)/OR$, where *f* is the frequency of the analysed factor in patients and OR, the odds ratio.

3. Results

3.1. Frequencies of mitochondrial haplogroups

In order to evaluate the potential genetic association between the mitochondrial haplogroups and end-stage heart failure in patients undergoing cardiac transplantation, the frequency of nine European mitochondrial haplogroups in control (n=206), heart donor (n=248) and heart allograft recipient (n=450) populations was estimated. Because of the low frequencies of several mtDNA variants we grouped the patients in seven haplogroups according to their proposed evolutionary proximity.¹¹ In this way, the

minimum expected frequency to ensure accuracy in the χ^2 -independence test is reached. The demographic characteristics of the study subjects are shown in Table 2. Different age and gender distribution was found among groups. These differences in some way were predictable because as it has been published, most of HT recipients are older age men and by contrary most of the donors are young men.¹ A one-way analysis of variance and contingency tables analyses demonstrated no variations in the distribution of haplogroups in relation with age ($p=0.32$) and sex ($p=0.55$) of the heart recipients. On the one hand, the frequencies observed in the control group and in the heart donors were similar (Table 3), and consistent with previous studies of Spanish^{12,14} and European^{7,15} populations. Frequencies obtained ranged from 40.3% in the control population and 44% in heart donors for the most common haplogroup H (Table 3). On the other hand, the frequency of haplogroup H (OR=1.75, [95% CI 1.25-2.45] $P=0.006$, etiologic fraction=23.2%) was significantly higher in cardiac transplant recipients than in controls (Table 3). This haplogroup also remained significantly higher in heart transplant patients than in controls after correcting for age and gender (Table 3). In both groups the most prevalent H subhaplogroups in Western Europe (H1, H3) and H* were also genotyped (data not shown). The results did not show a statistical significance (OR 0.93 [95% CI 0.76-1.13], $p=0.42$), and no H subhaplogroup has been significantly more frequent in patients than in controls. Besides, the frequency of haplogroups T and Uk was slightly but not significantly lower in heart allograft recipients than in controls (Table 3). This result might be attributed to the small number of patients carrying these haplogroups. To verify if this difference achieves statistical significance a larger cohort of patients would be necessary.

Furthermore, we also performed a χ^2 -independence test dividing our patients according to underlying cardiac disease, but only taking into account the most

predominant conditions leading to heart transplantation: ischemic heart disease and idiopathic dilated cardiomyopathy. As expected, the frequency of haplogroup H was higher in both subgroups of patients (OR 1.70 [95% CI 1.13-2.56], $P=0.001$ and OR 1.56 [95% CI 1.02-2.39], $P=0.03$ respectively) than in controls (adjusted values for age and gender: (OR 1.86 [95% CI 1.02-3.41], $P=0.044$ and (OR 1.58 [95% CI 0.97-2.59], $P=0.05$ respectively). However, it was independent of the underlying cause of the end-stage HF (OR 1.09 [95% CI 0.70-1.69], $p=0.71$) because it was similar in patients undergoing heart transplantation because of ischemic heart disease (53.4%) or dilated cardiomyopathy (51.4%) (Table 4).

3.2. Pretransplant risk factors for heart failure and mtDNA haplogroups

In order to elucidate if the high frequency of haplogroup H in the patients was associated with major risk factors for cardiovascular disease instead of the HF itself, we estimated the frequency of each haplogroup according to different pretransplant variables like body mass index (BMI), blood pressure, total cholesterol, and diabetes mellitus (Table 5a). No associations were found between mitochondrial haplogroups and risk factors for cardiovascular disease, indicating that the high frequency of haplogroup H is owed to the cardiac dysfunction itself. In addition, no associations were observed between HF severity, evaluated by LVEF and NYHA functional classes, and mitochondrial haplogroups (Table 5b).

3.3. Role of mitochondrial haplogroups in the transplant and post-transplant follow-up

To investigate the potential association between the mitochondrial genetic background and the post-transplant progress in the allograft recipients, contingency tables and

Kaplan-Meier analyses were performed. No significant differences were observed between haplogroups and the preoperative ischemic time (212.4 ± 62.3 min; range 70-430), or post-transplant tumor development (Table 6). Likewise, Kaplan-Meier analysis showed no significant differences between the post-transplant median survival years and the haplogroup frequencies (median survival [95% CI], 11.96 years [10.19 to 13.73; overall log rank, $p=0.82$], even when we compared post-transplant median survival years between patients with haplogroup H (H survival) and patients with the rest of haplogroups pooled together (non-H survival) (Figure 1). Finally, the presence of coronary allograft vasculopathy (CAV), the major factor limiting long-term survival after heart transplantation, was evaluated only in 320 patients and was diagnosed in 111 (34.7%) of them (Table 6). Interestingly, the frequency of haplogroup Uk (OR 4.1 [95% CI 1.49-11.24], $P=0.021$) was significantly higher in CAV than in non-CAV heart allograft recipients when each group was compared with all of the other haplogroups pooled together (Table 6). This association remained after adjusting for age and gender (Table 7). Besides, when we only compared the frequencies of haplogroup Uk and the most prevalent haplogroup H, a significantly higher frequency of haplogroup Uk in CAV heart allograft recipients was also observed (OR 3.93 [95% CI 1.40-11.00], $P=0.049$; adjusted values for age and sex (Table 7).

3.4. Heart donor haplogroups and post-transplant follow-up

The distribution of donor haplogroups in relation with age ($p=0.55$) and sex ($p=0.64$) of the heart donors was analyzed and, like in the heart recipient population, no variations were observed. Likewise, as it was expected, the frequency of haplogroup H (OR 1.51 [95% CI 1.10-2.06], $P=0.009$) was lower in the heart donor group than in cardiac

allograft recipients. Haplogroup H also remained significantly associated after correcting for age and gender (OR 1.47 [95% CI 0.99-2.19] $P=0.032$).

To study the influence of the heart donor haplogroups on morbidity and mortality after heart transplantation, we were able to recover only 248 genetic samples of heart donors. Of these, only 141 were matched with the allograft recipient haplogroups. Due to the low frequencies of the haplogroups in the 141 heart recipient-donor pairs we pooled the heart recipients in two groups: group H-H (58 patients), when heart recipients and donors exhibited the same haplogroup H, and group H-non H (83 patients), when heart recipients presented haplogroup H and heart donors exhibited a different haplogroup. Regarding to the development of CAV in the heart allograft recipients, no significant differences were observed when we compared H-H and H-nonH groups (OR 1.09 [95% CI 0.48-2.46], $p=0.82$), suggesting that the presence of CAV in the allograft recipients was independent of heart donor haplogroups. Likewise, the influence of heart donor haplogroups on the overall post-transplant mortality was evaluated, and no significant differences (OR 1.57 [95% CI 0.79-3.13] $p=0.19$) were found between the H-H versus H-nonH groups. In summary, the results reported here suggest that heart donor haplogroups have no influence on morbidity or mortality after heart transplantation.

4. Discussion

The contribution of the genetic background to health and disease are research areas of paramount interest. At this moment, there are several studies that report that common mitochondrial DNA haplotypes, not only in humans but also in mice, are associated with various phenotypes including learning performance and the penetrance of some mtDNA-linked diseases.^{5,16} However, the involvement of mitochondrial haplogroups in cardiovascular diseases remains unresolved and controversial and very few studies to

clarify this issue have been carried out. For example, it was reported that the N9b haplogroup may be considered protective against myocardial infarction in Japanese men,¹⁶ that haplogroup T is associated with coronary artery disease in a middle European population,¹⁸ and recently haplogroup H was associated with early onset myocardial infarction in patients from the North of Spain.²⁰ However, these results have not been reproduced and no association of mitochondrial haplogroups with risk of ischemic cardiovascular disease or acute coronary syndromes was observed in other European studies.^{20, 21}

In the present report, the genetic association between European mitochondrial haplogroups and end-stage HF was evaluated in 450 patients undergoing heart transplantation. The similarity of haplogroup distribution found among our control and heart donor groups to the distributions reported in different Spanish and European studies suggests that our control, heart donor and recipient populations are representative of the general population.¹

One of the most outstanding results that emerges from our study is the overrepresentation of mitochondrial haplogroup H in patients with refractory HF. This suggests that this haplogroup may have a role in heart energy metabolism, and thus contributes to HF progression. Until now, several articles have been published pointing out a biological role of this haplogroup under energetic and metabolic stress conditions. For instance, it has been reported that haplogroup H might be a predisposition factor for neurodegenerative diseases.^{7,5} It has also been associated with a better survival rate in patients with sepsis, and with protection against progression of the acquired immune deficiency syndrome (AIDS).⁵ Finally, a recent study by Palacin et al. supports an association of mitochondrial haplogroup H in a Spanish population with early onset of myocardial infarction.¹⁹ In spite of our study is broader and the underlying cardiac

disease in our cohort includes not only patients with ischemic heart disease but also with other cardiomyopathies, both studies coincide in the association of haplogroup H with cardiac disease.

The functional mechanism by which HF progression could be influenced by haplogroup H is still unknown. On the one hand, it has been reported that the capacity of the mitochondrial oxidative phosphorylation, when HF is established, is reduced and linked to a progressive mechanical dysfunction of cardiomyocyte sarcomeric proteins.⁴ In this sense, the higher frequency of haplogroup H found in patients with end-stage HF might be indicating a less efficient mitochondrial ATP production than in other haplogroups, and consequently a greater mechanical failure of the hearts of these patients. On the other hand, it has been observed that the activity of the electron transport chain (ETC) enzymes was normal in the ventricular myocardium of an animal HF model, and that the defect mainly resides in the assembly and function of a major ETC supercomplex (respirasome).²² In that way, haplogroup H may influence not only ATP and reactive oxygen species (ROS) production, but also ETC assembly kinetics when the respiratory demand increases in the heart, and this could be an additional contributing factor to the pathogenetic mechanism involved in end-stage HF. In fact, different assembly rates of respiratory chain supercomplexes belonging to different mitochondrial haplogroups have been shown to occur in a cellular model of mitochondrial disease.²³ However, further functional studies on a cardiomyocyte model are needed to elucidate this and other issues of the physiopathologic mechanisms of end-stage HF. Finally, a reduced mtDNA replication and a downregulation of the mitochondrial transcription cascade have recently been observed in human and experimental failing hearts.²⁴ This fact, combined with the decreased mtDNA copy number that has been observed *in vitro* in haplogroup H versus J cybrid cells, might support a reduced replication capacity of

haplogroup H under conditions of increased energy demand (like in cardiac dysfunction), thus exacerbating the constant progress of the HF.²⁵

Recently, it was suggested that mitochondrial haplogroups play a role in several risk factors for cardiovascular diseases such as hypertension, obesity, hyperlipemia, and metabolic syndrome.⁵ However, the lack of association between mitochondrial haplogroups and pretransplant risk factors for heart failure in our study is in accordance with other results found in European populations.²⁰ These risk factors are influenced by multi-genetic and environmental conditions, and conflicting results might reflect a modest role of mitochondrial haplogroups in the pathogenetic mechanisms of these late-onset diseases. In the same manner, we were unable to detect any association of mitochondrial haplogroups with the severity of HF, as indicated by LVEF and NYHA functional classes. However, a limitation of this study is the lack of patients in earlier stages of heart failure, long before undergoing cardiac transplantation. For that reason, to understand the involvement of mtDNA variants in the progress of failing heart, patients at early stages of HF need to be studied.

Additionally, although preliminar, this is the first study showing that mtDNA haplogroup Uk may be associated with an increased risk of CAV, a model of chronic rejection involving the entire length of the transplanted vessels.^{8,26} It is believed that CAV is caused by immunologic mechanisms and non-immunologic factors, including free radical-induced damage.²⁶ Mitochondrial haplogroup Uk, a subgroup of haplogroup U, has been considered as protective against developing idiopathic Parkinson's disease,⁷ AIDS progression, and to be associated with increased serum immunoglobulin E level.⁵ Although the mechanisms underlying these associations remain unclear, haplogroup-specific differences in immunologic pathways and ROS generation in mitochondria might be influencing the development of CAV. In turn, plasma levels of

proinflammatory cytokines are influenced by different genetic polymorphisms, and some of them are predictors of cardiovascular disease.²⁷ Haplogroup Uk might be related with a higher expression of cytokines and immune response than other mitochondrial haplogroups, and for that reason it could be overrepresented in allograft recipients with CAV. A further replication of this study in a higher number of cardiac allograft recipients with CAV is necessary to assess the reproducibility of these findings.

Finally, the influence of heart donor haplogroups on the morbidity or mortality observed after heart transplantation has been analysed. As we expected, the haplogroup distribution among heart donors was similar to that observed in our control population. For the same reason, the frequency of haplogroup H was lower in heart donors than in cardiac allograft recipients. However, the post-transplant course of the cardiac allograft recipients was independent of heart donor haplogroups, without any influence of mitochondrial haplogroups of the donors on CAV or mortality. Although an association between the recipient haplogroups with longevity has been found in several European and Asiatic studies,⁵ we and others²⁰ were unable to reproduce this association. This fact probably reflects the differences in the approach and design of the analyses.

In summary, the results reported here show mitochondrial haplogroups as risk factors for the progression of end-stage HF, and possibly for allograft complications in a Spanish cardiac transplant population. These results may provide a reliable starting point for further investigations on the relationships between end-stage HF, mitochondrial genetic background, and the pathological mechanisms that could influence the relentless evolution of the failing heart.

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Legends

Figure 1.- Cumulative survival of cardiac allograft recipients as a function of post-transplant years: 1A. In the overall allograft recipient population; 1B. Comparison of the post-transplant survival years between patients with haplogroup H (H survival) and patients with the rest of haplogroups pooled together (non-H survival).