CARDIOLOGY

Microvascular Function and Endothelial Progenitor Cells in Patients with Severe Hypercholesterolemia and the Familial Hypercholesterolemia Phenotype

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Abstract

Objective: To evaluate endothelial progenitor cells (EPCs) and systemic microvascular function in patients with severe hypercholesterolemia, comparing patients with the definite familial hypercholesterolemia (FH) phenotype (DFH) or probable/possible FH phenotype (PFH). There is a large spectrum of atherosclerotic disease between these two clinical phenotypes of FH, and to acquire further knowledge of the pathophysiology of vascular disease in both is desirable. *Methods:* Subjects with severe hypercholesterolemia, defined as low-density lipoprotein cholesterol (LDL-C) >190 mg/dL, were classified as DFH or PFH and underwent measurement of the number of EPCs by flow cytometry and evaluation of cutaneous microvascular reactivity using a laser speckle contrast-imaging system with iontophoresis of acethylcholine (ACh) or sodium nitroprusside. EPCs were defined as CD45– or CD45^{low}, CD34+CD133+CD309+ cells. Categorical variables were compared using Fisher test and continuous variables with Student t test or Mann-Whitney test, and a value of $p < 0.05$ was considered statistically significant. *Results:* Patients with DFH had higher LDL-C than those with PFH. There was no difference in the median number of EPCs between patients with DFH or PFH, but there was a significant reduction of endothelial-dependent, ACh-induced vasodilatation in the former. *Conclusion:* Patients with DFH have impaired microvascular endothelial-dependent vasodilatation compared to those with PFH, indicating more severe vascular disease in the former.

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Introduction

Hypercholesterolemia is a key risk factor for causing atherosclerotic vascular disease [1]. Patients with low-density lipoprotein cholesterol (LDL-C) >190 mg/dL are considered to have severe hypercholesterolemia [2], and in these cases, familial hypercholesterolemia (FH) must be considered [3].

FH is a frequent autosomal dominant disorder [4] whose clinical diagnosis (FH phenotype) is based on high LDL-C with physical stigmata such as tendon xanthomata, or evidence of these signs in first- or second-degree

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relatives, and personal or family history of premature coronary disease [5]. The FH phenotype is classified as definite FH (DFH), probable or possible FH (PFH) based on a score including clinical and laboratory data and DNA analysis [6]. Mutations are found in a majority of patients with DFH, and atherosclerosis is generally more severe in these patients compared to those with possible FH [7]. However, genetic testing is not widely available, and in many patients, diagnosis remains essentially clinical.

Normal endothelial function is a central component of vascular health and has been frequently evaluated by means of the assessment of microvascular reactivity [8]. Endothelial progenitor cells (EPCs) are circulating precursor cells that express cell surface markers of mature endothelium and of stem cells and assist in endothelial repair and angiogenesis [9]. EPCs comprise a heterogeneous cell population consisting of bone marrow-derived cells and endothelial precursors originating from monocytic cells, most commonly identified by flow cytometry by means of surface markers [10, 11]. The literature suggests different roles of EPCs in vasculogenesis, with the early EPCs contributing to neovasculogenesis mainly by secreting the angiogenic cytokines that help recruit resident mature endothelial cells and induce their proliferation and survival, whereas late EPCs enhance neovasculogenesis by providing a sufficient number of endothelial cells based on their high proliferation potency [12]. The idea that EPCs become dysfunctional during the course of a disease relies on the demonstration of their defective regenerative capacity or compromised ability to form colonies or migrate and form capillary-like structures. EPCs are subject to environmental and endogenous stressors, with aging being the most common cause of EPC dysfunction and reduction, but also hyperglycemia, atherosclerosis and others [13–15].

It is not known if there are significant differences in the extent of abnormalities of these indicators of vascular health among patients with the DFH or PFH phenotypes. Therefore, this study aimed to evaluate the number of EPCs and systemic microvascular function in patients with severe hypercholesterolemia, comparing the DFH and PFH phenotypes.

Methods

This was a prospective study of individuals attending the outpatient clinics of the Instituto Nacional de Cardiologia in Rio de Janeiro, Brazil, from July 2014 to March 2015. A screening of lipid panel results from all outpatients ≥18 years was performed ($n =$ 4,500), and patients with LDL-C >190 mg/dL ($n = 280$) were selected. Among 280 patients with severe hypercholesterolemia, 198 were considered ineligible because of the absence of personal or family history of hypercholesterolemia and/or cardiovascular disease. The remaining had their medical records checked for exclusion criteria, which included acute coronary syndromes or myocardial revascularization <30 days, autoimmune diseases, thyroid disorders, chronic renal failure, liver diseases, malignancy, steroid use, pregnancy or breastfeeding. Six patients were excluded due to recent surgery or hospital admission for acute coronary syndromes, and 1 due to cancer. Eligible patients were invited by phone call for further evaluation. Of these, 8 could not be reached by phone, 10 refused to participate, 3 died before the initial evaluation, and therefore, 54 participated in the study protocol. These patients signed an informed consent approved by the local Institutional Review Board. The study was undertaken in accordance with the Helsinki Declaration of 1975, revised in 2000. A flow diagram detailing patient inclusion and reasons for exclusion is in the Appendix.

The FH phenotype was classified as DFH, with >8 score points, or PFH, with 3–8 points [6]. Prior cardiovascular disease was defined as a history of myocardial infarction, >50% stenosis of any epicardial coronary artery at coronary angiography, myocardial revascularization (percutaneous or surgical) or stroke. Hypertension was defined as blood pressure ≥140/90 mm Hg and/or antihypertensive drug use. Diabetes mellitus was defined by history and the use of hypoglycemic medications, or fasting glucose >126 mg/dL. Body mass index was calculated as weight in $kg/(height)^2$.

Venous blood samples were obtained in the morning after 12 h of fasting. Serum triglyceride levels, total cholesterol, LDL-C, high-density lipoprotein (HDL) cholesterol (HDL-C) and glucose were evaluated.

The measurements of the number of EPCs and of microvascular reactivity were performed by investigators blinded to patient phenotype.

Quantification of EPCs
EPCs were all quantified by flow cytometry (BD FACSCanto; BD Biosciences, San Jose, CA, USA) and analyzed using Infinicyt software (Cytognos, Salamanca, Spain). EPCs were quantified using a 2-mL whole blood sample in which 2 million events were acquired. For the labelling of surface antigens, the following antibodies conjugated with fluorochromes were used: CD45 Percp, CD34 FITC, CD133/2 PE and CD309 (VEGFR-2/KDR) APC (Biotech, AL, USA). Flow-cytometric analysis was carried out in whole blood without any enrichment procedure to avoid enrichment artifacts. EPCs were defined as negative or "low" for hematopoietic marker CD45 and positive for markers CD34, CD133+, CD309+, excluding leukocytes (CD45+). We used a multiparametric analysis with sequential gating, employing Infinicyt software (Cytognos) to identify the phenotypes of the population of interest, as follows:

CD45low or CD45– were selected and the rest (CD45+) was excluded.

Among the CD45low or CD45– population, CD34+ cells were selected.

Among CD45low or CD45–CD34+ cells, we identified those with CD133+.

Among CD45low or CD45–CD34+CD133+ cells, we selected those with CD309+.

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Fig. 1. Flow cytometric panels showing representative patterns relative to the method utilized for the identification of endothelial progenitor cells (EPCs). **a** The analysis gate used to exclude platelets, dead cells and debris (shown in blue). **b** The EPCs (pink) in the total population analyzed (gray). **c** The gate used to exclude hematopoietic cells expressing the CD45 antigen applied to PerCP-labelled anti-CD45 vs. side scatter (SS) the CD45^{low} EPCs. **d** The positive applied to FITC-labelled CD34+CD45^{low} vs. side scatter (SS). **e** The positive applied to PE-labelled CD133/2, FITC-labelled CD34+CD45^{low} vs. side scatter (SS). **f** The positive applied to APC-labelled CD309+ (VEGFR2 or KDR), PE-labelled CD133/2+, FITC-labelled CD34+CD45^{low} vs. side scatter (SS). **g**, **h** The expression of antigens double-positive used to evaluate EPCs: **g** shows APC-labelled CD309+ (VEGFR2 or KDR) and PE-labelled CD133/2+ and **h** shows FITC-labelled CD34 and APC-labelled CD309+ (VEGFR2 or KDR).
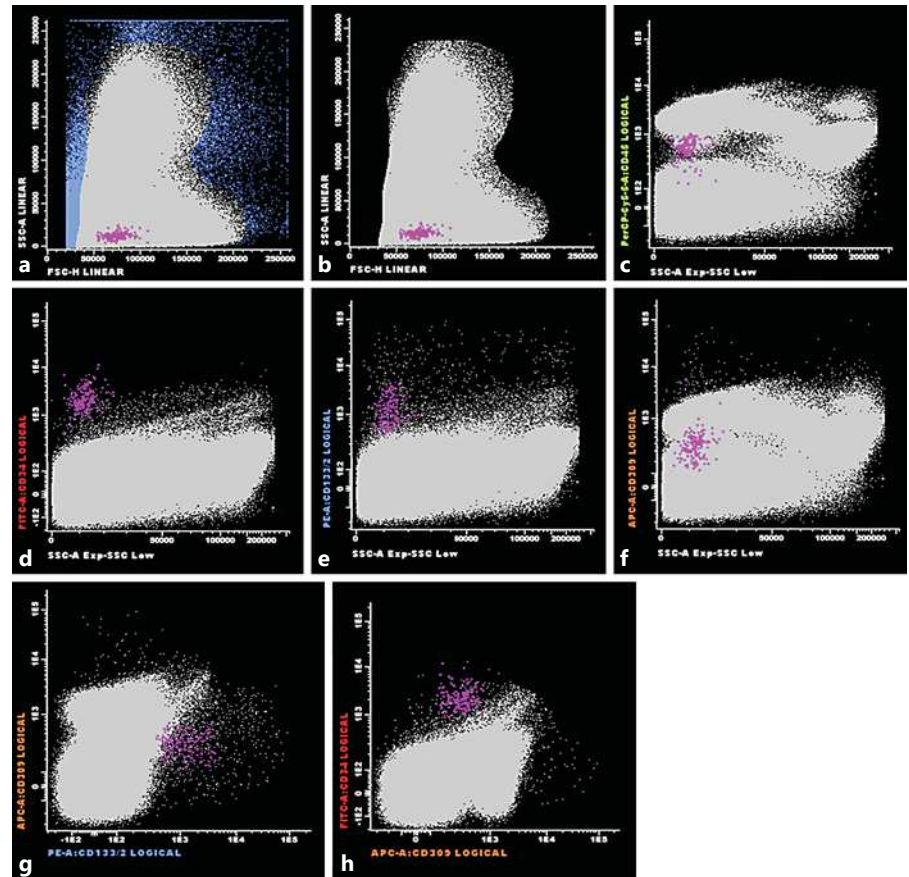
We finally checked the distribution at the FCS × SSC histogram of the cells with the phenotype CD45^{low} or CD45–CD34+CD133+CD309+.

A sequential strategy [16] was used to remove dead cells, platelet aggregates and debris, to exclude CD45+ and then follow the sequence described above (Fig. 1). The absolute number of EPCs was defined as a percentage relative to the white blood cell count assessed using a hematology cell analyzer.

Evaluation of Microvascular Reactivity

Microvascular reactivity was evaluated using a laser speckle contrast-imaging system (PeriCam PSI system; Perimed, Sweden) with iontophoresis of acetylcholine (ACh) or sodium nitroprusside (SNP) for the noninvasive and measurement of cutaneous microvascular perfusion changes measured in arbitrary perfusion units. Two skin sites on the forearm were chosen and two drug-delivery electrodes were installed using adhesive discs (LI 611; Perimed). ACh 2% w/v or SNP 2% w/v (Sigma Chemical Co., St. Louis, MO, USA) iontophoresis was performed with a micropharmacology system (PF 751 PeriIont USB Power Supply; Perimed). The results of the pharmacological tests were expressed as the area under the blood flow/time curve (representing the global flow response to different physiological and pharmacological stimuli) [16].

Statistical Analysis

Continuous data were analyzed using 2-tailed unpaired Student *t* test or Mann-Whitney test, and categorical variables with Fisher exact test. Results are displayed as mean ± SD, number and percentage or median and interquartile range. Analyses were performed with SPSS software version 21.0, and *p* values <0.05 were considered statistically significant.

Results

Table 1 shows demographic and clinical characteristics of patients with DFH or PFH. Physical stigmata of elevated cholesterol were more common in patients with DFH, although only corneal arch was significantly more frequent. Prior coronary artery disease was significantly more frequent and LDL-C was higher in patients with DFH. There was no significant difference in the median number of EPCs among patients with DFH or PFH.

Figure 2 depicts the AUC of ACh- or SNP-induced microvascular vasodilatation of patients with DFH or PFH. There was a significant reduction of endothelial-

Fig. 2. Area under the curve (AUC) of acetylcholine (ACh) or sodium nitroprusside (SNP) among patients with definite familial hypercholesterolemia (DFH) or probable familial hypercholesterolemia (PFH). APU, arbitrary perfusion units.
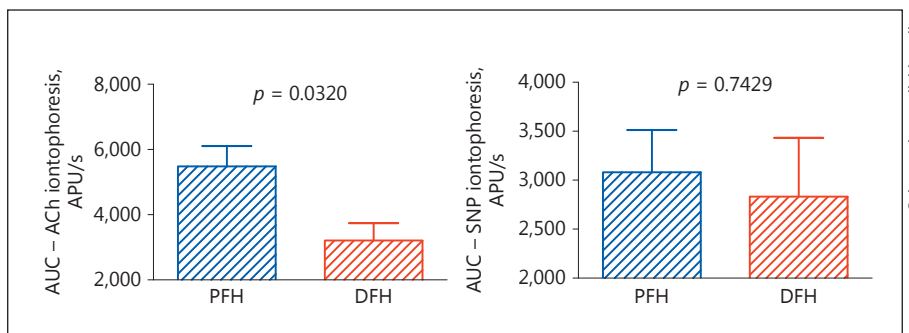
Table 1. Demographic, clinical and laboratory characteristics of patient with definite familial hypercholesterolemia (DFH) or possible/probable familial hypercholesterolemia (PFH)

	DFH ($n = 15$)	PFH ($n = 39$)
Age, years	52.3±15.4	54.9±12.0
Women	11 (73.3)	28 (71.8)
Hypertension	8 (53.3)	27 (69.2)
Diabetes	3 (20.0)	10 (25.6)
Smoking	0	5 (12.8)
Corneal arch	4 (26.7)	2 (5.6)*
Xanthelasma	2 (13.3)	2 (5.6)
Body mass index	27.1±6.0	28.3±5.4
Use of any hypolipemic medication	11 (73.3)	20 (51.3)
Statins		
Low dose	5 (33.3)	11 (28.2)
High dose	3 (20.0)	8 (20.5)
Statin + ezetimibe	3 (20.0)	1 (2.6)
Prior cardiovascular disease	15 (100.0)	27 (69.2)*
Total cholesterol, mg/dL	255.6±89.6	240.3±58.1
LDL-C, mg/dL	196.8±51.9	157.4±50.5 *
HDL-C, mg/dL	52.1±10.5	49.4±11.4
Triglyceride, mg/dL	125.0 (85.0–165.5)	134.0 (91.0–200.0)
Apo A1, mg/dL	141.4±22.6	137.2±19.7
Apo B, mg/dL	134.6±38.5	109.9±29.0*
Glycemia, mg/dL	115.9±73.6	109.9±48.3
EPC, %	0.026 (0.013–0.051)	0.034 (0.006–0.127)

* $p < 0.05$. Values are n (%), mean ± SD or median and interquartile range. EPC, endothelial progenitor cells; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

dependent, ACh-induced vasodilatation in patients with DFH. On the other hand, endothelium-independent vasodilation induced by SNP was not significantly different among DFH or PFH.

Discussion

FH is one of the causes of severe hypercholesterolemia and one of the most common monogenic inherited diseases [3]. Genetic diagnosis is not available for many pa-

tients, though, especially in developing countries. Therefore, knowledge on the association of the different phenotypes of FH with the degree of severity of atherosclerotic vascular disease is clinically relevant, as it may help differentiate them, besides optimizing patient management, directing resources to the more severely affected patients.

In this study, patients with DFH had higher LDL-C, more stigmata of hypercholesterolemia and an increased prevalence of prior cardiovascular disease than those with PFH, as previously demonstrated [3]. No significant dif-

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ference in the number of EPCs was found between patients with DFH or PFH. It has been shown that elevated LDL-C levels are associated with a reduced number and function of EPCs [18], possibly due to an acceleration of the senescence of these cells [19]. This may suggest that number of EPCs may be similarly affected by the elevated LDL-C in both FH phenotypes, without further reductions with the increased LDL-C of DFH patients. It is worth noting that HDL-C also has demonstrated an association with the number of EPCs, with lower serum HDL associated with a reduced number of EPCs [20, 21].

EPCs are a heterogeneous cell population consisting of bone marrow-derived cells and endothelial precursors originating from monocytic cells, most commonly identified by flow cytometry by means of surface markers [22, 23]. Problems with the definition of EPCs reflect the changing of surface markers as cells traffic from the bone marrow to the circulation and tissues, gradually losing monocytic and pan-leukocytic markers as they mature. Circulating, bone marrow-derived EPCs are $CD45^{low}$ [24, 25]. The consensus combination of markers include CD34 and CD133 [24], but problems in identifying EPCs remain, and thus the discovery of more specific markers is still necessary.

Patients with DFH had a significant decrease of ACh-induced vasodilatation, showing an impairment of endothelial-dependent microvascular vasodilatation. Masoura et al. [26] have found impairments in endothelial-dependent and endothelial-independent vasodilatation in patients with FH. Ultimately, it has been shown that this vascular dysfunction may be reversed by lipid-lowering drugs [27].

Our results underscore the increased severity of systemic microvascular derangement in patients with DFH. Despite the importance of the intensive treatment of severe hypercholesterolemia of any etiology, current results suggest that patients with DFH may deserve even more intensive management, to prevent ischemic heart disease and its consequences. In fact, the importance of the alterations of coronary endothelial microvascular function in the pathophysiology of ischemic heart disease is currently widely recognized [28, 29].

In some cases, microvascular dysfunction can be considered an independent risk marker, as well as a causal factor of myocardial ischemia, even in the absence of detectable coronary atherosclerotic lesions [28]. Thus, prevention through intense control of cardiovascular risk factors has a central role in the management of cardiac microvascular dysfunction and subsequent ischemic disease [29].

Limitations

This is a study of a highly selected population, which is a consequence of the very large number of factors that may influence the number of EPCs and had to be excluded. This limits the generalizability of the results, which ideally should be representative of a larger population presenting to physicians. However, if a truly "clinical" population was analyzed, it might be impossible to identify the isolated effect of a single risk factor on the studied variables. Besides that, a multivariable analysis to identify the independent predictors of endothelial dysfunction would be desirable, but we believe that the small number of patients limits this approach, as the number of candidate variables in the analysis would probably cause overfitting of the model and generate unreliable results.

Conclusions

Patients with DFH have impaired microvascular endothelial-dependent vasodilatation compared to those with PFH, but there is no significant difference in the number of EPCs. Larger studies are necessary to further elucidate these findings.

Appendix
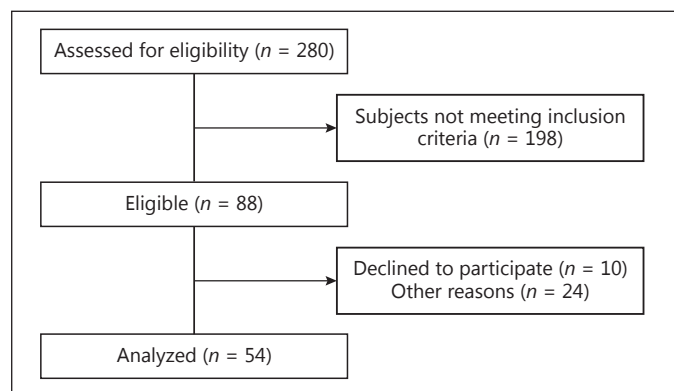
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