Chapitre 7 Impact de l'aphérèse des lipoprotéines avec l'adsorption au sulfate de dextran sur l'expression sanguine des gènes impliqués dans la santé cardiovasculaire chez des patients avec hypercholestérolémie familiale homozygote

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Title page

Impact of lipoprotein apheresis with dextran-sulfate adsorption on the expression of genes involved in cardiovascular health in the blood of patients with homozygous familial hypercholesterolemia

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Running title

Homozygous familial hypercholesterolemia and lipoprotein apheresis

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Abbreviations

Apo: apolipoprotein;

BCL3: B-cell lymphoma 3-encoded protein;

C: cholesterol;

CAD: coronary artery disease;

DSA: dextran sulfate adsorption;

EGR: early growth response protein;

hoFH: homozygous familial hypercholesterolemia;

LA: lipoprotein apheresis;

LDLR: LDL receptor;

MMP9: matrix metallopeptidase 9.

Résumé

L'AL avec l'ASD est une méthode fiable permettant de diminuer les concentrations de C-LDL chez des patients avec HFHo. L'objectif de cette étude était d'évaluer l'impact de l'AL avec ASD sur les niveaux d'expression sanguine (*whole blood mRNA levels*) des gènes associés à la santé cardiovasculaire chez des patients avec HFHo.

Des échantillons sanguins ont été collectés avant et après un traitement d'AL avec ASD chez 9 patients avec HFHo. L'expression sanguine de plus de 30 000 gènes a été analysée sur des puces d'ADN avant et après un traitement d'AL avec ASD.

Des réductions dans les concentrations plasmatiques de C-LDL (diminution médiane : -73,8% ; étendu : -55,9 à -82,0 ; *P*=0,0001) et de Lp(a) (diminution médiane : -74,1% ; étendu : -65,6 à -84,1 ; *P*=0,003) ont été induites par le traitement d'AL. L'AL avec ASD n'a pas eu d'impact sur l'expression sanguine de la plupart des gènes associés au métabolisme du cholestérol, des acides gras et des lipoprotéines. Cependant, l'AL avec ASD a augmenté significativement l'expression sanguine de l'EGR (*early growth response protein*) 1 (1,94 fois l'expression pré-AL, *P*=0,02) et 3 (1,56 fois l'expression pré-AL, *P*=0,0008) et de la BCL3 (*beta-cell lymphoma 3 encoded protein*) (1,25 fois l'expression pré-AL, *P*=0,03).

En conclusion, cette étude démontre qu'un seul traitement d'AL avec ASD n'a qu'un impact très limité sur l'expression d'un large spectre de gènes associés à la santé cardiovasculaire. Ces résultats suggèrent que le contact entre les cellules sanguines et la membrane de séparation primaire du système d'AL avec ASD ou avec la circulation extracorporelle aurait induit l'augmentation de l'expression des gènes *EGR1*, *EGR3* et *BCL3* dans les cellules sanguines.

Abstract

Lipoprotein apheresis (LA) with dextran sulfate adsorption (DSA) is a reliable method to decrease LDL-cholesterol (C) concentrations in patients with homozygous familial hypercholesterolemia (HoFH). The objective of the present study was to investigate the impact of LA with DSA on the mRNA expression of genes associated with cardiovascular health in the whole blood of HoFH patients. Blood samples were collected before and after LA treatment with DSA in 9 HoFH patients. Microarray analyses were performed to measure the whole blood expression of >30 000 annotated genes pre- and post-LA. Concomitant reductions in LDL-C (median -73.8%, range: -55.9 to -82.0, *P*= .0001) and lipoprotein (a) concentrations (median -74.1%, range -65.6 to -84.1, *P*= .003) were induced with LA treatment. LA with DSA did not impact the whole blood mRNA expression of most key genes involved in cardiovascular health, including those associated with cholesterol, fatty acid and lipoprotein metabolism. However, LA with DSA significantly upregulated the whole blood expression of early growth response protein (*EGR*)*1* (1.94-fold, *P*= .02), *EGR3* (1.56-fold, *P*= .0008) and B-cell lymphoma 3-encoded protein (*BCL3*; 1.25-fold, *P*= .03). In conclusion, this study demonstrated that a single LA treatment with DSA has very limited impact on the whole blood expression of a broad spectrum of genes associated with cardiovascular health. Our results suggest that contact between blood cells and the primary membrane or extracorporeal circulation could upregulate the expression of *EGR1*, *EGR3* and *BCL3* in blood cells.

Keywords: Homozygous familial hypercholesterolemia, Lipoprotein apheresis, Microarray analysis

Introduction

Homozygous familial hypercholesterolemia (HoFH) is caused, in most cases, by mutations in both alleles of the LDL receptor (*LDLR*) gene. Clinical features of HoFH include markedly high LDL-cholesterol (C) concentrations, tendon and skin xanthomas, extensive atherosclerosis, and extreme coronary artery disease (CAD) risk.[1] If untreated, CAD occurs during childhood or adolescence in HoFH patients.[1]

Repetitive long-term lipoprotein apheresis (LA) remains the gold-standard therapy for HoFH as it has been demonstrated to increase life expectancy in these patients.[2] Dextran sulfate adsorption (DSA) is a common LA technique that acutely decreases apolipoprotein (apo) B-containing lipoprotein concentrations by approximately 60%.[3] It is well recognized that the cardioprotective effects of LA in HoFH patients mainly result from the extracorporeal removal of cholesterol-rich apoB-containing particles. However, the impact of LA on the whole blood expression of genes associated with cardiovascular health remains unknown, thereby limiting our comprehension of the intravascular mechanisms induced by this therapy.

The objective of the present study was to investigate the impact of LA with DSA on the whole blood mRNA expression of a broad spectrum of genes associated with cardiovascular health using microarray analysis in HoFH patients. We hypothesized that LA with DSA beneficially regulates the whole blood mRNA expression of key genes associated with cardiovascular health among HoFH patients.

Materials and methods

Materials and methods are detailed in the **Supporting Information**. Briefly, nine HoFH subjects underwent one complete LA treatment using DSA (Liposorber® LA-15 system, Kaneka Corporation, Osaka, Japan) as part of their routine bi-monthly therapy. Blood samples were collected immediately pre- and post-LA. Microarray analyses were performed using GeneChip® Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) to assess the whole blood expression of >30 000 annotated genes involved in various metabolic pathways, including the cardiovascular system. Genes that whole blood expression was significantly modified post vs pre-LA with DSA were subsequently submitted to Ingenuity Pathway Analysis system (Ingenuity® software, www.ingenuity.com) to identify associated metabolic pathways.

Results

The median filtrated plasma volume during LA with DSA was 4400 mL (range: 3000-4900). LA with DSA significantly decreased LDL-C and lipoprotein (a) (Lp(a)) concentrations (**Table 1**). LA had no impact on the whole blood mRNA expression of most genes associated with cardiovascular health,

including key genes involved in cholesterol, fatty acid and lipoprotein metabolism (hydroxymethylglutaryl-CoA reductase, microsomal triglyceride transfer protein, LDLR, VLDL receptor, sterol regulatory binding protein 1 and 2 and peroxisome proliferator-activated receptors). However, as presented in **Figure 1**, LA with DSA significantly upregulated the whole blood expression of early growth response protein (*EGR*)1, *EGR3* and B-cell lymphoma 3-encoded protein (*BCL3*), while a statistical trend suggested an upregulation in the expression of the matrix metallopeptidase 9 (*MMP9*) (*P*0.09). These 4 genes were associated with 28 overrepresented pathways. Of those, 3 were associated to *BCL3*, 5 to *EGR1*, 19 to *MMP9* and 1 to *EGR1* and *MMP9* (**Supporting Information table 1**). Most pathways were related to endothelial activation or inflammation.

Discussion and conclusion

This study revealed that a single LA treatment using DSA had no impact on the whole blood expression of most genes associated with cardiovascular health, including those involved in cholesterol, fatty acid and lipoprotein metabolism, in patients with HoFH. These observations are consistent with the neutral impact of LA therapy on the secretion and clearance of apoB-containing particles as previously reported.[4] Accumulating evidence now suggests that LA therapy does not induce alterations in lipoprotein metabolism or a metabolic response to counterbalance the acute depletion of circulating lipoproteins. These observations suggest that the cardioprotective effects of LA therapy in HoFH patients are mainly related to the repetitive mechanical extracorporeal removal of cholesterol-rich apoB-particles from plasma rather than intravascular mechanisms induced by the therapy.

EGR1 and *EGR3* are involved in inflammation and endothelial dysfunction.[5, 6] *BCL3* is also involved in inflammation as a member of the nuclear factor-kappa-B family,[7] and *MMP9* is implicated in intimal thickening and plaque rupture.[8] Identified overrepresented pathways associated with these genes are also concordant with the function of these genes. Our results suggest that contact between the blood cells and the primary membrane or extracorporeal circulation could upregulate the expression of *EGR1*, *EGR3*, *BCL3* and *MMP9* in blood cells. Alternatively, it could not be excluded that the upregulation of these genes reflect acute endothelial activation, since gene expression assessment using peripheral blood is a valid marker of endothelium homeostasis.[9] This hypothesis is also concordant with a previous observation that elevations in plasma concentrations of interleukin-6, a cytokine that can be secreted from endothelial cells, were reported following LA with DSA in HoFH patients.[10, 11]

In the present study, it remains uncertain whether the upregulation in the expression of *EGR1*, *EGR3*, *BCL3* and *MMP9* was related to the DSA system or to the LA treatment *per se*. This effect was unlikely attributable to dextran-sulfate, as whole blood cells and plasma are separated in the LA system prior to the adsorption step. It also remains unlikely that the changes we observed were related to the

acute reduction in plasma lipids induced by LA with DSA. Moreover, it remains uncertain whether the expression of these genes increased or decreased in the days following LA. Nenseter *et al.*[12] previously compared serum levels and expression from peripheral blood mononuclear cells of MMP9 measured the day before LA and 15 days after LA in HoFH subjects and no difference was observed. In this context, in the present study, one can assume that the activation of the genes observed was attenuated in the days following the treatment. Further investigation is required.

In conclusion, this study demonstrated that a single LA treatment with DSA has very limited impact on the expression of a broad spectrum of genes associated with cardiovascular health. The results suggest that LA with DSA does not perturb overall patterns of gene expression. The results also suggest that contact between blood cells and the primary membrane or extracorporeal circulation could upregulate the expression of *EGR1*, *EGR3*, *BCL3* and *MMP9* in blood cells. Molecular mechanisms underlying the upregulation of *EGR1*, *EGR3*, *BCL3* and *MMP9* mRNA expression in whole blood remain to be identified.

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Authors' contributions and disclosures

PC and BL designed the research; JPDC, NL, and AJT conducted the research; JPDC, PC, and AJT analyzed the data; JPDC, AJT, JB, BL, and PC wrote the paper; and PC had primary responsibility for the final content. JPDC is the recipient of doctoral scholarships from the Canadian Institute of Health Research and the Fonds de Recherche du Québec – Santé. BL is the Chair of Nutrition at Laval University. This work was supported by an unrestricted grant from Kaneka Pharma LLC (Osaka, Japan).

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Table

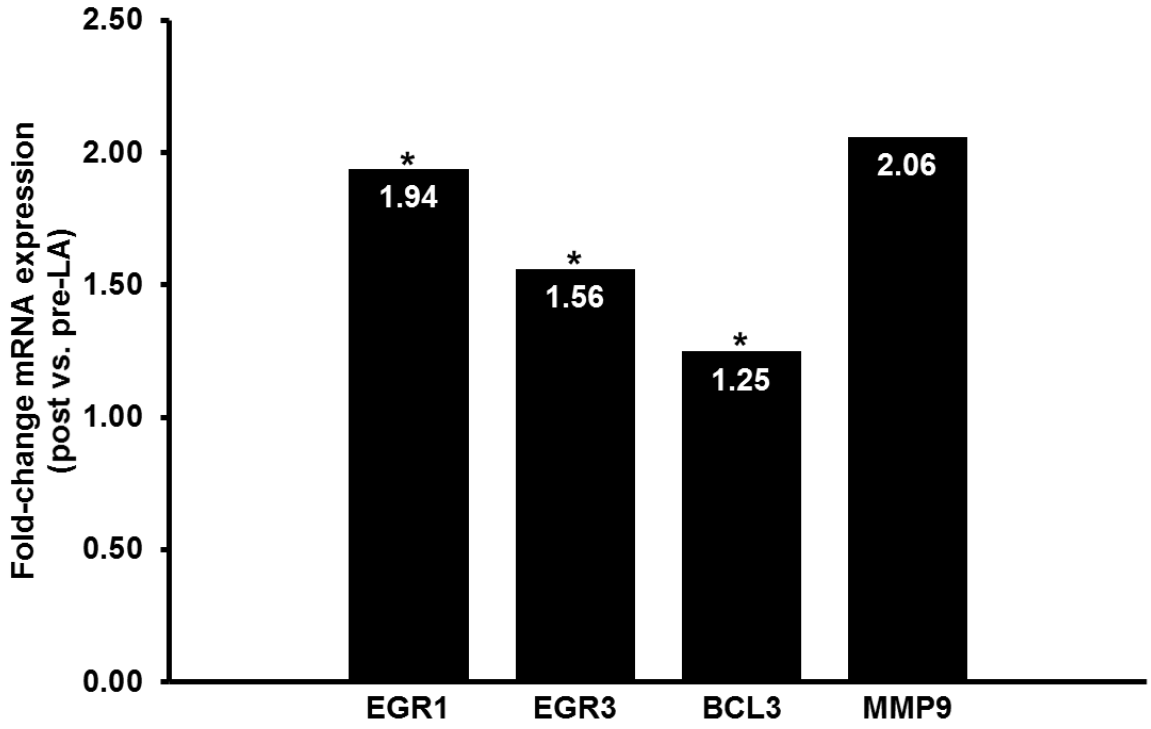
Table 7-1 Pre- and post-LA biochemical characteristics of patients with HoFH (n=9)

	Pre	**Post**	**%Δ**	***P***
Total-C (mmol/L)	11.87 (5.04-17.10)	4.03 (2.78-6.75)	-65.4 (-43.1; -72.9)	<.0001
Triglycerides (mmol/L)	2.05 (0.79-4.15)	1.03 (0.40-2.39)	-49.8 (-22.8; -69.6)	.003
HDL-C (mmol/L)	0.90 (0.72-1.68)	0.83 (0.59-1.40)	-9.3 (-6.9; -18.1)	.0006
LDL-C (mmol/L)	9.47 (2.97-14.87)	2.13 (1.27-5.61)	-73.8 (-55.9; -82.0)	.0001
Total-C/HDL-C	12.52 (3.00-21.65)	4.97 (2.05-10.07)	-59.4 (-31.7; -70.1)	.0003
Lp(a) (nmol/L)	90 (58-457)	37 (15-111)	-74.1 (-65.6; -84.1)	.003

Data are presented as the median (range). *P* values were calculated using mixed procedures for repeated measures. C: cholesterol; HoFH: homozygous familial hypercholesterolemia; LA: lipoprotein apheresis; and Lp(a): lipoprotein (a).

Figure

Figure 7-1 Fold change in the whole blood mRNA expression post- vs pre-LA with DSA



Fold change in the whole blood mRNA expression post- vs pre-LA with DSA. *****: *P*<.05, calculated with Benjamini-Hochberg correction for multiple comparisons. BCL: B-cell lymphoma 3-encoded protein; DSA: dextran sulfate adsorption; EGR: early growth response protein; LA: lipoprotein apheresis; and MMP: matrix metallopeptidase 9.

Supporting Information

Materials and methods

Study subjects

Nine HoFH subjects were recruited from a pool of patients who were being followed at the Lipid Research Center of the CHU de Québec-Université Laval in Quebec City. All participants were at least 18 years old (range: 18-55 y). None of the subjects were pregnant or nursing; had acute liver disease, hepatic dysfunction, or persistent elevations of serum transaminases; had a recent history of alcohol or drug abuse; had diabetes mellitus; had a history of cancer; or had undergone hormonal treatment. They were all treated with a maximally tolerated dose of statin and ezetimibe and were receiving LA bi-monthly for years.

All subjects were previously diagnosed with HoFH by screening for the nine most common mutations in the *LDLR* gene among the French-Canadian population using genomic DNA.[1-3] Four patients were compound heterozygous, carrying a >15 kb deletion at the 5' end of the gene (del15kb)[4] and the W66G mutation in exon 3.[5] One subject was compound heterozygous, carrying the del15kb mutation and the C646Y mutation in exon 14.[6] Two subjects were homozygous for the del15kb mutation,[4] and one was homozygous for the W66G mutation in exon 3.[5] One subject carried negative unidentified mutations and was diagnosed based on the clinical features of HoFH.

Study design

All subjects underwent one LA treatment using DSA (Liposorber® LA-15 system, Kaneka Corporation, Osaka, Japan) as part of their routine bi-monthly therapy. In the LA with DSA system, the plasma is pumped into one of the two dextran sulfate adsorption columns after primary separation. ApoB-containing lipoproteins electrostatically bind in the first column to the negatively charged dextran sulfate. The plasma is then transferred to the other column, and the first column is rinsed with NaCl to remove the apoB-containing particles. Both columns work in alternating cycles. ApoB-depleted plasma is then mixed with blood and passed through a blood warmer before it is reinjected into the patient. Heparin is used as an anticoagulant during treatment.[7, 8] The manufacturer's specifications recommend treating 1.5 patient plasma volumes during a single procedure. The plasma volume for each patient was estimated using the following equation: plasma volume (L) = 0.065 X weight (kg) X (1-hematocrit).[9] Blood samples were collected immediately pre- and post-LA.

Plasma lipoprotein concentrations

Venous blood samples were obtained from an antecubital vein. Serum was separated from blood cells by centrifugation at 2200 rpm (1100 g) for 10 minutes at 18°C. Serum cholesterol and TG

concentrations were determined with a Roche/Hitachi MODULAR analyzer (Roche Diagnostics, Indianapolis, IN, USA) using the proper reagents.

Microarrays analysis

Blood samples for microarray analyses were collected in PAXgene™ Blood RNA vacutainers (PreAnalytix, Hombechtikon, Switzerland), incubated at room temperature for 2 hours for RNA stabilization, stored at -20° C for at least 24 h and then frozen at -80° C until analysis. RNA was extracted from blood using the PAXgene™ Blood RNA System Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) was used to quantify and verify the total RNA quality prior to microarray analysis. Microarray analyses were performed at the McGill University and Génome Québec Innovation Centre (Montréal, Canada) using the GeneChip® Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. These platforms measure the expression of >30 000 annotated genes involved in various metabolic pathways, including cardiovascular health.

Genes that whole blood expression was significantly modified post vs pre-LA with DSA were submitted to Ingenuity Pathway Analysis system (Ingenuity® software, www.ingenuity.com) to identify associated metabolic pathways.

Statistical analyses

Statistical analyses of microarray data were conducted with the Limma package of the Bioconductor software (www.bioconductor.org).[10] This approach separately fitted a linear model for each gene. Microarray data were included in the model as a dependent variable, and treatment (post- vs pre-LA) was the independent variable. Benjamini-Hochberg correction was used for multiple comparisons in the microarray data. Post- and pre-LA lipoprotein concentrations were compared using mixed procedures for repeated measures, using pre-/post-LA as the fixed independent variable, subjects as the random effect and lipoprotein concentrations as the dependent variable.

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Supporting Information Table 7-1 Metabolic pathways associated with EGR1, EGR3, BCL3 and MMP9

Molecule(s)	Ingenuity Canonical Pathways	P
BCL3	IL-9 Signaling	.009
BCL3	TR/RXR Activation	.02
BCL3	PPAR/RXR Activation	.03
EGR1	CDK5 Signaling	.02
EGR1	Role of Tissue Factor in Cancer	.02
EGR1	GNRH Signaling	.02
EGR1	CXCR4 Signaling	.03
EGR1	B Cell Receptor Signaling	.03
EGR1, MMP9	Regulation of the Epithelial-Mesenchymal Transition Pathway	.0005
MMP9	Airway Pathology in Chronic Obstructive Pulmonary Disease	.002
MMP9	Inhibition of Angiogenesis by TSP1	.006
MMP9	Inhibition of Matrix Metalloproteases	.007
MMP9	Neuroprotective Role of THOP1 in Alzheimer's Disease	.008
MMP9	Glioma Invasiveness Signaling	.01
MMP9	Bladder Cancer Signaling	.02
MMP9	HIF1α Signaling	.02
MMP9	Pancreatic Adenocarcinoma Signaling	.02
MMP9	LXR/RXR Activation	.02
MMP9	Atherosclerosis Signaling	.02
MMP9	Ovarian Cancer Signaling	.03
MMP9	Relaxin Signaling	.03
MMP9	Granulocyte Adhesion and Diapedesis	.03
MMP9	Hepatic Fibrosis / Hepatic Stellate Cell Activation	.03
MMP9	Agranulocyte Adhesion and Diapedesis	.04
MMP9	ILK Signaling	.04
MMP9	IL-8 Signaling	.04
MMP9	Leukocyte Extravasation Signaling	.04
MMP9	Colorectal Cancer Metastasis Signaling	.046
MMP9	Axonal Guidance Signaling	.08

BCL3: B-cell lymphoma 3-encoded protein; EGR: Early growth response protein; MMP9: Matrix metallopeptidase 9.