Proprotein convertase subtilisin kexin type 9 (PCSK9) is a natural inhibitor of the low-density lipoprotein (LDL) receptor pathway. PCSK9 binds to the LDL receptor at the cell surface and targets it for lysosomal degradation after endocytosis. As a result, heterozygous carriers of PCSK9 loss-of-function mutations have naturally low LDL-C levels and a reduced incidence of cardiovascular events. Pharmacological inhibition of PCSK9, especially when used in combination with statins, reduces LDL-C to low levels (≈0.25g/L). A relationship between low LDL-C levels and the risk of hemorrhagic transformation after cerebral ischemia/reperfusion in mice with low levels of LDL-C resulting from proprotein convertase subtilisin kexin 9 (PCSK9) deficiency.

Methods—PCSK9−/− and PCSK9+/+ mice were fed with a high-fat/high-cholesterol (21%/0.15%) diet for 1 month. Plasma lipids were measured using colorimetric assays. PCSK9−/− and PCSK9+/+ mice (n=15 per group) were subjected to a 4-hour intraluminal occlusion of the middle cerebral artery followed by 20 hours of reperfusion. Spontaneous hemorrhagic transformation was assessed by quantification of hemoglobin in ischemic tissue. In vitro, a cell model of blood–brain barrier was used to test endothelial barrier integrity in response to decreasing concentrations of LDL-C from 1 to 0.25g/L in ischemia/reperfusion conditions.

Results—PCSK9−/− mice had lower LDL-C, high-density lipoprotein-cholesterol, and total cholesterol levels than PCSK9+/+ mice before and after 1 month on the high-fat/high-cholesterol diet. Hemoglobin concentration in ischemic cerebral tissue was not different between PCSK9−/− and PCSK9+/+ mice (31.5 [18.9–60.1] and 32.8 [14.7–69.9] ng/mg protein, respectively; P=0.81). Infarct volume was also similar in both groups (P=0.66). Incubation of human cerebral endothelial cells with decreasing concentrations of LDL-C under ischemia/reperfusion conditions did not alter blood–brain barrier permeability.

Conclusions—Low levels of LDL-C did not increase the risk of hemorrhagic transformation after cerebral ischemia/reperfusion in mice. Our observations suggest that PCSK9 inhibition, leading to LDL-C lowering, should not increase hemorrhagic complications after acute ischemic stroke. (Stroke. 2014;45:3086-3088.)

Key Words: hemorrhage ■ low-density lipoprotein cholesterol ■ mice ■ PCSK9 protein ■ stroke
were euthanized, and intravascular washout was performed by intracardiac perfusion of heparinized saline. The brain was removed and cut into 1-mm coronal slices using a brain matrix mold for evaluation of infarct volume and HT. HT was macroscopically scored on coronal brain slices by 3 independent observers blinded to the group status and confirmed by Masson trichrome staining. Hemoglobin was measured to assess HT in homogenates of ischemic brain tissue quantitatively (Hemoglobin Mouse ELISA Kit-Abcam). Infarct volume was determined using Image J software from coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride.7

In vitro, a cell model of blood–brain barrier, consisting of human cerebral microvascular endothelial cells/D3 donated by P.O. Couraud (Institut Cochin, Paris, France), was used to test the endothelial barrier integrity in response to decreasing concentrations of LDL-C. Blood–brain barrier integrity was measured as transendothelial electric resistance using the xCELLigence system (Roche, Basel, Switzerland) as follows: cells were seeded at 15×103 cells per well onto E-plates coated with collagen I. When transendothelial electric resistance reached a maximal plateau, the confluent cell monolayer was incubated with decreasing concentrations of LDL-C (isolated from human plasma), from 1 to 0.25 g/L, for ≥24 hours and subjected to oxygen-glucose deprivation for 4 hours. Glucose and oxygen were then resupplied to the cells for 20 hours to mimic reperfusion. Changes in transendothelial electric resistance are attributed to resistance variations because of modifications of paracellular junction tightness. Kinetics of transendothelial electric resistance were displayed as Cell Index (arbitrary units).

Data were analyzed using a Mann–Whitney test, and P values were 2-sided, with a significance level of 0.05. Results are expressed as medians (min–max).

Results
As anticipated,1 PCSK9−/− mice had lower LDL-C, high-density lipoprotein-cholesterol, and total cholesterol levels than PCSK9+/+ mice before (not shown) and after 1 month on the high-fat/high-cholesterol diet (Figure 1). Transient intraluminal MCAO was performed on 15 PCSK9−/− and 15 PCSK9+/+ mice. One mouse of each genotype died overnight after cerebral ischemia. Twenty-four hours after MCAO, hemoglobin concentration in ischemic cerebral tissue was not different between PCSK9−/− and PCSK9+/+ mice (31.5 [18.9–60.1] and 32.8 [14.7–69.9] ng/mg protein, respectively; P=0.81; Figure 2). Macroscopic and histological (Masson trichrome) qualitative evaluations of HT by blinded multiple-observer analysis confirmed this observation. Infarct volumes were also similar in PCSK9−/− and PCSK9+/+ mice (0.86 [0.70–1.07] and 0.90 [0.59–1.26] cm3, respectively; P=0.66).

In vitro, the transendothelial electric resistance of the human cerebral microvascular endothelial cells/D3 monolayer was unaffected by decreasing LDL-C concentrations under oxygen-glucose deprivation conditions (Figure 3).

Discussion
There are some inherent limitations to the experimental models used in the present study. Notably, mice are primarily an high-density lipoprotein-cholesterol species, whereas humans are LDL predominates. We have limited this important bias by feeding the animals a high-fat/high-cholesterol diet to increase their LDL-C levels by 100%. Another important limitation is that PCSK9 gene invalidation does not mimic the transient pharmacological inhibition of circulating PCSK9 in vivo. PCSK9 knockout mice may, for instance, adapt to the permanent lack of PCSK9 in a way that may affect the cell response to ischemia. Unfortunately, specific antismouse PCSK9 monoclonal inhibitors are not commercially available.
In addition, this experimental model does not account for the cumulative LDL-lowering effects resulting from the combined inhibition of PCSK9 by monoclonal antibodies and of hydroxymethylglutaryl-CoA reductase (HMGCoA) reductase by statins. Transient MCAO procedure is a clinically relevant model to study HT after cerebral ischemia/reperfusion that has been previously validated in rats.8 The blood–brain barrier human cerebral microvascular endothelial cells/D3 monolayer permeability model has also been validated in our laboratory9 and is appropriate to assess the functionality of brain endothelium. 10 Thus, the low circulating lipid levels of PCSK9 knockout mice do not aggravate HT after MCAO. Likewise, low levels of LDL-C do not alter blood–brain barrier permeability. Concentrations of LDL-C from 1 to 0.25 mg/dL are in the same range as those observed in patients treated with PCSK9 inhibitors. Despite the inherent limitations mentioned above, our observations suggest that PCSK9 inhibition leading to LDL-C lowering should not increase hemorrhagic complications after acute ischemic stroke. The effects of PCSK9 inhibitors alone or in combination with statins on the risk of HT after ischemic stroke should, however, be carefully monitored in the large outcome clinical trials currently underway.

Acknowledgments
We thank Olivier Thibaudeau (UMR Inserm 1152, Plateau de Morphologie) for histology.

Sources of Funding
This research was supported by the Agence Nationale de la Recherche: Project Grant ANR-JCJC1105 to O Meilhac and Project Grant ANR-Blanc-Bénéfices Thérapeutiques d’une Nouvelle Cible Thérapeutique hypolipémiant (BCNCT) to G. Lambert.

Disclosures
Dr Lambert has received honoraria and research funding from Sanofi-Regeneron, Amgen, and Pfizer. Dr Amarenco has received consulting fees from AstraZeneca, Bristol-Myers Squibb, Daiichi, Eli Lilly, GlaxoSmithKline, Guerbet, Negma, Novartis, Pfizer, Sankyo, Sanofi-Aventis, and Servier; lecture fees from AstraZeneca Bayer, Boehringer-Ingelheim, Bristol-Myers Squibb, Merck, Otsuka Pharmaceutical, Pfizer, Sanofi-Aventis, and Servier and grant support from Boehringer-Ingelheim, Bristol-Myers Squibb, Eisai, Merck, Astra-Zeneca, Pfizer, and Sanofi-Aventis. The other authors report no conflicts.

References

Figure 3. Effect of low-density lipoprotein (LDL) concentration on blood–brain barrier (BBB) permeability under oxygen-glucose deprivation (OGD)/reperfusion conditions. Human cerebral endothelial cells were treated with decreasing concentrations of LDL. Plots show Cell Index (CI). Results are mean CI values±SEM from 3 independent experiments performed in duplicate.
Low Levels of Low-Density Lipoprotein-C Associated With Proprotein Convertase Subtilisin Kexin 9 Inhibition Do Not Increase the Risk of Hemorrhagic Transformation
Alexy Tran-Dinh, Angélique Levoye, Gilles Lambert, Liliane Louedec, Clément Journé, Olivier Meilhac and Pierre Amarenco

Stroke. 2014;45:3086-3088; originally published online August 14, 2014; doi: 10.1161/STROKEAHA.114.005958
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/45/10/3086

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/