Lipodystrophy-Linked LMNA p.R482W Mutation Induces Clinical Early Atherosclerosis and In Vitro Endothelial Dysfunction

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Objective—Some mutations in LMNA, encoding A-type lamins, are responsible for Dunnigan-type-familial partial lipodystrophy (FPLD2), with altered fat distribution and metabolism. The high prevalence of early and severe cardiovascular outcomes in these patients suggests that, in addition to metabolic risk factors, FPLD2-associated LMNA mutations could have a direct role on the vascular wall cells.

Approach and Results—We analyzed the cardiovascular phenotype of 19 FPLD2 patients aged >30 years with LMNA p.R482 heterozygous substitutions, and the effects of p.R482W-prelamin-A overexpression in human coronary artery endothelial cells. In 68% of FPLD2 patients, early atherosclerosis was attested by clinical cardiovascular events, occurring before the age of 45 in most cases. In transduced endothelial cells, exogenous wild-type-prelamin-A was correctly processed and localized, whereas p.R482W-prelamin-A accumulated abnormally at the nuclear envelope. Patients’ fibroblasts also showed a predominant nuclear envelope distribution with a decreased rate of prelamin-A maturation. Only p.R482W-prelamin-A induced endothelial dysfunction, with decreased production of NO, increased endothelial adhesion of peripheral blood mononuclear cells, and cellular senescence. p.R482W-prelamin-A also induced oxidative stress, DNA damages, and inflammation. These alterations were prevented by treatment of endothelial cells with pravastatin, which inhibits prelamin-A farnesylation, or with antioxidants. In addition, pravastatin allowed the correct relocalization of p.R482W-prelamin-A within the endothelial cell nucleus. These data suggest that farnesylated p.R482W-prelamin-A accumulation at the nuclear envelope is a toxic event, leading to cellular oxidative stress and endothelial dysfunction.

Conclusions—LMNA p.R482 mutations, responsible for FPLD2, exert a direct proatherogenic effect in endothelial cells, which could contribute to patients’ early atherosclerosis. (Arterioscler Thromb Vasc Biol. 2013;33:2162-2171.)

Key Words: arteriosclerosis ■ endothelial cells ■ laminin A/C ■ oxidative stress ■ prelamin A ■ statins, HMG-CoA

A-type lamins are intermediate filaments and major components of the nuclear lamina, a filamentous network underlying the inner nuclear membrane that provides structural and mechanical stability for the nucleus in nearly all differentiated cells. A-type lamins interact with heterochromatin and transcriptional regulators, highlighting their important role in chromatin organization, gene expression, and DNA repair.1 The 2 main A-type lamin isoforms, lamin-A and lamin-C, arise from alternative splicing of the LMNA gene. The precursor of lamin-A, prelamin-A, undergoes a complex post-translational maturation comprising a step of C-terminal farnesylation followed by carboxymethylation and a proteolytic cleavage by the metalloprotease ZMPSTE24, resulting in the carboxymethylated C-terminal removal of the protein, including its farnesyl group, and in the release of mature lamin-A.3

LMNA mutations cause inherited diseases commonly named laminopathies, including muscular dystrophies, cardiomyopathies, progeroid phenotypes, and lipodystrophic syndromes.2 Among them, the Dunnigan-type familial partial lipodystrophy (FPLD2; OMIM #151660) is mainly attributable to LMNA p.R482 heterozygous substitutions.3,4 This syndrome is characterized by a gradual atrophy of subcutaneous adipose tissue in the extremities, gluteal, and truncal areas and his clinical manifestations include obesity, diabetes, dyslipidemia, and hypogonadism.

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occurring after puberty, whereas fat accumulates in the face and neck. Severe metabolic disturbances, such as insulin resistance, diabetes mellitus, hypertriglyceridemia, and low-density lipoprotein cholesterol, are associated with FPLD2.5–7

Previous reports stressed that precocious vascular diseases (coronary heart disease, stroke, and peripheral arteritis) could also be part of the FPLD2 phenotype.6,9 Otherwise, patients with the Hutchinson-Gilford progeria syndrome (HGPS), a premature aging disorder linked to different LMNA mutations, display very severe and precocious atherosclerosis leading to early mortality,9,11 in the absence of classical cardiovascular risk factors.12

The pathophysiological mechanisms involved in laminopathies associated with lipodystrophies are not fully understood. We, and others, have shown that FPLD2 is associated with abnormal prelamin-A accumulation, possibly because of the misrecognition of the mutated protein by the protease ZMPSTE24.13,14 Prelamin-A accumulation was associated with premature cellular senescence, mitochondrial dysfunctions, and oxidative stress in skin fibroblasts with FPLD2.14

HGPS result from the accumulation of progerin, a truncated and unprocessed form of prelamin-A that remains farnesylated.15,16 Interestingly, progerin is expressed in patients’ vascular wall cells,13,17,18 resulting in cellular senescence.19 Progerin and farnesylated prelamin-A are now considered as dominant-negative toxins that disrupt nuclear lamina integrity and lead to induction and persistence of DNA double-strand breaks (DSBs).20–23 In accordance, decreasing the amount of farnesylated progerin reduced the premature aging phenotype associated with HGPS in vitro and in vivo in mice,24–27 and improved vascular stiffness in children with HGPS.28

Endothelial cell dysfunction and senescence are considered as the first steps of vascular wall injury during the process of atherosclerosis.29,30 In endothelial cells, oxidative stress was shown to impair NO biodisponibility and to promote inflammation, leukocyte adhesion, and cellular senescence.31–34 In addition, DNA damages were observed in atherosclerotic lesions of patients with coronary heart diseases in vivo and linked with oxidative stress in vitro.35

Thus, we hypothesized that abnormal accumulation of farnesylated p.R482–prelamin-A in the cells of patients with FPLD2 could result in endothelial cell dysfunction and senescence that, in addition to metabolic risk factors, could be important pathogenic events leading to premature atherosclerosis in these patients.

In the present study, we analyzed the clinical cardiovascular events in 19 adult patients with LMNA p.R482–linked FPLD2, which highlighted their early and severe atherosclerosis. To determine whether FPLD2–associated LMNA mutation could directly induce endothelial defects, we overexpressed wild-type (WT) or FPLD2–associated p.R482W–prelamin-A in human coronary artery endothelial cells (HCAECs) in primary culture. p.R482W–prelamin-A in endothelial cells and patients’ fibroblasts was abnormally retained at the nuclear envelope. We also show, for the first time, that exogenous expression of p.R482W, but not WT–prelamin-A, resulted in endothelial dysfunction, DNA DSBs, and cellular senescence. Pravastatin, which decreases prelamin-A farnesylation, improved these in vitro defects, suggesting that accumulation of mutated and farnesylated prelamin-A could participate in FPLD2–associated premature atherosclerosis.

Patients and Methods

Details about patients and methods are available in the online-only Supplement.

Results

LMNA p.R482W/Q Patients Displayed Early and Severe Atherosclerosis

We analyzed cardiovascular and metabolic features of 19 consecutive patients from 14 unrelated pedigrees, aged >30 years, with FPLD2–associated LMNA p.R482W/Q mutations (Table). FPLD2 patients had a mean age of 49.5 years, and a mean body mass index of 23.8 kg/m². Diabetes mellitus was present in 84% of cases and was generally controlled with a mean HbA1c of 7.5%. Dyslipidemia was found in 94% of patients. The mean level of fasting triglycerides was increased (4.3 mmol/L±0.7), and that of high-density lipoprotein cholesterol (0.9 mmol/L±0.1) and apolipoprotein A1 (1.1 g/L±0.1) were decreased.

Table. Cardiovascular and Metabolic Characteristics of Patients With FPLD2 With LMNA p.R482W/Q Mutations

<table>
<thead>
<tr>
<th>LMNA p.R482W/Q</th>
<th>Number of women/number of cases 16/19 (84%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>49.5±2.8</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.8±0.9</td>
</tr>
<tr>
<td>Hypertension</td>
<td>7/17 (41%)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>4/15 (27%)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>4.2±0.7</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.9±0.4</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>ApoA1, g/L</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>ApoB, g/L</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Statin treatment</td>
<td>2/19 (11%)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>16/17 (94%)</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>8.6±0.8</td>
</tr>
<tr>
<td>Fasting insulin, pmol/L</td>
<td>211±52</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>7.5±0.3</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>16/19 (84%)</td>
</tr>
<tr>
<td>Clinical atherosclerosis</td>
<td>13/19 (68%)</td>
</tr>
<tr>
<td>Atherosclerosis before age 45</td>
<td>8/13 (62%)</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM or number of cases/total (%). Dyslipidemia was defined as LDL-cholesterol ≥4.1 mmol/L and/or HDL-cholesterol <1.0 mmol/L for men and <1.3 mmol/L for women, and/or triglycerides ≥1.7 mmol/L. Hypertension was defined as blood pressure >140/90 mmHg or antihypertensive treatment. Clinical atherosclerosis was attested by the presence of coronary heart disease (decreased myocardial perfusion at imagery, myocardial infarction, coronary artery bypass graft [CABG], or stent implantation), peripheral arteritis with vascular surgery or stent implantation, and stroke. The precise clinical atherosclerotic events for each affected patient are detailed in the Table I in the online-only Data Supplement. ApoA1 indicates apolipoprotein A1; ApoB, apolipoprotein B; FPLD2, Dunnigan-type familial partial lipodystrophy; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.
decreased. The mean concentration of low-density lipoprotein cholesterol was 2.5 mmol/L with apolipoprotein B at 0.9 g/L ± 0.1. Hypertension or antihypertensive treatment was referred in 41% of cases.

LMNA p.R482W/Q patients showed a very high prevalence of symptomatic atherosclerosis at early age, as attested by cardiovascular events in 13 of them (68%), occurring before age 45 in 62% of cases. The clinical and biological characteristics of the patients with cardiovascular events are given in the Table I in the online-only Data Supplement.

We measured additional plasma markers in 12 patients with FPLD2 (4 with previously known cardiovascular events), compared with 12 age- and sex-matched healthy volunteers. Patients with FPLD2 had significantly higher plasma levels of the systemic inflammatory markers, high-sensitive C-reactive protein (1.85 ± 0.24 versus 0.94 ± 0.27 mg/L; *P* = 0.005) and interleukin-6 (IL-6; 2.57 ± 0.97 versus 0.70 ± 0.13 pg/mL; *P* = 0.04), and the endothelial cell dysfunction marker intercellular adhesion molecule-1 (ICAM-1; 88.8 ± 10.1 versus 68 ± 4.7 ng/mL; *P* = 0.05). Plasma vascular cell adhesion molecule-1 was not significantly different between patients with FPLD2 and controls (998.1 ± 90.1 versus 1115.4 ± 35.6 ng/mL; *P* = 0.34).

**Overexpression of p.R482W Prelamin-A Led to Defects in Prelamin-A Maturation and in Nuclear Shape Abnormalities**

To determine the effects of p.R482W LMNA mutations on endothelial cells, we transiently transduced HCAECs with adenoviral vectors containing Flag-tagged WT or p.R482W-prelamin-A cDNA. Flag protein expression, analyzed 72 hours after transduction, was similar whatever the construction (Figure 1A). We observed a 2.5-fold increased expression...
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of lamin-A under these conditions (Figure 1A in the online-only Data Supplement).

As previously observed in patients’ skin fibroblasts, p.R482W LMNA mutation altered prelamin-A maturation, as shown by abnormal prelamin-A accumulation, whereas overexpressed WT-prelamin-A was properly processed to mature lamin-A (Figure 1B). This accumulation could result from a slower maturation rate as compared with WT-prelamin-A, as demonstrated in the skin fibroblasts of the patients with FPLD2 (Figure 1B in the online-only Data Supplement). In transduced endothelial cells, overexpressed WT and mutated lamin-A localized to the nucleus. However, although cells overexpressing Flag-WT lamin-A showed regularly shaped nuclei, overexpression of p.R482W-prelamin-A led to misshapen nuclei, with nuclear blebs and nuclear envelope invaginations (Figure 1C). Interestingly, although WT exogenous lamin-A was distributed within the nucleoplasm and the nuclear rim, p.R482W-prelamin-A was mostly localized at the nuclear periphery, as determined by the nuclear distribution of Flag fluorescence (Figure 1D). A similar abnormal lamin-A distribution was observed in patients’ skin fibroblasts bearing the LMNA p.R482W mutation (Figure 1C in the online-only Data Supplement). As it is known that lipid groups increase the anchoring of lamins to the nuclear envelope, this result suggests that p.R482W-prelamin-A remained farnesylated.

p.R482W but Not WT-Prelamin-A Expression Led to Endothelial Dysfunction With Increased Inflammation and Peripheral Blood Mononuclear Cells Adhesion to HCAECs

Endothelial dysfunction, during atherosclerosis process, is characterized by a reduced bioavailability of NO, produced by endothelial NO synthase (eNOS), and a proinflammatory status. p.R482W but not WT-prelamin-A overexpression was associated with a marked decreased expression of eNOS both at the mRNA and protein levels (Figure 2A; Figure IIA in the online-only Data Supplement). Accordingly, NO production was impaired in p.R482W prelamin-A overexpressing...
monocyte chemoattractant protein-1 (2–5-fold versus WT; Figure IIB in the online-only Data Supplement; Figure 2C). We also observed that p.R482W but not WT-prelamin-A expression increased the mRNA expression and secretion of the cell adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 (≈2-fold increase versus WT; Figure IIC in the online-only Data Supplement; Figure 2D). To assess the functional consequences of the proadhesive and proinflammatory state induced by p.R482W-prelamin-A, we performed an adhesion assay of human peripheral blood mononuclear cells (PBMCs), isolated from healthy donors, to transduced HCAECs. The number of adherent PBMCs to p.R482W but not WT-prelamin-A overexpressing HCAECs was increased (Figure 2E; Figure IID in the online-only Data Supplement).

These results suggested that p.R482W mutated prelamin-A directly induced a proinflammatory and proadhesive state of endothelial cells, which could participate to the recruitment of PBMCs to the vascular wall.

Figure 3. p.R482W but Not WT-Prelamin-A Expression Led to Increased Oxidative Stress, Accumulation of DNA DSBs, and Early Senescence in HCAECs

As oxidative stress promotes endothelial dysfunction,32 we measured the production of reactive oxygen species (ROS) and the mRNA expression of cell stress markers. p.R482W but not WT-prelamin-A overexpression led to increased ROS production (1.7-fold increase versus WT; Figure 3A) and C/EBP-homologous protein 10, cAMP-dependent transcription factor-3 and -4 (ATF-3, ATF-4) mRNA expression (Figure 3B; Figure IIIA in the online-only Data Supplement). Because oxidative stress could induce DNA damages,35 we evaluated the number of transduced HCAECs expressing the phosphorylated histone γ-H2AX, which is recruited after the induction of DNA DSBs.37 γ-H2AX foci were observed in 10% to 12% of control and WT-prelamin-A overexpressing cells, but in 39% of cells overexpressing p.R482W-prelamin-A (Figure 3C; Figure IIIB in the online-only Data Supplement). Furthermore, primary cultured fibroblasts from FPLD2 patients bearing LMNA p.R482W-mutations were more frequently γ-H2AX-positive than controls (Figure IIIC in the online-only Data Supplement). Therefore, LMNA p.R482W mutation is associated with an accumulation of DNA DSBs. In agreement, cells overexpressing p.R482W-prelamin-A presented a higher expression of p53, p21WAF1, and p16INK4a (Figure 3D), which are cell cycle arrest proteins activated during DNA DSBs response.38 Finally, senescence-activated β-galactosidase activity was enhanced in p.R482W but not in WT-prelamin-A expressing endothelial cells (Figure 3E).
prelamin-A in WT and p.R482W-prelamin-A overexpressing HCAECs (Figure 4A).

Moreover, pravastatin reduced the amount of p.R482W-prelamin-A present at the nucleus periphery and increased its amount in the nucleoplasm (Figure 4B). The nuclear distribution of pravastatin-treated p.R482W-prelamin-A was no longer different from that of nontreated WT-lamin A (Figure 1D). These data strongly support our hypothesis that overexpressed p.R482W-prelamin-A was farnesylated and remained tightly associated with the nuclear membrane. When its farnesylation was impaired by pravastatin, it was partly dissociated from the nuclear envelope.

Pravastatin prevented both oxidative stress and accumulation of DNA DSBs induced by p.R482W-prelamin-A in vitro (Figure 4C and 4D). Moreover, pravastatin decreased the p.R482W-prelamin-A-induced endothelial secretion of ICAM-1 and inflammatory markers (IL-6, IL-8, and monocyte chemoattractant protein-1; Figure 4E and 4F). In cells overexpressing WT-prelamin A, pravastatin was devoid of any effect on these parameters.

Altogether, these data suggested that LMNA p.R482W mutation induced farnesylated prelamin-A accumulation, which led to oxidative stress, DNA damages, and inflammation, and thus contributed to endothelial cell dysfunction.

**Antioxidant Treatment Improved Adverse Effects Induced by p.R482W-Prelamin-A Overexpression**

We then investigated the contribution of oxidative stress to LMNA p.R482W–induced endothelial dysfunction. As expected, treatment with the antioxidant molecule N-acetyl cysteine reversed ROS production (Figure 5A) and mRNA expression of the cell stress marker C/EBP-homologous protein-1 (Figure 5B) secretions (4–6 experiments) were measured in 24-hour culture HCAEC supernatants. Results from 8 to 9 experiments are expressed as mean±SEM. *P<0.05 for comparison with wild-type (WT)-prelamin-A overexpressing cells; #P<0.05 for comparison between vehicle-treated and pravastatin-treated conditions; §P<0.05 for comparison with pravastatin-treated WT-prelamin-A overexpression conditions. Light gray bars denote cells treated by the vehicle alone; and dark gray bars, pravastatin-treated cells. Nontrans indicates nontransduced HCAECs; empty vector, HCAECs transfected with adenovirus containing an empty vector; WT, HCAECs overexpressing wild-type prelamin-A; and R482W, HCAECs overexpressing p.R482W-prelamin-A.
p.R482W-prelamin-A (Figure 5D). N-acetyl cysteine treatment had no significant effect on both ROS production and inflammatory secretions in WT-prelamin-A overexpressing cells.

The occurrence of DNA DSBs was evaluated after long-term antioxidant treatment with reduced S-glutathione. Reduced S-glutathione significantly prevented the occurrence of DNA DSBs in p.R482W-prelamin-A–overexpressing HCAECs (Figure 5E), suggesting that oxidative stress contributed to DNA damages.

Taken together, these data suggest that oxidative stress, secondary to accumulation of farnesylated p.R482W-prelamin-A, induced endothelial dysfunction, DNA damages, and senescence.

**Discussion**

In the present study, we analyzed the cardiometabolic features of 19 patients with FPLD2-associated LMNA p.R482W/Q mutations and confirmed the high prevalence of atherosclerotic cardiovascular diseases, which occurred before the age of 45 for most of them.

Thus, we explored the hypothesis of a direct role of LMNA p.R482W mutation on endothelial cells, which dysfunction is considered as an early important step toward atherosclerosis. We observed that overexpressed p.R482W-prelamin-A directly triggered endothelial dysfunction and senescence, which could result from the accumulation of farnesylated mutated prelamin-A and increased oxidative stress. These data give new mechanisms concerning the pathophysiology of LMNA-linked atherosclerotic complications.

Although patients with FPLD2 frequently presented proatherogenic features, such as diabetes mellitus, high triglycerides levels, and low high-density lipoprotein cholesterol with decreased apolipoprotein A1, they did not show high levels of low-density lipoprotein cholesterol and apolipoprotein B. This
suggested that early atherosclerosis is part of FPLD2 phenotype and could be, at least in part, linked to a direct effect of the mutation, in addition to classical cardiovascular risk factors.

We also observed that patients with FPLD2, as compared with controls, had higher plasma levels of high-sensitive C-reactive protein, IL-6, and ICAM-1, all markers linked with cardiovascular risk in the general population. Interestingly, IL-6 and ICAM-1 are secreted by endothelial cells, and C-reactive protein production is enhanced by IL-6.39–42 Conversely, vascular cell adhesion molecule-1, which was not significantly increased in patients with FPLD2, was not clearly associated with increased incidence of coronary heart disease or stroke.39,41

To evaluate the effect of the p.R482W mutation in endothelial cells, we used HCAECs overexpressing either WT- or p.R482W-prelamin-A. Although WT-prelamin-A was correctly processed, p.R482W LMNA mutations resulted in abnormal prelamin-A accumulation, as previously shown in patients’ fibroblasts.13,14 Prelamin-A accumulation could result from a delay in the maturation process, as demonstrated in the skin fibroblasts of the patients with FPLD2. Remarkably, prelamin-A processing rate is also slower in fibroblasts issued from patients with HGPS.43 Interestingly, p.R482W-prelamin-A had a peculiar nuclear localization, being predominantly present at the nuclear envelope. We also confirmed this mislocalization in patients’ fibroblasts. The effect of the farnesylation inhibitor pravastatin, which prevented the abnormal nuclear distribution of p.R482W-prelamin-A in HCAECs, suggests that p.R482W-prelamin-A remained farnesylated, its farnesyl group acting as an anchor to the nuclear envelope.43

During the atherosclerosis process, early alterations include endothelial dysfunction with a reduced bioavailability of NO, produced by eNOS.30 Indeed, in human atherosclerotic plaques, eNOS expression and NO production are decreased.44 In HCAECs overexpressing p.R482W, but not WT-prelamin-A, we observed a markedly diminished eNOS expression and NO production. A previous study reported a decreased eNOS expression in senescent endothelial cells, attributable to telomere shortening. Accordingly, transfection of the telomerase catalytic subunit significantly prevented endothelial dysfunction associated with cellular senescence, thus improving eNOS expression and activity.29 In our study, the early senescence of p.R482W prelamin-A overexpressing HCAECs could partly explain the diminution of eNOS expression and NO production.

We also showed an increased expression and secretion of chemokines, cytokines, and cell-surface adhesion molecules leading to increased adhesion of PBMCs to HCAECs overexpressing p.R482W but not WT-prelamin-A. Taken as a whole, these data indicate that p.R482W-prelamin-A led to endothelial dysfunction in vitro.

Oxidative damage was detectable in >90% of cells in atherosclerotic plaques45 and was shown to play a major role in endothelial dysfunction during the atherosclerosis process.32 We observed that ROS production and expression of cell stress markers C/EBP-homologous protein 10, ATF-3, and ATF-4 were enhanced in HCAECs overexpressing p.R482W-prelamin-A.

Oxidative stress is known to induce an array of DNA adducts and single-and double-stranded breaks.8 DNA DSBs activate the DNA repair pathways, including serine phosphorylation of histone H2AX. Failure of DNA repair results in increased expression of p53 and of cell cycle arrest checkpoint kinases.38 DNA damages and increased H2AX phosphorylation were observed in atherosclerotic plaques.46 We show here, for the first time, that overexpression of p.R482W-prelamin-A led to an increased number of DNA DSBs, revealed by γH2AX phosphorylation, in endothelial cells. We confirmed this result in skin fibroblasts from patients with FPLD2. Accordingly, expression of p53 and of the cell cycle blockers p16INK4a and
p119AF1 was increased in HCAECs overexpressing p.R482W-prelamin-A, resulting in cellular senescence, which also contributes to atherosclerosis.29

Farnesylated prelamin-A was shown to induce vascular smooth muscle cells impairment, with DNA DSBs and senescence.22 Our group previously reported that HIV protease inhibitors, known to inhibit ZMPSTE24,47 induced an accumulation of farnesylated prelamin-A in endothelial cells and that the inhibition of prelamin-A farnesylation by pravastatin treatment improved endothelial dysfunction in this model.33 We demonstrated here that pravastatin treatment in vitro also decreased the amount of p.R482W farnesylated prelamin-A, improved endothelial dysfunction and oxidative stress, and reduced the occurrence of DNA damages in HCAECs. Moreover, we observed that antioxidant treatments reduced the secretion of inflammatory and adhesion molecules and decreased DNA DSBs in HCAECs overexpressing p.R482W-prelamin-A.

Our study suggests that endothelial cell inflammation, pro-adhesive and senescent states were mediated by oxidative stress in cells expressing farnesylated p.R482W-prelamin-A. A hypothetical scheme of the pathophysiological mechanisms leading from LMNA p.R482 mutations to endothelial dysfunction is proposed in Figure 6.

Our study has some limitations. The model of lamin-A overexpression does not reproduce the 1:1 ratio of mutated versus WT-lamin expected in heterozygous cells from patients with FPLD2. However, under our overexpression conditions, we had a 2.5-fold increased lamin-A level leading to a stoichiometry of ≈1.5 exogenous for 1 endogenous lamin-A. Furthermore, cultured endothelial cells are not submitted to their physiological environment and, in particular, to the blood flow. Because A-type lamins are involved in mechanotransduction signaling, endothelial responses to blood flow could also be altered in patients’ cells.48 In addition, the effect of metabolic disturbances, which contribute to endothelial dysfunction in atherosclerosis, was not evaluated.

In conclusion, we show here that severe atherosclerosis observed in FPLD2 could partly originate from primary endothelial dysfunction directly induced by LMNA mutations, in addition to classical cardiovascular risk factors. Our results support a role for farnesylated p.R482W-prelamin-A in the early atherosclerosis observed in patients with FPLD2.

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Disclosures
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References
34. The p.R482W mutation, through accumulation of farnesylated p.R482W-prelamin-A, exerts a direct proatherogenic effect on endothelial cells. Early and severe atherosclerosis observed in patients with FPLD2 could, in addition to classical cardiovascular risk factors, partly originate from these primary injuries.
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SUPPLEMENTAL MATERIAL
Supplemental Methods:

Adenovirus production
cDNA of WT or mutated p.R482W-prelamin-A were integrated in pAd5 plasmid vector, under the control of the cytomegalovirus (CMV) promoter. HEK 293 were transfected with pAd5 in order to produce adenoviral particles containing encoding sequences of WT or mutated p.R482W-prelamin-A or empty vector. Then, the recombinant adenoviruses were amplified in HEK 293 cells in order to obtain sufficient number of virus, and purified by two consecutive cesium chloride (CsCl) centrifugation steps. CsCl was removed by gel filtration through PD10 columns (Amersham Pharmacia Biotech SA, Les Ulis, France) and collected. Viruses stocks were stored at −80 °C in PBS containing 15% glycerol. The titer of virus stocks was determined by plaque assays on HEK 293 cells and expressed as plaque-forming units (PFU).

Lamin-A and flag nuclear distribution
HCAECs or fibroblasts were stained with antibodies respectively directed against Flag and lamin-A, which also recognizes prelamin-A, and revealed by using secondary antibodies coupled to Alexa fluor 568 or 488 (Invitrogen). Cells were visualized by Leica SP2 confocal microscope at 100X magnification. Images were acquired with Leica confocal software. Pictures were acquired with 0.12 µm increment on Z-axis, and sections through the nucleus were used for Flag or lamin-A fluorescence distribution analysis. Fluorescence was quantified every 0.15 µm in 21 µm sections of each nucleus diameter with ImageJ software in HCAECs and in cultured fibroblasts. The distribution of the nuclear fluorescence was quantified in at least 50-60 cells for each condition. We used HCAECs from four independent trasduction and fibroblasts issued from three healthy donors and two unrelated FPLD2 patients. Statistical differences were determined using unpaired student t-test. P<0.05 was considered as significant.

Prelamin A processing rate
Prelamin-A processing rate was assessed as previously described 1. Briefly, skin fibroblasts from three healthy donors and three unrelated FPLD2 patients were treated with farnesyl transferase inhibitor (FTI-277, Tocris Bioscience, Bristol, UK) at 10µM for 48 hours. Then cells were washed three times and treated with cycloheximide (10 µg/mL) for 3, 6 and 20 hours, in order to block protein and prelamin-A synthesis. Cells were lysed and processed for SDS-PAGE. Western-blots were probed with anti-lamin A/C antibody (sc-7292, Santa Cruz biotechnologies, Santa Cruz, CA, USA) in order to determine prelamin-A level and its maturation rate. Level of prelamin-A was determined by densitometry analysis with ImageJ software. The rate of prelamin-A process followed the linear function “y = a.t + b” (y= percentage of prelamin-A at time t; a= maturation speed; t= time; b= percentage of prelamin-A after 48 hours FTI treatment), with r>0.9.

Supplemental methods references:
# Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| HPRT-1    | F: 5'-TAATTGGTGAGATGATCTCTCTCAAC-3'  
            | R: 5'-TGCTGACCAAGGAAAGC-3' |
| eNOS      | F: 5'-GTGGCTGTCATGGACCT-3'   
            | R: 5'-CCACGATGGAGCTTGGCT-3' |
| IL-6      | F: 5'-CACACAGACACGCACTCACC-3'  
            | R: 5'-CATCCATCTTTTCAGCCATC-3' |
| IL-8      | F: 5'-AGACAGCAGACACACAAGC-3'   
            | R: 5'-ATGTTTCCTCCGGGTTG-3'   |
| MCP-1     | F: 5'-TCAGCCAGATGCAATCAATG-3'  
            | R: 5'-TCCTGAACCCACTTCTGCTT-3' |
| ICAM-1    | F: 5'-TGCTGACAGCAGCACTCATA-3'  
            | R: 5'-CTCCTCTCTCTCTGTGAATT-3' |
| VCAM-1    | F: 5'-CGCTGACAATGAAATCTTGTAGT-3'  
            | R: 5'-GTATCTTTGGGTGATATGACTCCTT-3' |
| CHOP-10   | F: 5'-AAACGGAACAGATGGTCTTTCCC-3'  
            | R: 5'-GTGGGATGGAGGTCAGCAATCTGTTGCA-3' |
| ATF-3     | F: 5'-AGTAGTACAGCTGACACTGCA-3'  
            | R: 5'-CTCCTCTTTGCAAGGCTTCTT-3' |
| ATF-4     | F: 5'-CTTGGGAGATGGATGG-3'   
            | R: 5'-GTCGTCTTATTAGTCTCCTGGAC-3' |

F: Forward primer; R: Reverse primer.
Supplemental Figures legends:

Supplemental Figure I:
A, Lamin-A expression levels in transduced HCAECs
Densitometry analysis of lamin-A protein expression, normalized to β-actin.
*P<0.05 for comparison with the condition of WT prelamin-A overexpression; ns: non-significant.

B, Prelamin-A maturation rate in control and LMNA-mutated fibroblasts
After 48 hours of farnesyl transferase inhibitor (FTI) treatment, prelamin-A accumulated in both control and FPLD2 fibroblasts. Following FTI washout and cycloheximide (CHX) treatment, prelamin-A maturation rate was slower in FPLD2 fibroblasts than in controls (2.13 ± 0.19 vs 1.45 ± 0.15 % per hour; r > 0.9; p=0.05).

C, Lamin-A distribution through the cell nuclei of patients’ fibroblasts
p.R482W lamin-A is predominantly localized at the nuclear periphery, similarly to p.R482W-prelamin-A overexpressing HCAECs. Results are mean ± SEM of 50-60 fibroblasts from three control subjects and two unrelated FPLD2 patients (LMNA p.R482W).
*P<0.05 for comparison to control subjects.

Supplemental Figure II: p.R482W but not WT prelamin-A triggered endothelial dysfunction.
A, B, C, Relative mRNA expression of eNOS (A) IL-6, IL-8 and MCP-1 (B) and ICAM-1 and VCAM-1 (C) (normalized to HPRT-1 expression). Results of 5 different experiments are expressed as mean ± SEM.

D, Representative pictures of adhesion assay of Peripheral Blood Mononuclear Cells (PBMCs) from healthy donors on transduced endothelial cells.
Non trans., non transduced HCAECs; Empty vector, HCAECs transfected with adenovirus containing an empty vector; WT, HCAECs overexpressing wild-type prelamin-A; R482W, HCAECs overexpressing p.R482W prelamin-A.
*P<0.05 for comparison with WT prelamin-A overexpression condition.

Supplemental Figure III: p.R482W but not WT prelamin-A increased cell stress markers expression and DNA double-strand breaks and fibroblasts from patients with LMNA p.R482W mutation showed increased incidence of DNA double-strand breaks.
A, Relative mRNA expression of the cell stress markers ATF-3 and -4. Results of 4 different experiments are expressed as mean ± SEM.
*P<0.05 for comparison with WT prelamin-A overexpression condition.
Abbreviations are as defined in Supplemental Figure 1.

B, Representative pictures of DNA double-strand breaks occurrence presented in Figure 3C (100X magnification). Transduced HCAEC were stained with γ-H2AX antibody (green), lamin A antibody (red) and counterstained with DAPI (blue).
Abbreviations are as defined in Supplemental Figure 1.

C, DNA double-strand breaks (DSB) were evaluated by the percentage of γ-H2AX positive cells after immunofluorescence staining in primary fibroblasts from five control subjects and two unrelated patients with LMNA p.R482W-linked FPLD2. Experiences were performed at least 4 times for each fibroblast culture at passages 4-7. Results are expressed as mean ± SEM.
*P<0.05 for comparison with control fibroblasts.
Supplemental Figure I

A) HCAECs

Lamin-A expression level

B) Prelamin-A maturation rate in fibroblasts from controls and FPLD2 patients

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LMNA p.R482W</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTI 3hr</td>
<td>68%</td>
<td>63%</td>
</tr>
<tr>
<td>FTI 6hr</td>
<td>54%</td>
<td>60%</td>
</tr>
<tr>
<td>FTI 20hr</td>
<td>22%</td>
<td>36%</td>
</tr>
</tbody>
</table>

C) Lamin A fluorescence distribution in fibroblasts nuclei from controls and FPLD2 patients

* indicates statistical significance.
Supplemental Figure II

A) eNOS

B) IL-6, IL-8, MCP-1

C) ICAM-1, VCAM-1

D) Adherent PBMCs to endothelial cells: green staining

Empty vector, WT, R482W
**Supplemental Table I**

**Cardiovascular features of patients with FPLD2 due to LMNA p.R482 heterozygous substitutions and clinical signs of atherosclerosis.**

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Sex</th>
<th>LMNA genotype</th>
<th>Clinical vascular disease</th>
<th>Type 2 diabetes</th>
<th>Hypertension</th>
<th>Smoking</th>
<th>Dyslipidemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>F</td>
<td>p.R482W</td>
<td>Silent myocardial ischemia at age 42</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>HyperTG Low HDL-C</td>
</tr>
<tr>
<td>43</td>
<td>F</td>
<td>p.R482W</td>
<td>Myocardial infarction before age 42</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>HyperTG Low HDL-C</td>
</tr>
<tr>
<td>46</td>
<td>M</td>
<td>p.R482W</td>
<td>Stroke before age 45</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>HyperTG</td>
</tr>
<tr>
<td>47</td>
<td>M</td>
<td>p.R482W</td>
<td>Myocardial infarction at age 40</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>HyperTG Low HDL-C</td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>p.R482W</td>
<td>Three coronary artery bypass grafts at age 44</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Low HDL-C</td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>p.R482W</td>
<td>Coronary artery bypass graft at age 50</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>NA</td>
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<tr>
<td>52</td>
<td>M</td>
<td>p.R482W</td>
<td>Two myocardial infarctions at age 45</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>HyperTG</td>
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<tr>
<td>53</td>
<td>F</td>
<td>p.R482Q</td>
<td>Stroke at age 52</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>HyperTG Low HDL-C</td>
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<tr>
<td>55</td>
<td>F</td>
<td>p.R482W</td>
<td>Two myocardial infarctions at age 43</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>HyperTG Low HDL-C</td>
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<tr>
<td>62</td>
<td>F</td>
<td>p.R482Q</td>
<td>Stroke and coronary stent implantation at age 59</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>HyperTG Low HDL-C</td>
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<tr>
<td>69</td>
<td>F</td>
<td>p.R482W</td>
<td>Four coronary artery bypass grafts at age 45</td>
<td>Yes</td>
<td>NA</td>
<td>NA</td>
<td>HyperTG</td>
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<tr>
<td>71</td>
<td>F</td>
<td>p.R482W</td>
<td>Carotid endarterectomy at age 67</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>HyperTG Low HDL-C</td>
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<tr>
<td>71</td>
<td>F</td>
<td>p.R482W</td>
<td>Coronary stent implantation at age 59</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Hypertension was defined as blood pressure >140/90 mmHg or antihypertensive treatment. Hypertriglyceridemia (HyperTG): ≥ 1.7 mmol/L; Low HDL-Cholesterol (HDL-C): <1.3 mmol/L for women or <1 mmol/L for men; High LDL-Cholesterol: > 4.1 mmol/L; NA: not available.
Materials and Methods
An expanded Methods section is part of the online Supplemental Materials.

Cardiovascular parameters of patients with FPLD2
We analyzed the cardiovascular and metabolic phenotype of nineteen consecutive FPLD2 patients from 14 unrelated pedigrees and over 30 years old bearing LMNA p.R482W/Q mutations.

The concentrations of inflammatory markers (high sensitive-CRP, IL-6) and endothelial cell adhesion molecules (ICAM-1, VCAM-1) were measured in the plasma of twelve patients with LMNA p.R482W/Q and twelve age- and sex-matched healthy volunteers. The assays were performed with commercially available ELISA kit (R&D Systems Europe, Abington, UK) according to manufacturer instructions.

Cell culture and treatment
HCAECs and endothelial cell growth medium were purchased from Promocell (Heidelberg, Germany). HCAECs were seeded on 0.2 %-gelatin-coated plastic dishes. When indicated, transduced cells were treated immediately with pravastatin (25 µM) or GSH (2 mM), or after 48 h with NAC (1 mM) (Sigma-Aldrich, St Quentin Fallavier, France). Vehicle-treated cells were used as controls.

Primary fibroblast cultures, established from skin biopsy from two unrelated patients with FPLD2 due to LMNA p.R482W mutations and four unrelated non-diabetic controls 1, were cultured in DMEM supplemented with 1 g/L glucose, 10 % fetal bovine serum and L-glutamine (Invitrogen, Cergy-Pontoise, France). All patients gave their informed consent according to our local ethics committee.

Adenoviral-mediated expression of WT or p.R482W-prelamin-A in HCAECs
Early-confluent HCAECs were transduced with a multiplicity of infection of 1 plaque-forming unit per cell with Flag-tagged recombinant adenovirus containing either WT- or mutated p.R482W-prelamin-A (see Supplemental Methods). Non-transduced cells (non-trans.) or cells transduced with empty adenoviral vector (empty vector) were used as controls. Transduction efficiency was assessed for each adenoviral construct by counting Flag-positive cells. Approximately 70 % of cells expressed the transgene, without statistical differences between the different adenoviral constructs. All the experiments were performed 72 h after cell transduction.

Prelamin-A maturation and nuclear shape
Protein extracts were subjected to SDS-PAGE and Western-blotting. Antibodies were directed against lamin-A/C (SC-7292), prelamin-A (SC-6214) (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA), Flag (F-1804) and β-actin (A-5441) (Sigma-Aldrich, St Quentin Fallavier, France). Protein bands were visualized by enhanced chemiluminescence (Pierce, Fisher Scientific, Illkirch, France).

For immunofluorescence studies, HCAECs grown on 0.2 %-gelatin-coated (Sigma-Aldrich) coverslips were fixed in methanol for 10 min at -20°C. Antibodies directed against Flag, lamin-A (SC-20680, Santa Cruz Biotechnologies Inc.) and prelamin-A were revealed by using secondary antibodies coupled to Alexa fluor 568 or 488 (Invitrogen, Cergy-Pontoise, France). Cells were visualized by Leica SP2 confocal microscope at 100X magnification. Images were acquired with Leica confocal software. Flag-fluorescence nuclear distribution was analyzed as described in Supplemental Methods.
**Endothelial dysfunction and inflammation**
Total RNA was isolated from cultured cells using RNeasy kit (Qiagen, Courtaboeuf, France), according to manufacturer instructions. mRNA expression of eNOS, IL-6, IL-8, MCP-1, VCAM-1, ICAM-1, CHOP-10, ATF-3, ATF-4 was analyzed by real-time-PCR (LC480, Roche Diagnostics, Meylan, France). HypoxanthinePhosphoRibosylTransferase-1 (HPRT-1) was used as an internal standard for mRNA expression. eNOS (NOS-3, SC-654, Santa Cruz Biotechnologies Inc.) protein expression was evaluated by Western blotting. IL-6, IL-8, MCP-1, VCAM-1, and ICAM-1 secretions were measured on 24-hour HCAECs culture supernatant with multiplexed bead-based immunoassays (Procarta, Affimetrix, Santa Clara, CA, USA) on a Bio-Plex 200 system (Bio-Rad laboratories Inc., Hercules, CA, USA), using Bio-Plex Manager 4.1 software. Results were normalized to the protein content. The detection limit was 10 pg/mL for all markers.

**Peripheral blood mononuclear cells adhesion assay**
PBMCs adhesion assay was performed as previously described with minor modifications. Briefly, fresh human PBMCs obtained from four different healthy blood donors were isolated by density gradient centrifugation (Ficollplaque plus, 17-1440-02, GE Healthcare, Aulnay-sous-Bois, France). PBMCs were counted and labeled for 30 minutes with fluorescent tracer calcein acetoxymethyl ester (10 μmol.L⁻¹) (Sigma-Aldrich). Labeled PBMCs were added for 1 hour at 37°C to previously transduced HCAECs, cultured since 24 hours in serum-free Endothelial Cell Basal Medium (Promocell). After PBS wash, adherent PBMCs were counted in 5 different fields (Leica TCS SP microscope, 40X magnification). Results were normalized to HCAECs protein level for each condition.

**Oxidative stress and nitric oxide assay**
The production of ROS or nitric oxide were respectively assessed by the oxidation of 5,6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) or 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM diacetate) (Invitrogen), according manufacturer instructions and normalized to the protein level.

**Senescence of endothelial cells**
Nuclear foci secondary to DNA DSBs were visualized by immunofluorescence with antibodies directed against Ser139-phosphorylated histone variant H2A (γ-H2AX, 05-636, Upstate, Millipore, Molsheim, France) in transduced HCAECs and primary cultured fibroblasts (Leica TCS SP microscope, 40X magnification). Nuclear DNA was stained with di-amidino-2-phenylindole hydrochloride staining (DAPI, Sigma-Aldrich). Cellular senescence was evaluated by the Senescence-associated (SA) β-galactosidase activity and the protein expression of cell cycle arrest markers p53 (ab80645, Abcam, Paris, France), p21^WAF1^ (#2947, Cell Signaling Technology, Ozyme, Saint-Quentin-en-Yvelines, France) and p16^INK4a^ (55154, BD Pharmingen, Becton Dickinson, Le Pont-De-Claix, France), as previously described.

**Statistical analysis**
All experiments were performed at least four times on HCAECs issued from three different donors. All results are expressed as means ± SEM. Statistical significance was determined using non-parametric Kruskal-Wallis test to compare all conditions and non-parametric Mann-Whitney-Wilcoxon test to determine differences between p.R482W and WT-prelamin-A overexpressing cells. P<0.05 was considered as significant. Statistical analyses were performed using R software (www.r-project.org).
References


