Nitric oxide synthase-3 deficiency results in hypoplastic coronary arteries and postnatal myocardial infarction

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Received 9 May 2012; revised 13 July 2012; accepted 16 August 2012; online publish-ahead-of-print 9 October 2012

Aims
Hypoplastic coronary artery disease is a rare congenital abnormality that is associated with sudden cardiac death. However, molecular mechanisms responsible for this disease are not clear. The aim of the present study was to assess the role of nitric oxide synthase-3 (NOS3) in the pathogenesis of hypoplastic coronary arteries.

Methods and results
Wild-type (WT), NOS3−/−, and a novel cardiac-specific NOS3 overexpression mouse model were employed. Deficiency in NOS3 resulted in coronary artery hypoplasia in foetal mice and spontaneous myocardial infarction in postnatal hearts. Coronary artery diameters, vessel density, and volume were significantly decreased in NOS3−/− mice at postnatal day 0. In addition, NOS3−/− mice showed a significant increase in the ventricular wall thickness, myocardial volume, and cardiomyocyte cell size compared with WT mice. Lack of NOS3 also down-regulated the expression of Gata4, Wilms tumour-1, vascular endothelial growth factor, basic fibroblast growth factor and erythropoietin, and inhibited migration of epicardial cells. These abnormalities and hypoplastic coronary arteries in the NOS3−/− mice were completely rescued by the cardiac-specific overexpression of NOS3.

Conclusion
Nitric oxide synthase-3 is required for coronary artery development and deficiency in NOS3 leads to hypoplastic coronary arteries.

Keywords
Nitric oxide synthase • Coronary artery development • Congenital heart disease

Introduction
Congenital coronary anomalies affect up to 5% of the general population.1,2 Most of them do not have clinical signs or symptoms, and are often unrecognized. However, severe congenital coronary artery malformation is associated with myocardial ischaemia, infarction, and sudden cardiac death.3 Hypoplastic coronary artery disease (HCAD), a rare congenital coronary abnormality, is defined by malformation of one or more major branches of the coronary arteries with a marked decrease in the luminal diameter and length.4–6 Hypoplastic coronary artery disease can be asymptomatic, but is often associated with myocardial infarction and sudden cardiac death under stress, such as during intense physical activity.3,7 However, the molecular mechanisms responsible for the embryonic development of HCAD are still unknown.

Nitric oxide (NO) is produced from the guanidino group of L-arginine in an NADPH-dependent reaction catalysed by a family of NO synthase (NOS) enzymes.8 Originally identified as a vasodilatory agent, NO is now recognized as an important signalling molecule involved in a wide range of physiological processes including apoptosis, angiogenesis and cell growth.8,9 There are three distinct isoforms of NOS: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). Interestingly, NOS3 is expressed in cardiomyocytes during early mammalian embryonic heart development.10 Immunohistochemical analysis revealed that the heart starts to express...
NOS3 at E9.5 and the expression remains high until E13.5. Starting from E14.5 the levels of NOS3 expression decrease in both atria and ventricles. The expression of NOS3 in the heart peaks during coronary artery development, suggesting its potential importance. However, the role of NOS3 in embryonic coronary artery development is still not clear.

The signalling milieu within the developing heart is critical to coronary vasculogenesis. Transcription factors, such as Gata4...
and Wilms tumour-1 (Wt1),12,13 as well as growth factors, including the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and erythropoietin (EPO),14,15 are essential in the formation of coronary arteries.11,15 We have previously shown a key role of NOS3 to capillary vessel development in the heart.16 In the present study, we hypothesized that NO production from NOS3 within the embryonic heart is a critical signalling molecule in the development of coronary arteries and deficiency in NOS3 results in hypoplastic coronary arteries.

Methods
For complete methods, see Supplementary material online.

Results
Characterization of NOS3Tg and NOS3Tg;NOS32/2 mice
To study the specific role of NOS3 transgenic mouse (NOS3) in coronary artery development, a cardiac-specific NOS3Tg was generated under the control of a β-myosin heavy chain promoter.17 Expression of human NOS3 was detected only during embryonic development and specifically in the heart of NOS3Tg mice (Figure 1A–D). The NOS3Tg mice were then crossed with NOS32/2 to create the NOS3Tg;NOS32/2 mouse, an animal that lacks NOS3 in all organs except the heart during embryogenesis (Figure 1B and D). cGMP, a critical downstream signalling molecule of NOS3, was significantly decreased in the NOS32/2 mice.

Figure 2 Myocardial infarction and postnatal survival in NOS32/2 mice. (A) Evidence of spontaneous myocardial infarction in NOS32/2 mice at P0. Triphenyltetrazolium chloride (TTC) staining shows a large area of tissue death near the apex of the heart. The images are adjacent cross sections of the heart near the apex. Troponin I staining shows a significant loss of troponin I in the infarct myocardium. Haematoxylin/eosin staining shows the waviness of fibres near the border of the myocardium with intense eosinophilic cytoplasm. ECG tracing shows significant ST-elevation (arrows) and QRS inversion (arrowheads), representing signs of myocardial ischaemia, and possible cardiac hypertrophy, respectively. White bar: 1 mm. Black bar: 40 μm. (B) Spontaneous myocardial infarction in NOS32/2 animals at P0. *P < 0.01 vs. NOS3+/+, NOS3Tg;NOS32/2 and NOS3Tg mice. Tg;2/2 and Tg;+/+ indicate NOS3Tg;NOS32/2 and NOS3Tg, respectively. (C and D) Heart function assessed by echocardiography in NOS3+/+, NOS32/2, NOS3Tg;NOS32/2, and NOS3Tg mice at P0; n = 5–6 mice per group. *P < 0.01 vs. NOS32/2 mice. †P < 0.01 vs. NOS32/2. (E) Thirty-day survival after birth in NOS3+/+, NOS32/2, NOS3Tg;NOS32/2, and NOS3Tg mice. *P < 0.001 vs. NOS3+/+, NOS3Tg;NOS32/2, and NOS3Tg mice.
compared with NOS3+/+ hearts at postnatal day 0 (P0) (data not shown).

**Deficiency in NOS3 results in spontaneous myocardial infarction**

Deficiency in NOS3 resulted in 73% mortality within the first 4 days after birth (Figure 2E). To assess if myocardial infarction was the cause of death, P0 hearts were subjected to triphenyltetrazolium chloride (TTC), troponin I, and haematoxylin/eosin (H/E) staining. Triphenyltetrazolium chloride staining showed that the NOS3+/− mice had a large area of tissue death near the apex of the heart (Figure 2A). Troponin I is a part of the troponin complex that is integral to muscle contraction and decreased levels of troponin I represent cardiomyocyte death.18 Our data showed troponin I immunostaining was decreased in the area of infarct in the NOS3+/− mice (Figure 2A). Haematoxylin/eosin staining showed typical wavy fibres near the border of the infarct area with disappearance of nuclei and intense eosinophilic cytoplasm (Figure 2A), which...
are signs of acute myocardial infarction. ECG monitoring showed ST-elevation and QRS inversion in neonatal NO3/2/2 mice, indicating myocardial ischaemia and possible cardiac hypertrophy, respectively (Figure 2A). Spontaneous myocardial infarction was seen in 8/15 (53%) NO3/2/2 animals at P0 (Figure 2B). Consequently, left ventricular ejection fraction and fractional shortening were significantly decreased in the NO3/2/2 compared with NO3/+/+ mice (P < 0.05, Figure 2C and D). These abnormalities in the NO3/2/2 mice including myocardial infarction, cardiac dysfunction, and postnatal mortality were all rescued by cardiac-specific overexpression of NO3 (NO3Tg;NO3/2/2 mice, Figure 2A–E).
Deficiency in NOS3 impairs coronary artery development

NOS3−/− and NOS3+/+ hearts at P0 showed less branching and significant decrease in the left and right coronary artery diameters (Figure 3A–D). Three-dimensional reconstructions of the heart showed a significant decrease in the coronary volume in the NOS3−/− mice (P < 0.01, Figures 3E and 4A and Supplementary material online, Videos S1–3). Cardiomyocyte-specific NOS3 overexpression completely rescued these defects in the NOS3−/− mice (Figures 3A–E and 4A and Supplementary material online, Videos S4 and S5).

Figure 5 Coronary artery development at E15.5. (A) Three-dimensional reconstruction of E15.5 hearts. Coronary arteries shown in green were less abundant in NOS3−/− compared with NOS3+/− and NOS3+/+ mice. Cardiac-specific overexpression of the NOS3 restored coronary artery abundance in the NOS3−/− mice. Atria were excluded from the reconstruction in order to view the origins of the coronary arteries. (B–E) Coronary artery vessel diameter, abundance, and volume were significantly decreased in the NOS3−/− mice compared with NOS3+/− and NOS3+/+, which were restored in NOS3Tg;NOS3−/− mice; n = 5 hearts per group. Tg; −/− and Tg;+/+ indicate NOS3Tg;NOS3−/− and NOS3Tg, respectively. (F) Ventricular wall thickness was significantly increased in NOS3−/− compared with NOS3+/− and NOS3+/+ mice. Cardiomyocyte-specific overexpression of NOS3 (NOS3Tg;NOS3−/−) restored myocardial thickness to similar levels as in the NOS3+/+ mice; n = 4 hearts per group. For (B)–(F), *P < 0.01 vs. NOS3+/+ mice, †P < 0.01 vs. NOS3−/−.
Since a decrease in the coronary artery volume may have been a result of a smaller myocardial mass, myocardial volume, and thickness were analysed. However, both myocardial volume and thickness were increased in NOS3<sup>−/−</sup> and NOS3<sup>+/-</sup> compared with the NOS3<sup>+</sup>/+ mice (<sup>P</sup>, 0.01, Figure 4B and C). To determine whether the increased myocardial volume and thickness are a result of cardiac hypertrophy, cardiomyocyte cell size and nuclear size of P0 hearts were measured. The cardiomyocyte cell size and nuclear size were progressively increased in NOS3<sup>−/−</sup> and NOS3<sup>+/-</sup> compared with the NOS3<sup>+</sup>/+ mice (<sup>P</sup> < 0.01, Figure 4D and E). Therefore, our data showed an increased myocardial mass accompanied by severe coronary artery hypoplasia. These defects in the NOS3<sup>−/−</sup> mice were completely rescued by cardiomyocyte-specific NOS3 overexpression (<sup>P</sup> < 0.01, Figure 4D–E).

The coronary artery network is established by E15.5 in mice. To assess the onset of the coronary artery malformations, we

Figure 6  Myocardial mRNA expression of transcription and growth factors. (A–D) E12.5 hearts were collected and mRNA levels of Gata4, VEGFa, bFGF, and EPO were analysed by real-time RT–PCR. The mRNA levels of Gata4, VEGFa, bFGF, and EPO were significantly decreased in NOS3<sup>−/−</sup> compared with NOS3<sup>+/+</sup> mice, which were restored by cardiac-specific NOS3 overexpression in the NOS3<sup>Tg</sup>+/NOS3<sup>−/−</sup> hearts; <i>n</i> = 6–7 per group. *<sup>p</sup> < 0.01 vs. NOS3<sup>+/+</sup> mice. †<sup>p</sup> < 0.01 vs. NOS3<sup>−/−</sup> mice. Tg<sup>−/−</sup> and Tg<sup>+/+</sup> indicate NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup> and NOS3<sup>Tg</sup>, respectively. (E–H) E12.5 ex vivo heart cultures were used to investigate NOS3 signalling. Cultured NOS3<sup>+/+</sup> and NOS3<sup>−/−</sup> hearts were treated with 100 µM ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) a soluble guanylate cyclase inhibitor, or 2 mM 8-Br-cGMP, a cGMP analogue, for 6 h. ODQ decreased Gata4 and Wt1 mRNA levels in NOS3<sup>+/+</sup> hearts, whereas 8-Br-cGMP restored Gata4 and Wt1 mRNA levels in NOS3<sup>−/−</sup> hearts; <i>n</i> = 3–4 heart cultures per group. *<sup>p</sup> < 0.05 vs. controls.
Figure 7  The characterization and quantification of Wt1+ epicardial progenitor cells. (A) E12.5 hearts were immunostained for Wt1 and representative images from NOS3+/+, NOS3−/−, NOS3Tg;NOS3−/−, and NOS3Tg hearts are shown. The majority of Wt1+ cells are in the epicardium with limited expression in the myocardium. Scale bar = 60 μm. (B) Quantitative analysis showed a significant reduction in Wt1+ epicardial cells in NOS3−/− compared with NOS3+/+ hearts, which was restored by cardiac-specific NOS3 overexpression in the NOS3Tg;NOS3−/− hearts; n = 4–5. (C) Wt1 mRNA levels were analysed in E12.5 hearts by real-time RT–PCR. Wt1 mRNA expression was significantly decreased in the NOS3−/− compared with NOS3+/+ hearts, which was restored in NOS3Tg;NOS3−/− hearts; n = 6–7 per group. For (B) and (C), *P < 0.01 vs. NOS3+/+ mice, †P < 0.01 vs. NOS3−/−. Tg−/− and Tg+/+ indicate NOS3Tg;NOS3−/− and NOS3Tg, respectively.
further analysed coronary artery development at E15.5. Coronary artery abundance, vessel diameter, and volume were all significantly decreased, whereas the myocardial thickness was increased in NOS3$^{-/-}$ compared with the NOS3$^{+/+}$ and NOS3$^{+/+}$ mice at E15.5 ($P < 0.01$, Figure 5A–F). These results suggest that the onset of the defect is prior to E15.5. Consistent with our P0 data, these defects were completely rescued by cardiomyocyte-specific NOS3 overexpression ($P < 0.01$, Figure 5A–F). Since coronary artery formation at E15.5 was not impaired in the NOS3$^{+/+}$ mice, all subsequent molecular analyses on early coronary artery development were carried out in NOS3$^{+/+}$ in comparison with the NOS3$^{-/-}$ mice.
Down-regulation of transcription and growth factors in E12.5 NOS3−/− hearts

Capillary networks in the embryonic heart begin to develop at E12.5, and are regulated by transcription and growth factors that are critical to heart morphogenesis and coronary artery formation. Our data showed that the mRNA levels of Gata4, VEGFα, bFGF, and EPO were significantly decreased in the NOS3−/− compared with NOS3+/+ hearts, and were completely restored in the NOS3Tg;NOS3−/− hearts (P < 0.05, Figure 6A–D). Treatment with ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one, a soluble guanylyl cyclase inhibitor, significantly decreased Gata4 and Wt1 mRNA expression in the NOS3+/+ hearts (P < 0.05, Figure 6E and F). Conversely, NOS3−/− hearts treated with 8-bromo-cGMP, a cGMP analogue, showed a significant increase in Gata4 and Wt1 mRNA levels (P < 0.05, Figure 6G and H). These data suggest that NOS3 regulates the expression of Gata4 and Wt1 through a cGMP-dependent signalling pathway.

Deficiency in NOS3 decreases epicardium-derived cells migration

Epicardium-derived cells (EPDCs) express Wt1 and their migration into the myocardium is essential to the formation of coronary arteries. The number of Wt1+ epicardial cells was significantly decreased in NOS3−/− compared with NOS3+/+ hearts at E12.5 (P < 0.01, Figure 7A and B). In addition, myocardial Wt1 mRNA levels were also significantly decreased in NOS3−/− compared with the NOS3+/+ mice (P < 0.01, Figure 7C). The decreased myocardial expression of Wt1 and number of Wt1+ epicardial cells were rescued in the NOS3Tg;NOS3−/− mice (P < 0.01, Figure 7A–C). To study the role of NOS3 in EPDC migration, a coculture system was employed (Figure 8A). Epicardium-derived cells were isolated from E13 embryos overexpressing enhanced green fluorescence protein (eGFP). The cultured EPDCs were typical cobble stone shape and 100% positive for epicardin and Wt1. Cardiomyocytes were stained by α-actinin staining (Figure 8B), eGFP+ EPDCs were then co-cultured with either E13 NOS3+/+ or NOS3−/− cardiomyocytes treated with adenoviral lacZ or adenoviral NOS3 for 72 h. Our data showed that the migration of eGFP+ EPDCs was significantly decreased towards the NOS3−/− compared with the NOS3+/+ cardiomyocytes (Figure 8C and D). However, EPDC migration was increased in cardiomyocytes treated with adenoviral NOS3 compared with adenoviral lacZ controls (Figure 8C and D). Finally, to assess the migration and proliferation of EPDCs in the developing heart, ex vivo heart explants were employed. Our data showed that ex vivo EPDC migration and proliferation were significantly decreased in the NOS3−/− compared with NOS3+/+ hearts (P < 0.01, Figure 8E and F).

Discussion

Growing evidence suggests that NO plays an important role in embryonic heart development. To this end, we and others have previously shown that lack of NOS3 results in congenital septal defects and bicuspid aortic valves in mice. In addition, impaired capillary and microvessel development has been shown in the heart and lungs of the NOS3−/− mice, respectively. The present study was carried out to further examine the role of NOS3 in coronary artery development. We demonstrated for the first time that the deficiency of NOS3 leads to hypoplastic coronary arteries. These results show that NOS3 plays a critical role in normal coronary artery development (Figure 8G). To rescue the coronary phenotype in the NOS3−/− mice, we generated a mouse with cardiomyocyte-specific overexpression of NOS3 during embryonic development. The NOS3Tg mice had a normal cardiac and coronary artery phenotype. Importantly, cardiomyocyte-specific overexpression of NOS3 completely rescued the abnormal coronary artery development, cardiac hypertrophy, and postnatal survival in the NOS3−/− mice. The data strongly suggest that normal coronary artery development is driven by local NO signaling from endothelial cells and/or cardiomyocytes within the developing heart.

A classic pathway by which NO mediates its biological function is through cGMP-dependent protein kinase G (PKG) signalling. Activation of PKG has been shown to up-regulate Gata4 and Wt1. Thus, a deficiency in NOS3 decreases NO and cGMP production, and may lead to decreased PKG activation and downregulation of Gata4 and Wt1 expression. Gata4 is a master regulator of heart development, and controls the expression of many growth factors including VEGFα, bFGF, and EPO, that are

Figure 8 Migration of epicardium-derived cells. (A) Epicardium-derived cells were isolated from E13 hearts and co-cultured with cardiomyocytes as shown. The number of epicardium-derived cells migrated towards the cardiomyocytes was determined. (B) A representative confocal tiled image of the epicardium-derived cells and cardiomyocyte co-culture. Epicardium-derived cells (green) and cardiomyocytes were isolated from enhanced green fluorescence protein transgenic and C57BL6 wild-type mice, respectively. Cardiomyocytes were stained with cardiac α-actinin (red). (C) Quantitative analysis from (D) shows that epicardium-derived cell migration was significantly decreased in NOS3−/− compared with NOS3+/+ cardiomyocyte co-cultures, which was restored by adenoviral NOS3 treatment; n = 3–5 per group. **P < 0.01 vs. NOS3+/+ mice. (D) NO3 expression in cardiomyocytes promoted epicardium-derived cell migration. Representative confocal tiled images of eGFP+ epicardium-derived cells co-cultured with NOS3+/+ and NOS3−/− cardiomyocytes, which were treated lacZ or NOS3 adenoviral constructs. (E) Representative images of the epicardium-derived cell outgrowth of E12.5 NOS3+/+ and NOS3−/− hearts. Scale bar = 250 μm. (F) Quantitative analysis shows that maximum epicardium-derived cell migration distance was significantly decreased in NOS3−/− hearts compared with NOS3+/+ hearts; n = 7–8, *P < 0.01 vs. NOS3+/+ heart. (G) The proposed signalling pathway of NOS3 on coronary artery development. NOS3 promotes the expression of Gata4 and Wt1 via cGMP-dependent mechanisms. The production of growth factors (vascular endothelial growth factor-α, basic fibroblast growth factor, and erythropoietin) by Gata4 and epicardium-derived cells migration by Wt1 contribute to the critical role of NOS3 on coronary artery development.
important for coronary vessel formation. We showed that deficiency in NOS3 down-regulated the expression of Gata4, VEGFa, bFGF, and EPO in the embryonic heart. Additionally, Wt1, a transcription factor critical for epithelial and mesenchymal transition and coronary artery formation, was also down-regulated and the number of EPDCs was decreased in the NOS3-/- hearts. Furthermore, lack of NOS3 decreased EPDCs migration towards the cardiomyocytes. Our results suggest that NOS3 promotes normal coronary artery development via increases in the expression of transcription and growth factors, and EPDCs migration into the myocardium (see Figure 8G).

NOS3-/- mice have a high rate of postnatal mortality. We have previously shown that within 10 days after birth, mortality was 85, 38, and 13% for NOS3-/-, NOS3-/-, and NOS3+/+ mice, respectively. Most animals died within the first 3 days after birth. Consistent with our finding, Han et al. showed that 40% of NOS3-/- offspring succumbed within the first hour of birth. However, they did not monitor their animal survival beyond 1 hour of birth. In the present study, the mortality of the NOS3-/- mice was 73% in the first 4 days after birth, which is in agreement with the previous studies. Additionally, we demonstrated a dose-dependent cardiac dysfunction at P1 with the loss of one or both NOS3 alleles. Interestingly, this gene dose-dependent response was also observed in the coronary artery formation of NOS3-/-, NOS3-/-, and NOS3+/+ mice at P0 in the present study. These data suggest that hypoplastic coronary arteries may contribute at least in part to postnatal cardiac dysfunction and mortality in the NOS3-/- mice.

Hypoplastic coronary artery disease is often associated with myocardial infarction and sudden cardiac death when the heart is stressed. In the present study, coronary artery malformation is accompanied by spontaneous myocardial infarction in postnatal NOS3-/- mice. Our study demonstrated that NOS3 deficiency results in hypoplastic coronary arteries, a condition that mirrors HCAD in humans. Interestingly, recent studies have shown that a common 894G>T single nucleotide polymorphism, which reduces NOS3 activity, is associated with an increased risk of congenital heart disease, especially conotruncal heart defects. In addition, environmental factors and maternal conditions including psychological stress, hypertension, and diabetes, which decrease NOS3 expression and/or activity, are associated with increased risks of congenital heart disease. Thus, it is possible that decreased NOS3 signalling may promote the development of congenital heart disease in patients with these environmental and maternal conditions. Although further studies are required to analyse NOS3 gene mutation in patients with HCAD, the present study suggests that NOS3 is critical to coronary artery development and deficiency or mutation of NOS3 gene may lead to HCAD. Our study is the first to implicate NOS3 deficiency in the pathogenesis of HCAD and may help to design strategies in the diagnosis, prevention, and treatment of HCAD in humans.

Supplementary material
Supplementary material is available at European Heart Journal online.

Funding
This work was supported by grants from Heart and Stroke Foundation of Ontario (HSFO, NA-6774) and the Canadian Institutes of Health Research (CIHR) to Q.F., who is a HSFO Career investigator.

Conflict of interest: none declared.

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Renal artery fibromuscular dysplasia: in vivo optical coherence tomography insights

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A 66-year-old woman with long-term essential hypertension presented with poorly controlled blood pressure in spite of six antihypertensive drugs. Percutaneous renal denervation was indicated as a treatment of resistant arterial hypertension. Renal angiography showed ‘strings of beads’ appearance characteristic of fibromuscular dysplasia (FMD) in the mid-segment of the right renal artery (Panel A).

Optical coherence tomography (OCT) confirmed the diagnosis of medial fibroplasia showing the ‘strings and beads’ appearance in the longitudinal reconstruction (Panel B). Cross-sectional images showed several luminal stenosis due to media layer hyperplasia and fibrosis, with areas of intimo-media layer dissections and aneurysm formations, alternating with segments of normal three-layer appearance (Panels C–I). (Panel O) Three-layer appearance of the arterial wall (intima: white arrow, media: blue arrow, adventitia: yellow arrow) at the bottom and medial fibroplasia (asterisks) with an intimal dissection (red arrow) communicating with a little aneurysm formation at the top. Medial fibroplasia contains areas of low backscattering probably related with muscle cells and areas of high backscattering in the inner half of the media corresponding with collagen deposition. (Panel D) Three-layer appearance. (Panel E) intimo-medial dissection (red arrow). (Panel F) Luminal stenosis secondary to focal medial fibroplasia (asterisk). (Panel G) Ruptured medial fibroplasia with an aneurysm formation. (Panel H) Another intimo-medial dissection (red arrow). (Panel I) Small medial fibroplasia that protrudes into the lumen of the renal artery. As this case illustrates, OCT provides unique insights on the underlying pathology of FMD, showing detailed ‘in vivo’ histology information.

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