Role of genetics in peripheral arterial disease outcomes; significance of limb-salvage quantitative locus-1 genes

Emmanuel Okeke and Ayotunde O Dokun

Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Tennessee Health Sciences Center, Memphis, TN 38163, USA

Corresponding author: Ayotunde O Dokun. Email: adokun@uthsc.edu

Impact statement

Peripheral artery disease (PAD) is a major health care problem with significant morbidity and mortality. Individuals with similar atherosclerosis burden do display different severity of disease. This review outlines some of the progress made up-to-date in unraveling the molecular mechanisms underlining differential PAD severity with a focus on the role of the **L**imb **S**alvage-associated **Q**uantitative trait locus 1 (LSq-1), a key locus in adaptation to ischemia in PAD.

Abstract

Peripheral artery disease is a major health care problem with significant morbidity and mortality. Humans with peripheral artery disease exhibit two major and differential clinical manifestations – intermittent claudication and critical limb ischemia. Individuals with intermittent claudication or critical limb ischemia have overlapping risk factors and objective measures of blood flow. Hence, we hypothesized that variation in genetic make-up may be an important determinant in the severity of peripheral artery disease. Previous studies have identified polymorphism in genes, contributing to extent of atherosclerosis but much less is known about polymorphisms associated with genes that can influence peripheral artery disease severity. This review outlines some of the progress made up-to-date to unravel the molecular mechanisms underlining differential peripheral artery disease severity. By exploring the recovery phenotype of different mouse strains following experimental peripheral artery disease, our group identified the limb salvage-associated quantitative trait locus 1 on mouse chromosome 7 as the first genetic modifier of perfusion recovery and tissue necrosis phenotypes. Furthermore, a number of genes within LSq-1, such as ADAM12, IL-21Rα, and BAG3 were identified as genetic modifiers of peripheral artery disease severity that function through preservation of endothelial and skeletal muscle cells during ischemia. Taken together, these studies suggest manipulation of limb salvage-associated quantitative trait locus 1 genes show great promise as therapeutic targets in the management of peripheral artery disease.

Keywords: A disintegrin and metalloproteinase 12, Bcl-2-associated athanogene-3, hind limb ischemia, interleukin-21 receptor, limb salvage quantitative loci 1, peripheral arterial disease

Experimental Biology and Medicine **2018; 243: 190–197. DOI: 10.1177/1535370217743460**

Introduction

Peripheral artery disease (PAD) is a major health care problem with increasing morbidity and mortality.[1–5] PAD is characterized by impaired blood flow to tissues outside the heart due to atherosclerosis and this most frequently occurs in the lower extremities.[2,3,6] Humans with PAD exhibit two major and differential clinical manifestations: Intermittent claudication (IC), which is defined as mobility or exercise-triggered leg pain usually relieved by rest; and critical limb ischemia (CLI), which is defined as leg pain at rest and is usually accompanied by tissue loss or necrosis.[2,6,7] The risk factors for developing PAD include diabetes, hypertension, hypercholesterolemia, and smoking.[3,6] These risk factors are associated with development of both IC and CLI.[8] Although in a number of studies, diabetes was shown to be more common among individuals with CLI compared with those with IC, many people with IC also have diabetes.[8–11] Additionally, while many people with CLI tend to have low ankle-brachial index values (<0.5) many individuals with IC also have low ankle brachial index.[8,12,13] Taken together, this suggests that individuals with CLI or IC have overlapping risk factors and objective measures of blood flow. Hence, we hypothesized that variation in genetic make-up may be an important

Copyright © 2017 by the Society for Experimental Biology and Medicine

determinant of PAD severity.[2,14] The role of genetic influence on PAD outcomes can be considered in two major ways. (1) Genetic polymorphisms may contribute to the development of lower extremity atherosclerosis; examples of this include Apo lipoprotein E, interleukin-6, and thrombin.[9,15–17] (2) Alternatively, following vessel occlusion due to atherosclerosis, genetic polymorphisms may influence disease severity such as extent of blood flow recovery, necrosis, or tissue loss.[18,19] The role of inflammation in the development of atherosclerosis is well known.[20] Numerous studies have identified polymorphism in genes, contributing to extent of atherosclerosis[2,9,15–17] but much less is known about polymorphism in genes that can influence PAD severity.[18,19,21] The hind limb ischemia (HLI) model is a well-known preclinical model of PAD that involves surgical ligation and excision of the femoral artery to induce limb ischemia.[22–29] The application of this experimentally induced PAD technique has significantly advanced our knowledge of the pathophysiology of PAD and some of the molecular mechanisms involved in the adaptation to ischemia.[14,23,25,29–32]

Similar to the findings in humans where PAD severity differs despite overlapping risk factors, extent of blood flow recovery and presence or absence of tissue necrosis differs considerably among different strains of mice following HLI.[14,27,33,34] Studies by Fukino *et al.*,[27] were one of the earliest to show following induction of HLI in the C57BL/6 and Balb/c strains perfusion recovery was quite different between the 2 strains.[27] The C57BL/6 strain typically showed favorable perfusion recovery with little or no tissue necrosis, whereas the Balb/c strain typically showed poor perfusion recovery with profound tissue necrosis.[14,27,34] Our group and others have since confirmed these findings and showed that following induction of HLI in different mouse strains perfusion recovery and extent of limb necrosis differed considerably.[14,33,34]

Identification of LSq-1, the first genetic modifier of PAD outcomes

We took advantage of the difference in the recovery phenotype between the C57BL/6 and Balb/c strains to identify the first quantitative trait locus (QTL) associated with perfusion recovery and tissue necrosis in mice following HLI.[14] Initial studies to identify the LSq-1 QTL involved generating F1 mice by crossing C57BL/6 and Balb/c mice. The F1 exhibited no significant difference in perfusion recovery or necrosis from the C57BL/6 parental strain. This finding is consistent with a dominant allele(s) in C57BL/6 being responsible for the perfusion recovery and lack of tissue loss. To map the allele in C57BL/6 mice, we backcrossed the F1 to the Balb/c and generated 105 N2 progeny. The N2 progeny was genotyped for the 239 SNPs that were informative in this cross. The average spacing between SNPs was 6.8 ± 1.6 cM, affording full coverage of the mouse genome. All but one of the chromosomes (chromosome 7) showed linkage profiles that were essentially flat, suggesting no other strongly acting loci maps to the other chromosomes. This single locus on chromosome 7 spanned $\approx$31 Mb with significant linkage to tissue necrosis and day 21 perfusion ratios. The peak LOD scores were 7.96 and 3.71, respectfully, at marker *rs13479513*. Interestingly, the linkage peak for necrosis was even more significant, far surpassing the $P = 0.001$ threshold. The linkage peaks for perfusion ratio and necrosis were almost identical in chromosomal position, differing only in the magnitude of the strength of linkage.[14]

Hence, we identified, to our knowledge, the first QTL-1 associated with the extent of perfusion recovery following experimental PAD. However, the mechanism by which this locus modifies perfusion recovery or tissue preservation was not known. We postulated that this locus may impact preexisting collateral vessels, arteriogenesis, development of new collateral vessels via angiogenesis, adaptation to tissue ischemia, or some combination of these factors to contribute to differential recovery after ischemia or that genes within this locus could work through mechanisms not yet postulated.[14]

Identifying specific LSq-1 genes involved in adaption to tissue ischemia

To home in on the genes within the LSq-1 capable of modifying PAD severity, experimental PAD was performed on six inbred mouse strains shown previously to demonstrate favorable (C57Bl/6J, C57BlKS/J, CBA/J, DBA2, and FVB/nJ) or poor (129SI/Svlm) recovery in an ischemic stroke model[35] and their perfusion recovery phenotype was determined. These mice strains were then classified as showing favorable recovery (C57Bl/6-like) or showing poor recovery (Balb/c-like). Haplotype blocks based on single nucleotide polymorphism (SNP) alleles, common in strains that recover well but absent in strains that recover poorly were generated.[14] For this analysis, two additional mouse strains with already known perfusion recovery phenotype were included, i.e. Balb/c and A/J. Hence, our haplotype block analysis was based on SNPs in C57Bl/6J = C57BlKS/J = CBA/J = DBA2/J but $\neq$ Balb/cJ, A/J, 129SI/SvlmJ, FVB/nJ. Using this approach, five haplotype blocks containing 25 genes were identified.[34] See Table 1, adapted from Dokun *et al.*[34]

Experimental validation of the expression profiles of the 25 genes housed within the five-haplotype blocks in non-ischemic mouse hind limb muscles (gastrocnemius) was performed.[34] Additionally, day 3 post ischemic expression was also assessed in mouse hind limbs and four genes were found to be significantly upregulated in the C57BL/6 strain compared to the Balb/c strain.[34] These genes are **ADAM12** (**A** **D**isintegrin **A**nd **M**etalloproteinase 12),[34] **IL-21Rα** (Interleukin-21 receptor),[34] **IL-4Ra** (Interleukin-4a receptor),[34] and **INPP5F** (inositol polyphosphate-5-phosphatase F).[34] ADAM12 was put forward as the lead candidate gene modifier of PAD severity because it showed the most increase in mRNA and protein fold expressions (>3 fold) in C57BL/6 compared to Balb/c following hind-limb ischemia. The role of ADAM12 in perfusion recovery following experimental PAD was assessed using gain-of-function approach in Balb/c strain (which normally has poor recovery) and loss-of-function approach in C57BL/6 strain (that normally has good recovery), we demonstrated that ADAM12

Table 1 Summary of genes within the five haplotype blocks and their mRNA expression profile

Haplotype Block	Gene (Assay ID)	Expression in Mouse Hind limbs	Upregulated in Ischemia	C57BL/6 > Balb/c	Balb/c > C57BL/6
Block 1	3100003L05Rik (Mm01277666_m1)	No	No	No	No
	4933440M02Rik (Mm01277432_m1)	No	No	No	No
	4930571K23Rik (Mm03807449_s1)	No	No	No	No
	Jmjd5 (Mm00513079_m1)	Yes	No	No	No
	Nsmce1 (Mm00471126_m1)	Yes	No	No	No
	Il4ra (Mm01275139_m1)	Yes	Yes	Yes (<2-fold)	No
	Il21a (Mm00600319_m1)	Yes	Yes	Yes (=2-fold)	No
	Gtf3c1 (Mm01278522_m1)	Yes	No	No	No
	D430042009Rik (Mm00525471_m1)	Yes	No	No	No
	Gsg1l (MM01278522_m1)	No	No	No	No
	Xpo6 (Mm00503902_m1)	Yes	No	No	No
	Sbk1 (Mm00455133_m1)	Yes	No	No	No
	Lat (Mm00456761_m1)	Yes	Yes	No	No
	Spns1 (Mm00470041_m1)	Yes	No	No	No
	Nfatc2ip (Mm00803049_m1)	Yes	No	No	No
	Cd19 (Mm00515420_m1)	No	No	No	No
Block 2	Bag3 (Mm00443474_m1)	Yes	No	No	No
	Inpp5f (Mm00724391_m1)	Yes	Yes	Yes (<2-fold)	No
Block 3	ADAM12 (Mm00475719_m1)	Yes	Yes	Yes (>3-fold)	No
	D7Ertd443e (Mm01131413_m1)	Yes	No	No	No
	Dock1 (Mm01269874_m1)	Yes	Yes	No	No
Block 4	9430038I01Rik (Mm03991735_g1)	Yes	Yes	No	Yes
Block 5	Gpr123 (Mm00623786_m1)	No	No	No	No
	Kndc1 (Mm006278522_m1)	No	No	No	No
	Utf1 (Mm00447703_g1)	No	No	No	No

Note: No expression = no amplification or comparative threshold > 40. Adapted from Dokun *et al.*[34]

mediated favorable perfusion recovery and rescued limb blood flow following hind-limb ischemia as measured using laser Doppler imaging technology.[34] Moreover, we showed ADAM12 upregulation in ischemic hind limbs occurred primarily in endothelial cells.[34] *In vitro* studies in pooled HUVECs (Human Umbilical Vein Endothelial Cells) also corroborated the *in vivo* finding of ADAM12 upregulation in ischemic endothelial cells. We further showed knock down of ADAM12 in ischemic endothelial cells decreased cell proliferation, angiogenesis (tube formation), while increasing apoptosis.[34] These findings affirm the notion – that ADAM12 is a genetic modifier of murine peripheral arterial disease at least in part through regulation of endothelial cell function in ischemia.[34]

ADAM12 is a key modulator of PAD in mice and men

ADAM12 is a member of the metalloproteinase family of enzymes. A multi-domain, multifunctional, typically membrane-bound and zinc-dependent proteases.[36,37] There are two alternative splicing variants of human ADAM12 protein – L-form also known as the membrane bound form and S-form also known as the secreted form. ADAM12-L is an orthologue of mouse ADAM12, which is the variant of ADAM12 in mouse.[38,39] ADAM12-L is composed of N-terminal pro-domain, metalloproteinase domain, disintegrin domain, cysteine-rich domain, EGF-like domain, transmembrane region and cytoplasmic tail. The structural difference between the L-form and the S-form is the absence of the transmembrane region and cytoplasmic tail in the S-form.[40,41] ADAM12 has been shown to induce Epidermal Growth Factor Receptor EGFR (RTKs; Receptor Tyrosine Kinase) activation via proteolytic regulation of the ligand (HB-EGF to EGF) activation in epithelial cells.[42,43] Additionally, *in vitro* studies by Frohlich *et al.*,[44] showed ADAM12 regulates ectodomain shedding/cleavage of several endothelial cell membrane proteins including Tie2 and VEGFR2.[44] However, whether this regulation of membrane proteins could occur *in vivo* was not known. Recently, our group extended these studies to show that ADAM12 cleaves Tie2 in ischemic mouse hind limbs *in vivo*.[34] Moreover, we found no evidence of VEGFR2 cleavage by ADAM12 *in vivo*.[34] We showed ADAM12 regulation of endothelial cell proliferation, survival, and angiogenesis (tube formation) in ischemia is via Tie2 shedding and receptor activation.[34] These studies suggest that robust upregulation of ADAM12 in ischemia is a key mechanism involved in post ischemic angiogenesis and ADAM12 functions at least in part through Tie2 cleavage and activation.[34]

Regulation of ADAM12

ADAM12 expression is subject to multiple order of regulation by other proteins.[34,38,45] Notch signaling (i.e. activation of Notch receptor) regulates ADAM12 expression at both mRNA and protein levels, and this requires active NF-κB signaling cue in cultured mammalian cells.[38] Diaz *et al.*,[46] showed in human epithelial cancer cells that hypoxia-induced Notch signaling was responsible for increase in

ADAM12 expression. This study also went on to show that increase in HIF-1α expression (the master regulator of hypoxia) parallels increases in ADAM12 expression.[46] Moreover, our study showed ADAM12 upregulation in ischemia is regulated by HIF-1α.[34]

Going beyond protein regulation of ADAM12 in ischemia, recent, studies from our group provided one of the first evidence of microRNA regulation of ADAM12 *in vivo* in the setting of ischemia.[45] MicroRNAs are important post-transcriptional regulators of gene expression.[28,47–51] MiR29 family consists of three closely related members, namely miR29a, miR29b, and miR29c, and all three members have the same seed sequence.[38,52,53] Prior data from Li *et al.*,[38] and colleagues, had showed a regulatory link between miR29a and ADAM12 in fibroblasts *in vitro*;[38] however, less was known about ADAM12 regulation by miRNAs in ischemia. Recently, studies from our group showed using gain-of-function and loss-of-function approaches that ischemia-induced upregulation of ADAM12 expression is mediated through ischemia-induced suppression of miR29a.[45] This is the first study to establish miR29 regulation of ADAM12 as a critical part of the endothelial cell adaptation to ischemia. Moreover, it established a key role for this signaling cue in post ischemic angiogenesis and perfusion recovery in experimental PAD.[45] Lastly, our data further suggest that impaired perfusion recovery following vessel occlusion in diabetes may be due in part to hyperglycemia-related impaired downregulation of miR29a leading to suboptimal upregulation of ADAM12 in ischemia.[45] Taken together, the regulation of ADAM12 expression appears to involve a plethora of different regulatory tiers that includes ligands, receptors, and miRNA[43–45] (i.e. Notch signal axis, HIF-1α, NF-κB, miR29a).

Genetic variation in *ADAM12* is associated with CLI in a human cohort

To investigate the possible role of ADAM12 in PAD severity in humans, we assessed whether SNPs in *ADAM12* are associated with clinical outcomes in a cohort of humans with PAD (CATHGEN bio-repository).[54] In this cohort with 135 CLI and 544 IC individuals, 96 linkage disequilibrium (LD) tagged SNPs were genotyped and tested for association with CLI using multivariable logistic regression, corresponding to good (69% of SNPs with r2 of 0.7) coverage of the gene. Only one SNP, RS1471244, was strongly associated with CLI (odds ratio 2.4 95% CI [1.5–4.1]), $P = 0.0006$ for dominant model; odds ratio 2.2 [1.3–3.6], $P = 0.002$ for additive model), even after adjustment for the presence of diabetes mellitus, age, race, and smoking.[55] Based on these findings, targeting ADAM12 directly or at the level of miR29a offers a promising therapeutic option in the management of peripheral arterial disease.

IL-21Rα in endothelial cell adaptation to ischemia

A review of the result of our gene expression studies also identified IL-21Rα as a gene within LSq-1 that was more highly upregulated in the C57BL/6 mice (strain with good recovery phenotype) compared to Balb/c (strain with poor recovery phenotype) following experimental PAD.[34] IL-21Rα was originally discovered in immune cells, where it was reported to control lymphoid and myeloid cell proliferation and differentiation.[56,57] IL-21Rα is a cell membrane cytokine receptor that forms a heterodimeric receptor complex with the common cytokine receptor γ chain (γc).[56,57] The activation of IL-21Rα by its cognate ligand IL-21 is known to play an essential role in both innate and adaptive immune responses by regulating classical signaling pathways such as STAT3, ERK1/2 and AKT-1 (Protein Kinase B) signal axes, which in turn enhances cell survival and angiogenesis.[56,57]

To better understand the role of IL-21Rα in post ischemic angiogenesis, Wang *et al.*[58] explored in which cell type ADAM12 upregulation was occurring and found that the upregulation of IL-21Rα occurred primarily in endothelial cells. Moreover they showed *in vitro* treatment of ischemic endothelial cells with IL-21 increased proliferation, migration, and tube formation.[58] *In vivo* mice lacking IL-21Rα showed impaired perfusion recovery following experimental PAD.[58] IL-21Rα-mediated improved perfusion recovery is accomplished by preventing endothelial cell apoptosis, enhancing endothelial cell survival, and promoting angiogenesis via STAT3 signaling pathway and increased anti-apoptotic BCL-2/BAX ratio.[58]

The significance of IL-21Rα signaling was further investigated in humans with PAD. In good agreement with data from preclinical studies, Wang *et al.*[59] showed that the expression of IL-21Rα was higher in skeletal muscle endothelial cells from calf muscle biopsies in human patients with PAD compared to control subjects. In addition, blood samples from patients with PAD had higher levels of circulating IL-21 ligand compared to age and gender-matched healthy subjects. Taken together, the data from both preclinical and human studies suggest that the upregulation of IL-21 and its cognate receptor IL-21Rα, and activation of IL-21Rα indeed contributes to robust perfusion recovery and muscle adaptation to ischemic insult.

Beyond the endothelium, BAG3 is an LSq-1 gene involved in skeletal muscle adaptation to ischemia

Following the initial identification of the LSq-1 locus, we speculated that genes within this locus may impact perfusion recovery or skeletal muscle adaption to ischemia.[14] Recent studies have highlighted the notion that the preservation of both endothelial cells and skeletal muscle cells within the mouse hind limb is critical for tissue preservation and favorable perfusion recovery following experimental PAD.[31] Briefly, *in vitro* study showed that both murine primary skeletal muscle cells and endothelial cells were susceptible to simulated ischemia (hypoxia and nutrient deprivation or HND).[31] In addition, *in vivo* study showed that Balb/c strain exhibited greater skeletal muscle atrophy and apoptosis compared to C57BL/6 strain following hind-limb ischemia.[31]

We previously identified a Bcl-2-associated athanogene-3 (BAG3) as one of the 25-gene cohort located on LSq-1 of mouse chromosome 7, using haplotype block analysis.[34] BAG3 is known to play a significant role in skeletal muscle biology and myofiber integrity.[60–62] However, unlike the observed upregulation of ADAM12 in day 3 post ischemic mouse hind limbs, BAG3 was not found to be upregulated at this time point.[34] Note emphasis is on three days post-ischemia analysis. In our original LSq-1 gene expression studies, we used samples from day 3 post-ischemic hind limbs for gene expression analysis, because at this time point perfusion recovery was comparable among mouse strains (C57BL/6 vs. Balb/c) following induction of experimental PAD.[14,34]

McClung *et al.*[63] recently revisited the possible role of BAG3 in post ischemic perfusion recovery with a focus on skeletal muscle. In this study, the investigators explored expression of BAG3 mRNA at day 1, 2, and 3 post induction of experimental PAD and found greater upregulation in C57BL/6 strain than in Balb/c strain on day 1 and 2. Interestingly, the study detected the greatest increase in BAG3 mRNA expression (>3 fold) on day 1 post hind-limb ischemia with no detectable upregulation of BAG3 mRNA on day 3 post-induction of experimental PAD when compared to non-ischemic controls. This study went further to identify that C57BL/6 BAG3 is different from Balb/c BAG3 due to a point mutation at residue 81 (Isoleucine to Methionine substitution at residue 81; Iso81Met). Additionally, they showed the Iso81Met mutation contributed to impaired muscle regeneration and limb blood flow in Balb/c following experimental PAD.[63] Upregulation of BAG3 after hind-limb ischemia and expression of the functional BAG3 (Isoleucine 81) are both critical for the role of BAG3 in preventing ischemic limb necrosis (skeletal muscle atrophy) and rescuing limb blood flow. At the molecular level, BAG3 stimulates muscle regeneration by increasing myofiber differentiation and muscle precursor cell fusion in ischemia via autophagy regulation.[63]

Non-LSq-1 genes associated with PAD and PAD severity

PAD is the result of atherosclerosis in the lower extremities,[2,3,6] hence genes involved in the development of atherosclerosis are likely to be associated with severity of PAD. For example, increased concentrations of circulating inflammatory biomarkers such as C-reactive protein (CRP)[64–67] and Serum amyloid A (SAA)[67,68] have been reported to be associated with PAD severity and inversely correlates with ABI (reviewed in Hazarika *et al.*,[69]). Also, HMGB1 (High-mobility group box 1 protein) a transcriptional regulatory factor as well as an inflammatory mediator has been shown to be elevated in the plasma of patients with PAD compared to patients without PAD.[70] Additionally, osteopotegerin (OPG) a member of the tumor necrosis factor (TNF) receptor family has been implicated in vascular diseases and its levels were correlated with presence and severity of PAD.[70] Previously, work by Sachdev *et al.*[71] also showed that pre-treatment with anti-HMGB1 antibody increased muscle necrosis following HLI, and as a result the authors suggested that HMGB1 and TLR4 protect against muscle necrosis following HLI.[71] Beyond the involvement of genes associated with the pathogenesis of atherosclerosis and PAD severity, microRNAs such as miR93 have been shown to regulate mouse perfusion recovery following HLI.[28]

The above-mentioned genes may serve as biomarkers for PAD and PAD severity; however, less is known about whether these genes actually play key roles in adaptation to ischemia and possible molecular mechanisms involved. Additionally, whether polymorphism in these genes may play a role in PAD severity is not known.

Conclusion

Recent research advancements in the study of preclinical model of PAD (PAD) have uncovered a number of molecular pathways that are important in the outcome of PAD severity. The survival of both endothelial cells and skeletal muscle cells following hind-limb ischemia is critical for tissue preservation and perfusion recovery.[2,31,34,63] Current experimental evidence from preclinical model of PAD indicates that on one hand, ADAM12 and IL-21Rα are genetic modifiers of endothelial cell functions: cell proliferation, survival, and angiogenesis.[34,58] On the other hand, BAG3 is a genetic modifier of skeletal muscle fate and functions: muscle precursor cell differentiation and fusion.[63] There is also data showing polymorphism in ADAM12 is associated with CLI in humans with PAD.[34,55] IL-21Rα and IL-21 expression is higher in tissue samples from humans with PAD compared to the level in tissue from individuals without PAD.[59] Mutations in BAG3 gene are present in humans with muscular dystrophy.[61,72] These clinical observations are consistent with the findings in our preclinical studies, which show these LSq-1 genes play key roles in perfusion recovery and tissue preservation following experimental PAD.[34,45,58,63] Taken together, ADAM12 and IL-21Rα in endothelial cells, and BAG3 in skeletal muscle cells are potential therapeutic targets in the management of PAD in humans.

The identification of LSq-1 and our studies of some of the genes within this QTL has significantly improved our understanding of the pathophysiology of PAD severity. Nevertheless, our understanding of tissue adaptation to ischemia and the molecular mechanisms critical to limb survival following vessel occlusion is still quite limited. Future studies to explore the role of other LSq-1 genes in tissue adaptation to ischemia would likely be very informative. Findings from the current studies suggest manipulation of ADAM12, IL-21Rα, and BAG3 provide promise as therapeutic targets that may improve PAD outcomes in humans.

Authors' contributions: EO and AOD wrote and prepared the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

This work was supported by a Robert Wood Johnson Foundation, Harold Amos Medical Faculty Development Program award, NIH NHLBI, 3 R01 HL101200–01S1 and R01HL130399.

REFERENCES

1. Ouriel K. Peripheral arterial disease. *Lancet* 2001;**358**:1257–64
2. Dokun AO, Annex BH. Peripheral arterial disease. In: Huntington FW and Geoffrey G (eds) *Genomic and Personalized Medicine*. Cambridge, MA: Academic Press, 2009, pp.773–80
3. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FGR. Inter-society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg* 2007;**45**:S5–67
4. Fowkes FGR, Rudan D, Rudan I, Aboyans V, Denenberg JO, McDermott MM, Norman PE, Sampson UKA, Williams LJ, Mensah GA, Criqui MH. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. *Lancet* 2013;**382**:1329–40
5. Annex BH, Beller GA. Towards the development of novel therapeutics for peripheral artery disease. *Trans Am Clin Climatol Assoc* 2016;**127**:224–34
6. Fowkes FGR. Peripheral vascular disease: a public health perspective. *J Pub Health* 1990;**12**:152–9
7. Norgren L, Hiatt W, Dormandy J, Nehler M, Harris K, Fowkes F, Bell K, Caporusso J, Durand-Zaleski I, Komori K, Lammer J, Liapis C, Novo S, Razavi M, Robbs J, Schaper N, Shigematsu H, Sapoval M, White C, White J, Clement D, Creager M, Jaff M, Mohler E, Rutherford R, Sheehan P, Sillesen H, Rosenfield K, Group TIW. Inter-society consensus for the management of peripheral arterial disease (TASC II). *Eur J Vasc Endovasc Surg* 2007;**33**:S1–75
8. Aquino R, Johnnides C, Makaroun M, Whittle JC, Muluk VS, Kelley ME, Muluk SC. Natural history of claudication: long-term serial follow-up study of 1244 claudicants. *J Vasc Surg* 2001;**34**:962–70
9. Resnick HE, Rodriguez B, Havlik R, Ferrucci L, Foley D, Curb JD, Harris TB. Apo E genotype, diabetes, and peripheral arterial disease in older men: the Honolulu Asia-aging study. *Genet Epidemiol* 2000;**19**:52–63
10. Hirsch A, Haskal Z, Hertzer N, Bakal C, Creager M, Halperin J. ACC/AHA 2005 Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): executive summary a collaborative report from the American Association for vascular surgery/society for vascular surgery, society for cardiovascular angiography and interventions, society for vascular medicine and biology, society of interventional radiology, and the ACC/AHA task force on practice guidelines (writing committee to develop guidelines for the management of patients with peripheral arterial disease) endorsed by the American Association of cardiovascular and pulmonary rehabilitation; national heart, lung, and blood institute; society for vascular nursing; transatlantic inter-society consensus; and vascular disease foundation. *J Am Coll Cardiol* 2006;**47**:1239–312
11. Wattanakit K, Folsom AR, Selvin E, Weatherley BD, Pankow JS, Brancati FL, Hirsch AT. Risk factors for peripheral arterial disease incidence in persons with diabetes: the Atherosclerosis Risk in Communities (ARIC) Study. *Atherosclerosis* 2005;**180**:389–97
12. Rajagopalan S, Mohler ER, III, Lederman RJ, Mendelsohn FO, Saucedo JF, Goldman CK, Blebea J, Macko J, Kessler PD, Rasmussen HS, Annex BH. Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation* 2003;**108**:1933–8
13. Lederman RJ, Mendelsohn FO, Anderson RD, Saucedo JF, Tenaglia AN, Hermiller JB, Hillegass WB, Rocha-Singh K, Moon TE, Whitehouse MJ, Annex BH. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. *Lancet* 2002;**359**:2053–8
14. Dokun AO, Keum S, Hazarika S, Li Y, Lamonte GM, Wheeler F, Marchuk DA, Annex BH. A quantitative trait locus (LSq-1) on mouse chromosome 7 is linked to the absence of tissue loss after surgical hindlimb ischemia. *Circulation* 2008;**117**:1207–15
15. Flex A, Gaetani E, Pola R, Santoliquido A, Aloi F, Papaleo P, Dal Lago A, Pola E, Serricchio M, Tondi P, Pola P. The −174 G/C polymorphism of the interleukin-6 gene promoter is associated with peripheral artery occlusive disease. *Eur J Vasc Endovasc Surg* 2002;**24**:264–8
16. Lane DA, Grant PJ. Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. *Blood* 2000;**95**:1517–32
17. Monsalve MV, Robinson D, Woolcock NE, Powell JT, Greenhalgh RM, Humphries SE. Within-individual variation in serum cholesterol levels: association with DNA polymorphisms at the apolipoprotein B and AI-CIII-AIV loci in patients with peripheral arterial disease. *Clin Genet* 1991;**39**:260–73
18. Dokun A, Annex BH. *Genetic polymorphisms in peripheral arterial disease, role of genomic methodologies*. USA: Elsevier, 2009
19. Annex KABAODaB. Genomics and molecular based therapy in peripheral arterial disease. In: Huntington W and Geoffrey SG (eds) *Genomics and personalized medicine*. 2nd ed. USA: Elsevier, 2012, p.2
20. Libby P, Ridker PM, Hansson GK. Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol* 2009;**54**:2129–38
21. Leeper NJ, Kullo IJ, Cooke JP. Genetics of peripheral artery disease. *Circulation* 2012;**125**:3220–8
22. Couffinhal T, Silver M, Zheng L, Kearney M, Witzenbichler B, Isner J. Mouse model of angiogenesis. *Am J Pathol* 1998;**152**:1667–79
23. Waters RE, Terjung RL, Peters KG, Annex BH. Preclinical models of human peripheral arterial occlusive disease: implications for investigation of therapeutic agents. *J Appl Physiol* 2004;**97**:773–80
24. Hazarika S, Dokun AO, Li Y, Popel AS, Kontos CD, Annex BH. Impaired angiogenesis after hindlimb ischemia in type 2 diabetes mellitus: differential regulation of vascular endothelial growth factor receptor 1 and soluble vascular endothelial growth factor receptor 1. *Circ Res* 2007;**101**:948–56
25. Dokun AO, Chen L, Lanjewar SS, Lye RJ, Annex BH. Glycaemic control improves perfusion recovery and VEGFR2 protein expression in diabetic mice following experimental PAD. *Cardiovasc Res* 2014;**101**:364–72
26. Park B, Hoffman A, Yang Y, Yan J, Tie G, Bagshahi H, Nowicki PT, Messina LM. Endothelial nitric oxide synthase affects both early and late collateral arterial adaptation and blood flow recovery after induction of hind limb ischemia in mice. *J Vasc Surg* 2010;**51**:165–73
27. Fukino K, Sata M, Seko Y, Hirata Y, Nagai R. Genetic background influences therapeutic effectiveness of VEGF. *Biochem Biophys Res Commun* 2003;**310**:143–7
28. Hazarika S, Farber CR, Dokun AO, Pitsillides AN, Wang T, Lye RJ, Annex BH. MicroRNA-93 controls perfusion recovery after hindlimb ischemia by modulating expression of multiple genes in the cell cycle pathway. *Circulation* 2013;**127**:1818–28
29. Scholz D, Ziegelhoeffer T, Helisch A, Wagner S, Friedrich C, Podzuweit T, Schaper W. Contribution of arteriogenesis and angiogenesis to post-occlusive hindlimb perfusion in mice. *J Mol Cell Cardiol* 2002;**34**:775–87
30. Katwal A, Dokun A. Peripheral arterial disease in diabetes: is there a role for genetics? *Curr Diab Rep* 2011;**11**:218–25
31. McClung JM, McCord TJ, Keum S, Johnson S, Annex BH, Marchuk DA, Kontos CD. Skeletal muscle specific genetic determinants contribute to the differential strain-dependent effects of hindlimb ischemia in mice. *Am J Pathol* 2012;**180**:2156–69
32. Li Y, Hazarika S, Xie D, Pippen AM, Kontos CD, Annex BH. In mice with type 2 diabetes, a vascular endothelial growth factor (VEGF)-activating transcription factor modulates VEGF signaling and

induces therapeutic angiogenesis after hindlimb ischemia. *Diabetes* 2007;**56**:656–65
33. Helisch A, Wagner S, Khan N, Drinane M, Wolfram S, Heil M, Ziegelhoeffer T, Brandt U, Pearlman JD, Swartz HM, Schaper W. Impact of mouse strain differences in innate hindlimb collateral vasculature. *Arterioscler Thromb Vasc Biol* 2006;**26**:520–6
34. Dokun AO, Chen L, Okutsu M, Farber CR, Hazarika S, Jones WS, Craig D, Marchuk DA, Lye RJ, Shah SH, Annex BH. ADAM12: a genetic modifier of preclinical peripheral arterial disease. *Am J Physiol* 2015;**309**:H790–803
35. Keum S, Marchuk DA. A locus mapping to mouse chromosome 7 determines infarct volume in a mouse model of ischemic stroke/clinical perspective. *Circulation* 2009;**2**:591–8
36. Kveiborg M, Albrechtsen R, Couchman JR, Wewer UM. Cellular roles of ADAM12 in health and disease. *Int J Biochem Cell Biol* 2008;**40**:1685–702
37. Nyren-Erickson EK, Jones JM, Srivastava DK, Mallik S. A disintegrin and metalloproteinase-12 (ADAM12): function, roles in disease progression, and clinical implications. *Biochim Biophys Acta* 2013;**1830**:4445–55
38. Li H, Solomon E, Duhachek Muggy S, Sun D, Zolkiewska A. metalloprotease-disintegrin ADAM12 expression is regulated by notch signaling via MicroRNA-29. *J Biol Chem* 2011;**286**:21500–10
39. Wewer UM, Albrechtsen R, Engvall E. ADAM12. In: Hooper NM and Lendeckel U (eds) *The ADAM Family of Proteases. Proteases in Biology and Disease, vol 4*. Boston, MA: Springer, 2005, pp.123–46
40. Duffy MJ, McKiernan E, Donovan N, McGowan PM. Role of ADAMs in cancer formation and progression. *Clin Cancer Res* 2009;**15**:1140
41. Duhachek-Muggy S, Zolkiewska A. ADAM12-L is a direct target of the miR-29 and miR-200 families in breast cancer. *BMC Cancer* 2015;**15**:93
42. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat Med* 2002;**8**:35–40
43. Diaz B, Yuen A, Iizuka S, Higashiyama S, Courtneidge SA. Notch increases the shedding of HB-EGF by ADAM12 to potentiate invadopodia formation in hypoxia. *J Cell Biol* 2013;**201**:279–92
44. Frohlich C, Klitgaard M, Noer JB, Kotzsch A, Nehammer C, Kronqvist P, Berthelsen J, Blobel C, Kveiborg M, Albrechtsen R, Wewer UM. ADAM12 is expressed in the tumour vasculature and mediates ectodomain shedding of several membrane-anchored endothelial proteins. *Biochem J* 2013;**452**:97–109
45. Chen L, Okeke E, Ayalew D, Wang D, Shahid L, Dokun AO. Modulation of miR29a improves impaired post-ischemic angiogenesis in hyperglycemia. *Exp Biol Med (Maywood)*. 2017;**242**:1432–43.
46. Diaz B, Yuen A, Iizuka S, Higashiyama S, Courtneidge SA. Notch increases the shedding of HB-EGF by ADAM12 to potentiate invadopodia formation in hypoxia. *J Cell Biol* 2013;**201**:279–92
47. Ambros V. The functions of animal microRNAs. *Nature* 2004;**431**:350–5
48. Chen Z, Lai T-C, Jan Y-H, Lin F-M, Wang W-C, Xiao H, Wang Y-T, Sun W, Cui X, Li Y-S, Fang T, Zhao H, Padmanabhan C, Sun R, Wang DL, Jin H, Chau G-Y, Huang H-D, Hsiao M, Shyy JYJ. Hypoxia-responsive miRNAs target argonaute 1 to promote angiogenesis. *J Clin Invest* 2013;**123**:1057–67
49. Chen X, Liang H, Zhang J, Zen K, Zhang C-Y. Secreted microRNAs: a new form of intercellular communication. *Trends Cell Biol* **22**:125–32
50. Chamorro-Jorganes A, Araldi E, Penalva LOF, Sandhu D, FernÃ ¡Ndez-Hernando C, SuÃ ¡, Rez Y. MicroRNA-16 and MicroRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. *Arterios Thromb Vasc Biol* 2011;**31**:2595–606
51. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, Chavakis E, Potente M, Tjwa M, Urbich C, Zeiher AM, Dimmeler S. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science* 2009;**324**:1710–3
52. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* 2010;**79**:351–79
53. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiol Genom* 2012;**44**:237–44
54. Shah SH, Bain JR, Muehlbauer MJ, Stevens RD, Crosslin DR, Haynes C, Dungan J, Newby LK, Hauser ER, Ginsburg GS, Newgard CB, Kraus WE. Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events: clinical perspective. *Circulation* 2010;**3**:207
55. Dokun Aoo M, Craig D, Shah SH, Jones WS, Marchuk DA, Lye RJ, Annex BH. *ADAM12 modifies severity of periperal arterial disease; evidence from preclinical and human studies*. Paper presented at American Heart Association Scientific Session, 2013
56. Fröhlich A, Marsland BJ, Sonderegger I, Kurrer M, Hodge MR, Harris NL, Kopf M. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. *Blood* 2007;**109**:2023
57. Leonard WJ, Wan C-K. IL-21 Signaling in immunity. *F1000Res* 2016;**5**: F1000 Faculty Rev-224
58. Wang T, Cunningham A, Dokun AO, Hazarika S, Houston K, Chen L, Lye RJ, Spolski R, Leonard WJ, Annex BH. Loss of interleukin-21 receptor activation in hypoxic endothelial cells impairs perfusion recovery after hindlimb ischemia. *Arterios Thromb Vasc Biol* 2015;**35**:1218–25
59. Wang T, Cunningham A, Houston K, Sharma AM, Chen L, Dokun AO, Lye RJ, Spolski R, Leonard WJ, Annex BH. Endothelial interleukin-21 receptor up-regulation in peripheral artery disease. *Vasc Med* 2016;**21**:99–104
60. Kostera-Pruszczyk A, Suszek M, Płoski R, Franaszczyk M, Potulska-Chromik A, Pruszczyk P, Sadurska E, Karolczak J, Kamińska AM, Rędowicz MJ. BAG3-related myopathy, polyneuropathy and cardiomyopathy with long QT syndrome. *J Muscle Res Cell Motil* 2015;**36**:423–32
61. Selcen D, Muntoni F, Burton BK, Md EP, Sewry C, Bite AV, Engel AG. Mutation in BAG3 causes severe dominant childhood muscular dystrophy. *Ann Neurol* 2009;**65**:83–9
62. Hishiya A, Kitazawa T, Takayama S. BAG3 and Hsc70 interact with actin capping protein CapZ to maintain myofibrillar integrity under mechanical stress. *Circ Res* 2010;**107**:1220–31
63. McClung JM, McCord TJ, Ryan TE, Schmidt CA, Green TD, Southerland KW, Reinardy JL, Mueller SB, Venkatraman TN, Lascola CD, Keum S, Marchuk DA, Spangenburg EE, Dokun AO, Annex BH, Kontos CD. A BAG3 coding variant in mice determines susceptibility to ischemic limb muscle myopathy by directing autophagy. *Circulation* 2017;**136**:281–96
64. Pasceri V, Willerson JT, Yeh ETH. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* 2000;**102**:2165
65. McDermott MM, Lloyd-Jones DM. The role of biomarkers and genetics in peripheral arterial disease. *J Am Coll Cardiol* 2009;**54**:1228–37
66. Hogh AL, Joensen J, Lindholt JS, Jacobsen MR, Ostergaard L. C-Reactive protein predicts future arterial and cardiovascular events in patients with symptomatic peripheral arterial disease. *Vasc Endovasc Surg* 2008;**42**:341–7
67. Maksimovic M, Vlajinac H, Radak D, Marinkovic J, Jorga J. Relationship between high-sensitivity C-reactive protein and risk factors in patients with peripheral arterial disease – a cross-sectional study. *Angiology* 2012;**64**:230–6
68. Vidula H, Tian L, Liu K, Criqui MH, Ferrucci L, Pearce WH, Greenland P, Green D, Tan J, Garside DB, Guralnik J, Ridker PM, Rifai N, McDermott MM. Biomarkers of inflammation and thrombosis as predictors of near-term mortality in patients with peripheral arterial disease: a cohort study. *Ann Intern Med* 2008;**148**:85–93
69. Hazarika S, Annex BH. Biomarkers and genetics in peripheral artery disease. *Clin Chem* 2017;**63**:236–44
70. Giovannini S, Tinelli G, Biscetti F, Straface G, Angelini F, Pitocco D, Mucci L, Landolfi R, Flex A. Serum high mobility group box-1 and osteoprotegerin levels are associated with peripheral arterial disease

and critical limb ischemia in type 2 diabetic subjects. *Cardiovasc Diabetol* 2017;**16**:99

71. Sachdev U, Cui X, Tzeng E. HMGB1 and TLR4 mediate skeletal muscle recovery in a murine model of hindlimb ischemia. *J Vasc Surg* 2013;**58**:460–9
72. Odgerel Z, Sarkozy A, Lee H-S, McKenna C, Rankin J, Straub V, Lochmüller H, Paola F, D'amico A, Bertini E, Bushby K, Goldfarb LG. Inheritance patterns and phenotypic features of myofibrillar myopathy associated with a BAG3 mutation. *Neuromusc Disord* 2010;**20**:438–42