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Genetics of Peripheral Artery Disease

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The importance of peripheral arterial diseases

Broadly defined, peripheral vascular disease refers to disease of the extra-cardiac blood vessels, including diseases of the arteries, veins and lymphatics. Peripheral arterial disease (PAD) refers to disease affecting non-coronary arteries, but is most often used to describe disease of the arteries supplying the limbs. Peripheral arterial disease is most commonly due to atherosclerosis, but may also be secondary to cardiac or vascular embolism, vasculitis, hypercoagulopathy, vascular dissection, vascular compression syndromes, and other less common disorders. In addition, peripheral arterial diseases include those characterized by fixed or dynamic stenoses, as well as aneurysmal disease such as abdominal aortic aneurysm (AAA). In this review, we focus on atherosclerotic arterial occlusive disease affecting the vessels supplying blood flow to the lower extremities (PAD), and discuss our current understanding and the future directions of PAD genetics.

PAD is a significant public health problem, and a major source of morbidity and mortality that affects approximately 8 million Americans. PAD contributes to impaired quality of life (eg. intermittent claudication reducing mobility), morbidity (eg. non-healing ulcers and ischemic rest pain) and mortality (generally due to its association with coronary and carotid artery disease). PAD is responsible for approximately half a million hospitalizations and 100,000 angiograms annually [1, 2]. Due in part to a general unfamiliarity with these diseases amongst the primary care community, PAD patients receive suboptimal treatment compared to patients with coronary artery disease (CAD), being prescribed therapeutic doses of statins, anti-hypertensive medicines, and antiplatelet agents less commonly than patients with CAD [3-6]. Much remains unknown about the biological origins of this disease and how to effectively identify and treat affected individuals.

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Using genetics to identify pathophysiological pathways and novel therapeutic targets

A greater understanding of how genetic variation influences susceptibility to PAD may inform the development of novel therapeutics. High-throughput, whole-genome technologies efforts have recently made inroads toward these goals (Table). The advent of genome-wide association studies (GWAS, discussed below) and cDNA microarrays (which measure the mRNA expression levels of all coding genes) has allowed for the unbiased detection of pathways which are differentially activated in affected versus unaffected individuals. Whereas these are the contemporary tools now driving genetic investigations of PAD, it is important to first put this recent work into a historical context.

Earlier studies documenting the heritability of PAD

PAD is a complex disorder from a genetic standpoint. Unlike monogenic vascular syndromes such as Marfan and Loey's Dietz that manifest a Mendelian inheritance pattern[14], atherosclerotic PAD likely results from dozens or hundreds of genes interacting with each other and the environment to cause disease[7]. Epidemiological studies suggest that over 50% of the population burden of PAD is attributable to classical risk factors such as smoking, diabetes mellitus, dyslipidemia and hypertension[15]. The remaining risk is thus accounted for by other unmeasured environmental or genetic components.

Several studies indicate a heritable component to PAD. In a study of patients with premature PAD (onset before age 50), half of the subjects' asymptomatic siblings had occult lower extremity atherosclerosis as determined by duplex ultrasonography[16]. A recent Swedish twin study[17]used discharge diagnosis to define PAD, and is likely more representative of patients with symptomatic claudication or critical limb ischemia. Genetic effects accounted for 58% (95% CI, 50% to 64%) and non-shared environmental effects for 42% (95% CI, 36% to 50%) of the phenotypic variance among twins in this report. Three studies conducted to date estimate that 21% of the inter-individual variation in ABI is attributable to inherited factors. These studies include the National Heart, Lung, and Blood Institute (NHLBI) Twin Study[18], the Genetic Epidemiology Network Arteriopathy Study (GENOA)[19], and a study performed in the Framingham Offspring Cohort[20]. Many of the participants in these studies had ABI values in or near the reference range. While sibling studies frequently overestimate attributable genetic risk because of shared environmental risk factors within families[21], these reports each provided evidence that ABI is at least moderately inheritable, and justify the search for responsible gene variants. Historical methods included case-control approaches and linkage analyses.

Earlier studies to identify genetic determinants of PAD

Candidate Gene Studies

Using the candidate gene approach, one searches for an association between a specific variant in a specific gene (eg. a single nucleotide polymorphism, or SNP) and a clinical phenotype (generally defined by a low ABI in PAD patients). Such polymorphisms may alter a gene's expression by altering the binding of its required transcription factors, impairing the stability or intracellular trafficking of its mRNA transcript, or limiting its ability to be translated into a functional protein. Often, the polymorphism may be linked to another gene that is responsible for the disease. Collections of cases and controls are assembled and genotyped for variants in a suspected pathway [22]. Driven by our understanding of atherosclerotic disease, most efforts thus far have focused on genes that are known or suspected to be related to modulating lipids, blood pressure, vasomotor tone, inflammation or thrombosis. Association studies have included genes related to coagulation

and platelet aggregation (prothrombin, Factor V Leiden), leukocyte activation (IL-6, E-Selectin, ICAM), and endothelial function (NOS3), amongst others (reviewed in [7, 8, 23]).

Unfortunately, these studies have shed little light onto the pathophysiology of PAD. Plagued by small sample sizes and inadequate statistical power, many of the originally positive studies have not been successfully replicated in independent cohorts, suggesting the original association was falsely positive [24]. Concerns over inadequate matching of racial/ethnic groups (who are known to have different rates of PAD as well as different allele frequencies) are justified by the possibility that 'population stratification' may lead to spurious associations [25]. Moreover, a significant proportion of the candidate gene studies reported to date do not conform to Hardy-Weinberg equilibrium (i.e. the genotype was not distributed across the population as predicted by classical genetics) suggesting systematic genotyping errors or selection bias [23]. Perhaps most concerning, a number of these studies appear to have been un-blinded, additionally casting doubt on their conclusions.

Taken together, these candidate gene studies are inconclusive. Certain variants that have been identified appear promising and warrant additional investigation, such as polymorphisms in the homocysteine pathway regulating enzyme MTHFR[26-29], the inflammatory cytokine IL-6 [30, 31], and the vascular adhesion molecule ICAM[30, 32]. Finally, a major limitation of candidate gene studies is that they are not likely to uncover novel mechanisms, as the choice of gene variants is typically determined by previous observations indicating a putative role for the gene in atherosclerosis.

Linkage Studies

Family-based linkage studies have also been applied to understanding vascular diseases [33]. In this approach, the genome is scanned at a low resolution (every 10 centimorgans) for markers known as microsatellites that are co-inherited with the phenotype of interest (typically several million base pairs between tags). Once a marker has been firmly associated within the affected members of the study families, the surrounding region of the genome is fine-mapped to identify the causal gene which is 'linked' to the microsatellite and the disease. This method is conceptually superior to the candidate gene approach in that it does not require an a priori hypothesis about which gene is responsible for the disease and it scans the entire genome. This approach has been powerful in a number of Mendelian diseases, both vascular (e.g. *NOTCH3* signaling in the autosomal dominant stroke syndrome, CADASIL[34, 35]) and otherwise (e.g. sarcomeric proteins in hypertrophic cardiomyopathy [36]).

In the realm of PAD, however, the results have been somewhat disappointing. To date, only one positive linkage study has been reported. This study of 116 extended Icelandic families (884 subjects) identified a locus on chromosome 1p31 that was associated with angiographically or surgically documented PAD [9]. This locus, known as PAOD1, had a logarithm of odds (LOD) score of 3.93 (> 3 is significant). Moreover, this locus was independent of other vascular risk factors and the association was strengthened when stroke patients were removed. Together, these findings suggest that the associated gene may specifically predispose patients to vascular disease in the lower extremity vascular bed. Unfortunately, the causative gene on Chromosome 1 has not yet been identified and this association has not been replicated by other investigators. The only other sizable linkage study performed to date, which utilized only ~ 1/3 the number of microsatellite makers studied in the preceding study, failed to definitively identify a significant genomic locus [19].

To be effective, linkage studies require extended family pedigrees and genes with large effect sizes [37]. As PAD tends to affect older adults, it is difficult to compile large collections of affected families. Further, this disease is polygenic and results from a number of factors

each with a modest effect on risk, rather than one dominant gene that will be easily detected. Together, it is not surprising that this approach has not been met with more success in PAD.

GWAS Studies

Recent major advances in human genetics promise to overcome each of these deficits [38]. In 2001, the Human Genome Project was completed, fully codifying the 3.1 billion nucleotides that make up our genetic code. Perhaps even more importantly, the International HapMap project provided a 'catalog' of common polymorphisms across the genome in 2004, allowing us for the first time to study the natural variation that makes each individual unique. These tools, combined with technological advances that have enabled the relatively inexpensive genotyping of millions of 'tag' SNPs simultaneously, have revolutionized modern genomics research. Employing the genome-wide association study (GWAS) approach, researchers can now scan the full genomes of large cohorts of patients to identify variants that are over-represented in subjects with a given disease compared to unaffected controls (see review in [38, 39]). Unlike the candidate gene approach, the GWAS platform allows for an agnostic approach where no prior knowledge is required to implicate novel biological pathways [40]. Further, the genome-wide approach allows for the consideration of polymorphisms in so-called 'gene-deserts' that do not encode any of the known ~20,000 protein coding genes. These areas may contain non-coding elements that can modify gene expression, such as long-noncoding RNAs.

Unlike conventional linkage analysis using microsatellites, the GWAS technology scans the genome at much higher resolution and with greater fidelity. Moreover, late-onset diseases with high mortality rates (such as PAD) can be studied without the need for extended family pedigrees, and genes with modest effect size (OR of 1.2) can be detected. One drawback is that this approach requires large numbers of subjects (thousands), and because of multiple comparisons, the level of significance must be very high for a variant to be reliably associated with disease. Nevertheless, this approach has already provided revolutionary insights in several fields, as typified by the implication of the complement pathway in macular degeneration, autophagy in Crohn's disease, and hedgehog signaling in human height [41-43].

Since 2006, multiple loci have been definitively associated with cardiovascular disease[44]. These lead SNPs have been replicated by several consortia in multiple racial ethnic groups. The strongest and most consistent association with cardiovascular disorders is with the intergenic portion of Chromosome 9p21 ($p = 1.6 \times 10^{-25}$) [11], which has been queried in well over 100,000 patients [45, 46]. Variants at this locus have been reported to be responsible for as much as 21% of one's lifetime risk of myocardial infarction (MI) [47]. Importantly, these same polymorphisms also correlate with risk in the periphery, including aneurysmal disease, stroke and arterial stiffness [12, 48]. The link to peripheral vascular disorders is most robust for AAA and intracranial aneurysms (accounting for 26% of the attributable risk for these diseases), and persists even after removing patients with a history of MI. A follow up study on the representative 9p21 SNP rs1333049supported this association with PAD status as well as severity of ABI in a cohort of older individuals (mean age 76) [49]. The association persisted after controlling for diabetes, smoking, lipid levels, prior MI and hypertension, suggesting a novel pathophysiological mechanism at play.

The fact that the 9p21 SNPs correlate with disease status independently of known traditional risk factors has increased the enthusiasm for understanding the biology mediated by these SNPs. It is notable that these same SNPs also predict risk of the intracranial non-atherosclerotic berry aneurysm [12]. Taken together, we and others have postulated that 9p21 likely does not alter vessel wall inflammation, thrombosis or lipid accumulation- but rather regulates the structural integrity of the artery itself. Given the particularly strong link to

aneurysmal disorders, it is likely that the dominant role of 9p21 variants *in vivo* will center around vascular smooth muscle cell physiology and cell-fate decision making [50].

While much work has focused on the 9p21 hits described above, it is worth highlighting that a number of other GWAS polymorphisms have been implicated as significant at the genome-wide level. As the majority of these localize to genes not previously implicated in vascular disease, they certainly warrant investigation and should also yield novel biological targets. For example, one of the most exciting leads comes from a recent GWAS which found an associated SNP within a cluster of genes that encode nicotinic acetylcholine (nACh) receptors [13]. This variant not only correlated with PAD (10% of the attributable risk for disease), but also predicted nicotine dependence and number of cigarettes smoked per day. It may be that the link to PAD occurs indirectly (i.e. by increasing lifetime smoking burden) or possibly by directly modulating the effect of tobacco on the vasculature. In this regard, we have shown that nicotine can directly accelerate plaque neovascularization and atherosclerosis by stimulating vascular nAChreceptors [51, 52].

Whereas the genetic variations discovered by GWAS have been predictive of CAD as well as PAD, it is likely that genetic variations that are more specific for PAD ultimately will be uncovered. Indeed, it is already apparent that the conventional risk factors have different predictive value for PAD, with tobacco exposure and diabetes mellitus being stronger risk factors for PAD than is dyslipidemia [53, 54]. Consistent with these differences in pathophysiological determinants is the observation of a different proteomic profile in patients with PAD and CAD (PAD/CAD), than those patients with CAD alone. Specifically, beta 2 microglobulin, cystatin C and C-reactive protein are each found in higher levels in PAD/CAD, and can be used together to stratify the risk of PAD in a susceptible population [55, 56].

Use of genetics in PAD detection and treatment

A greater understanding of the genetic underpinnings of PAD could enhance our capacity to detect those at risk for disease. PAD is underdiagnosed, and these patients are not receiving medication known to reduce morbidity and mortality. Only 10-30% of PAD patients manifest the ‘classic’ symptom of intermittent claudication [3, 57]. While the ABI test, which measures the ratio of blood pressure in the lower and upper extremities, is a simple and useful office-based technique to detect PAD [58], evidence indicates that this test is underutilized. In fact, the PARTNERS (Peripheral Arterial Disease Detection, Awareness, and Treatment in Primary Care) ABI screening study of nearly 7,000 adult general medicine patients found that over half of those with PAD had been previously undetected [3]. Even when widely implemented, the ABI does not correlate with functional status, and only poorly with disease progression [59, 60]. A blood test that could identify those at greatest risk for PAD could focus additional screening toward those populations.

Currently, genetic screening to assess the risk or progression of PAD is far from being realized. However, the possibility of such screening in the future is foreshadowed by recent advances in cardiac transplantation. In cardiac transplant recipients, the AlloMap test (which measures the expression of 11 genes in peripheral blood samples) has been shown to accurately predict allograft rejection [61]. This blood test has the potential of replacing the current screening methodology which is invasive and expensive (ie. frequent endomyocardial biopsies and echocardiograms). Clearly, standardized, off-the-shelf blood tests that will obviate the need for technical imaging studies would greatly enhance our ability to rapidly identify at-risk individuals, initiate therapies early in the course of disease, and prognosticate with greater accuracy. Given the fact that as many as 60 million

Americans technically meet the guideline criteria for a screening ABI, it is clear that an effective PAD panel could focus health care resources on those most at risk [55].

More powerful tools, that will arise from our forthcoming ability to rapidly and cheaply sequence the entire genome, will certainly provide further insights, point out additional targets and allow for therapies tailored to an individual's personal genetic makeup. To this final point, the field of pharmacogenomics, which studies the role of genetic differences in the response to drug therapy, has been rapidly expanding. A clear example relevant to PAD comes from the discovery that carriers of the common loss-of-function cytochrome p-450 enzyme polymorphism, *CYP2C19*, metabolize clopidogrel (the most efficacious antiplatelet drug in PAD [62]) significantly more slowly than unaffected controls (reviewed in [63]). These patients have reduced platelet inhibition on standard clopidogrel doses and experience a higher risk of major adverse cardiovascular events. As such, the FDA has provided a “black-box” warning for this agent in so-called “poor-metabolizers”. We do not yet know if higher doses of clopidogrel will reduce the risk of future event (without increasing bleeding). However, it is clear that clinicians will soon need to become facile with the concept of pharmacogenomics when choosing a drug for a particular patient [64]. Before long, we anticipate that a patient's genetic variations will be documented and will factor into drug selection similarly to other current considerations, such as age, weight and concurrent medications.

The future of PAD genetics

Gene-by-environment interactions

Gene-by-environment (GxE) interactions are hypothesized to play an important role in the expression of disease, and have spurred investigations into the pathophysiological synergy of genomic and environmental exposures. Recently, this topic was approached with the first Environment-Wide Association Study (“EWAS”) which identified the pesticide heptachlor epoxide as being associated with type II diabetes [65]. Application of similar ‘environment-wide’ scans will likely also be useful in PAD.

Epigenetic factors

Additionally, we will become more sophisticated in our evaluation of noncoding RNAs and other epigenetic modifications that promote peripheral arterial disease. The microRNAs are diffusible ~ 22 nucleotide single-stranded RNAs that target coding mRNA transcripts for degradation [66]. Though discovered less than a decade ago, they are now known to regulate upwards of 50% of the entire genome. This remarkable pathway, which consists of only ~ 1000 genes, has already been implicated in a number of pathways relevant to PAD including impaired diabetic neoangiogenesis, smooth muscle remodeling, and circulating endothelial progenitor cell number [67-70]. The advent of microarrays that can analyze the small RNA subfraction will certainly identify other microRNAs which are relevant to peripheral arterial disease.

Beyond microRNAs, tools now also exist to measure other epigenetic factors in PAD, such as histone modification, chromatin remodeling and DNA methylation [71]. These processes induce structural modifications to the DNA molecule, rather than alterations in the DNA sequence, itself. Changes in DNA methylation or chromatin modifications make a given gene more or less amenable to transcription, and thus can alter the expression of that gene. Epigenetic alterations may occur postnatally and reproducibly localize to certain regions of the chromosome in disease. Detecting these patterns can provide insights that will implicate causative genes. It is possible that epigenetic ‘signatures’ can even be inherited across families, and that traits acquired due to environmental exposure can be passed to offspring to contribute to the familial concentration of disease. While most of the cardiovascular

epigenetic research completed to date has focused on pathways related generally to atherosclerosis and vascular biology [72], interesting studies are forthcoming in AAA disease [73] and should also prove informative in PAD.

Acquired mitochondrial genetic alterations

There is accumulating evidence for a mitochondriopathy in PAD that contributes to the impairment in functional capacity[74]. The mitochondriopathy may be due in part to acquired alterations in mitochondrial DNA (mtDNA). In the patient with PAD, intermittent claudication is associated with ischemia-reperfusion, a known stimulus for generation of reactive oxygen species[75]. Regular bouts of ischemia-reperfusion may damage mtDNA, which is particularly vulnerable due to its proximity to reactive oxygen species generated by the electron transport chain (ETC), and because of its lack of protective histones. Indeed, by comparison to limbs of age-matched subjects without PAD, the skeletal muscle mitochondria in the most affected limb of patients with PAD have almost 20-fold greater frequency of the 4977-bpmtDNA deletion[76].These mitochondrial genetic abnormalities may explain in part the mitochondriopathy of PAD. Notably, the only ETC protein activity which seems unaffected in PAD skeletal muscle is that of complex II, which is encoded by nuclear, rather than mitochondrial, DNA[74]. However, similar mtDNA alterations can be seen in the unaffected limb in patients with unilateral PAD, suggesting that systemic oxidative stress or other factors may be contributing to the mtDNA abnormalities in PAD[77]. In any event, medical therapy to reduce mitochondrial injury or enhance mitochondrial function may represent an interesting therapeutic avenue in PAD[78].

Comparative genomics

Known differences in susceptibility to hindlimb ischemia between mouse strains are also now being exploited to advance our understanding of peripheral arterial disease and angiogenesis. Using an elegant comparative genomics approach, Dokun and colleagues performed a linkage analysis on mice from 6 genetic backgrounds that had undergone femoral artery ligation [10]. They identified a quantitative trait locus on chromosome 7 that influences wound healing in situations of tissue hypoxia and critical limb ischemia. Other investigators have found that this same locus also appears to control over 50% of the variability in infarct burden in a mouse model of ischemic stroke [79]. While the molecular mechanisms downstream of this region have yet to be defined, this example highlights the power of leveraging the natural differences that occur due to genetic variation between inbred animal lineages.

Exomic and whole genome sequencing

The most important advances, however, are likely to occur to as a result of the even more powerful genetic tools on the horizon. While the GWAS platform has provided new insights that will reshape our approach to PAD genetics, this tool is not without limitations [38, 80, 81]. High-density genotyping chips now can identify up to 2.5 million individual markers per subject, but rely on imputation to predict the remaining SNPs in our genome. Rare, ‘personal’ variants (i.e. occurring with a frequency of less than 1% across the population) are not accurately cataloged for consideration with the GWAS approach. Moreover, historical GWAS genotyping has had little if any power to detect so-called structural variants such as insertions, deletions and copy number variants - all of which have been implicated in vascular disease. The advent of exome and truly full genome sequencing, where all existing nucleotides are codified, will address these limitations. Companies such as Complete Genomics and Illumina now provide full genome sequencing services for as little as a few thousand dollars per patient (depending on order size [82]), and can provide this information in a matter of days (versus the $3 billion and 10 years required to sequence the first genome in the Human Genome Project). As Moore's Law on the declining cost of

computing continues to be outstripped by the falling price of sequencing, it will not be long until standard academic laboratories will have bench-top sequencers capable of scanning entire genomes for $1000 or less per genome[83].

PAD collaborations and advanced informatics

Most importantly, this generation will see the creation of larger and more successful collaborations focused on the investigation of PAD. With superior phenotypic characterization and longer term follow-up, these international groups will organize studies specifically intended for combining datasets and optimizing future meta-analyses [23]. Novel approaches will focus on collecting 'genetically enriched' subjects to examine the extremes of phenotypes. For example, our group has collected subjects with very early onset of atherosclerotic disease (<45 years old) in the ADVANCE study [84], and others have chosen to study the opposite end of the spectrum with subjects over 80 who have no medical comorbidities (the "Wellderly").

In addition to the creation of consortia for conducting genetic studies of complex diseases such as PAD, there is considerable interest in leveraging the electronic medical record (EMR) for high throughput phenotyping to facilitate such studies. The Electronic Medical Records and Genomics (eMERGE) network (www.gwas.org) was established in 2007 to develop and implement methods for leveraging biorepositories linked to EMR systems for large-scale genomic research [85, 86]. One of the participating sites in this network, Mayo Clinic, is conducting a GWAS of PAD that includes 1,648 cases and 1,675 controls[87] recruited in the clinical setting and with linkage to the EMR. Mining of structured data from the EMR as well as natural language processing of unstructured text was used to obtain relevant covariates. [87]

Conclusions

PAD is an important and highly prevalent condition with a heritable component. Vascular biologists and geneticists have begun to make inroads on this complex disease by applying increasingly sophisticated genetic approaches (Figure). We look forward to the next decade of research, as evolving technologies and interdisciplinary collaborations promise to finally provide insights on this often morbid condition.

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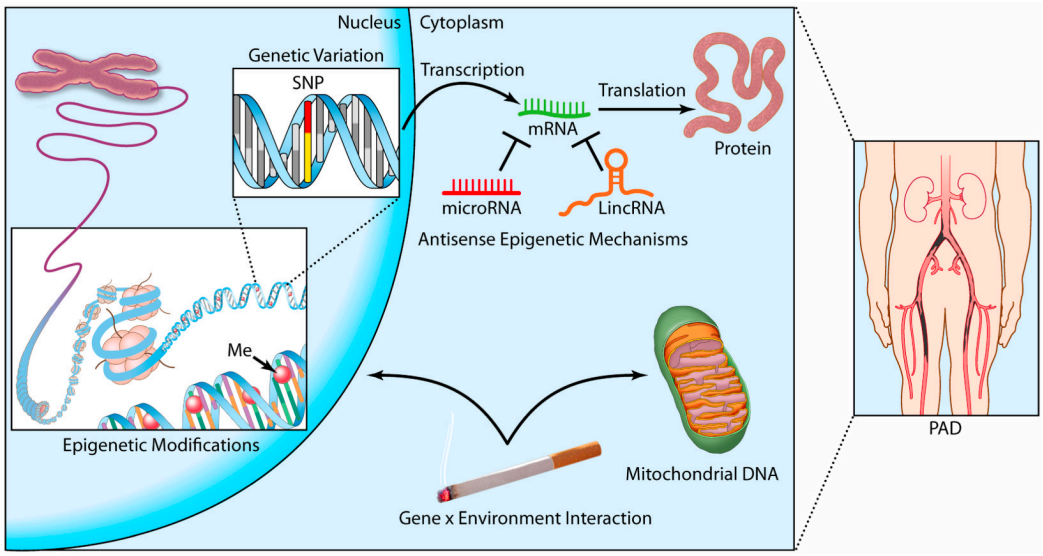

Figure 1.

The Genetics of PAD. Factors associated with risk of peripheral arterial disease include variation in the genetic code (e.g. single nucleotide polymorphisms or SNPs) and epigenetic DNA modifications (e.g. cytosine methylation). Post-transcriptional regulation by non-coding RNAs may also alter expression of gene products relevant to vascular homeostasis. Finally, recently appreciated mitochondrial DNA variation and complex gene-by-environment interactions may also be involved in PAD pathogenesis, as discussed in the text.

Table

Historical approaches to PAD genetics: The Candidate Gene Approach, Linkage Analysis, and Genome-Wide Association Studies.

	Candidate Gene Approach	Linkage Analysis	Genome Wide Association Study (GWAS)
Description	Typically a case-control approach which searches for a statistical association between a specific genetic variant (i.e. a SNP) and a disease of interes	A family-based approach where the genome is scanned for pre-specified DNA markers that are known to be highly variable (i.e. microsatellites). Regions that are found more commonly in the diseased members of the family are said to be 'linked' to the causative gene, which is then pursued with fine mapping -Also known as 'positional cloning'	A novel approach where single nucleotide polymorphisms (SNPs) are genotyped across the entire genome in subjects with and without a given disease. SNPs which differ in frequency between cases compared to controls are 'associated' with disease
Strengths	Can detect genes with small effect sizes Useful for 'complex' or polygenic conditions (such as PAD) Does not require extended families, thus simplifying the recruitment of large numbers of cases and controls	Requires no prior knowledge of the causative genes Is particularly useful for single-gene, Mendelian disorders Can be executed with a relatively small number of well-pedigreed families Scans the entire genome	Like linkage studies, is 'agnostic' and does not rely on assumptions about relevant disease Detects common variants with small effect sizes Is useful for non-Mendeliancomplex conditions Scans the 'entire' genome Does not require the collection of extended families afflicted with the disease of interest Has much higher resolution than linkage analysis
Weaknesses	Relies on our pre-existing knowledge about which genes and pathways are relevant to a disease (i.e. is a 'best-guess' approach) Does not have the potential to identify novel pathways Frequently is underpowered (especially in the context of a low pre-test probability) and confounded by natural differences amongst various racial ethnic groups (i.e. population stratification) Must be confirmed by independent investigators to be considered valid	Can only detect alleles with large effect sizes (i.e. is not likely to be useful in multifactorial conditions such as PAD) Requires families with affected individuals across multiple generations (i.e. is more difficult to perform in late onset and highly morbid conditions such as PAD)	To date, mostly disease susceptibility SNPs have been identified; less success with 'structural' variation (i.e. copy number variants, insertions, deletions, etc.) Requires relatively large sample sizes which can be confounded by incomplete phenotyping of patients (i.e. patients who have subclinical atherosclerosis may be erroneously identified as normals). Typically not powered to detect associations for rare variants (i.e. polymorphisms with allele frequencies <1%)
Examples related to PAD	~ 20 identified to date, reviewed in [7, 8] Thrombosis related • Factor II, V, VII; Fibrinogen; MTHFR; P2Y12 platelet receptor Atherosclerosis related • Interleukin-6; Angiotensin converting enzyme; CCR5 and CX3CR1 chemokine	Rare examples in the literature including: Human • PAOD - Chromosome 1p31 - causative gene remains unidentified [9] Mouse • LSq-1- Mouse chromosome 7 - identified with comparative genomics approach	~ 30 replicated associations identified to date for CAD/PAD?[11] • 9p21.3 locus correlates with PAD, CAD and AAA – likely related to *CDKN2A, CDKN2B*and *ANRIL*expression [12] • 15q24 locus correlates with smoking burden and PAD – likely related to nicotinic Ach receptor biology [13]

	Candidate Gene Approach	Linkage Analysis	Genome Wide Association Study (GWAS)
	receptors; ICAM-1; eNOS; etc	- causative gene remains unidentified [10]	