Genomic mapping in detection of vascular disorders

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Abstract:
Term vascular dysfunctional refers to a wide spectrum of vascular abnormalities including pathogenesis of tumors, their proliferation and leading to malfunctioned conditions. The treatment of most of the vascular anomalies including peripheral arterial disease and cardiac disease is multi factor procedure which include the embolic therapy, laser-based treatments and coagulation are all plating important role in managing the disease associated with vascular breakdown. The research proposal defines the treatment and diagnosis procedures involved in treatment of vascular abnormalities with a deep emphasis on techniques, efficiency and complications resulted from various other procedure and use of mapping and sequencing techniques based on genetics of variants of selected genes PIk3CA which will ultimately be more effective.

Keywords
Genetic Mapping, Vascular system, Embryogenesis, Vascular disorder, PIk3CA gene.
Introduction:

Vascular system consists of blood vessels and lymph vessels formed during early stage of the embryogenesis and are specialized for carrying nutrients, gases, wastes and hormones towards or away from the metabolically active tissues (Mulliken, Fishman, & Burrows, 2000). Formation during embryogenesis initiates with the formation of capillaries by endothelial cells which further undergoes branching or angiogenesis to form a complex network. Further development related to muscle development in formation of arteries occur in next phase thought to have their own function and properties (Hanson & Gottesman, 2005). These vessels contain a fluid called as lymph which contain essential nutrients and minerals for growth of lymph related organs during embryo development. Any damage to these vessels as a result of any change in body will lead to a condition referred as vascular system abnormalities that is enlargement or increase in number of vessels which entangled to form large clump (Wassef et al., 2015). These were further classified into two categories described as malfunctioning’s and tumor cells. Vascular type include hemangiomas, hemangioendothelioma mass which are most widely seen abnormalities seen in vascular disorders. These abnormalities are not visible in early days of birth but after 4 to 5 weeks they become visible and then proliferate, progress and at last regress (Paltiel, Burrows, Kozakewich, Zurakowski, & Mulliken, 2000).

Very little information is available for hemangiomas till now and then after few years of study the locus was being observed in the plasmid or chromosome 5q35 (FITZSIMONS, Gurwin, & Bird, 1987). In addition to it, mutation P1147s and another mutation p854s in VEGFR3 or receptor in VEGF was also investigated. In addition molecular genetics experts have also observed and found disease causing gene in the variety of vascular anomalies (Kim et al., 1993).

Rapid advances in field of molecular biology has revolutionized the field of treatment of vascular diseases by enabling the introduction of new techniques of molecular sciences towards understanding the disorder its diagnosis or prognosis. It is being also elected that knowledge in molecular biology will further be increased in next few years (Webb & Bohr, 1981). But instead of this advancement there is no practical applications involving the use of these techniques in hospitals and laboratories. Moreover, genetic human model was being presented by the researchers produced by selective breeding in order to reduce inbred which is thought to possess the characteristics of interested genotypes. The approach has presented the models of arthrosclerosis...
and hypertension. Similarly the recombinant techniques have also enabled the change in genotype of organism which will be helpful in altering phenotype (Kim et al., 1993).

The reason behind the study is that the many vascular malformations are major cause of mortality and morbidity in humans, but the reasons behind the diseases is yet uncovered and much potential has not been provided in this field. Genetic implementation will result in providing of new ways of treating and studying pathogenesis of disease including exploring the molecular mechanism which us behind the disease, specific therapies behind this abnormalities (Olofsson et al., 1996).

Numerous classic actions have been proposed by several researchers with regards to classifying and treatment. The work done by Malkein and glowchi in 1982; helped scientists in classifying the term into abnormalities and malformations in order to remove confusion. Original work was based on classifying them on the basis of histological properties such as arteries, veins and capillaries etc. A useful strategy behind this strategy is to use magnetic imaging to classify lesions into fast flowing and slow flowing based on extent of flow of lesion. Contrast enhanced tomography abbreviated as CT case are being studied in case of vascular malfunctioning (Hicklin & Ellis, 2005). The anatomy, enhancement, calcification, thrombus formation and involvement of adjacent structures is mediated by using multi detector CT systems. CT provides high resolution and results are easy to interpret. As for example hepatic hemangiomas is diagnosed with contrast head CT for hypo attenuating lesion on sequence along with peripheral globular increment on both arteries and veins which persists on delayed images. But CT is not as useful and advantageous as other techniques because it has limited detection capacity and use ionization radiations. On the other hand, MR (magnetic resonance) and UV (ultraviolet radiations are most early used noninvasive methods being used in treatment at early stage of disease (Iida, Kishi, & Hagimura, 1999).

Although UV are just used to differentiate the fast flowing from slow flowing lesions but having less benefits but magnetic resonance has been found to be more useful because of its reproducibility. due to these benefits and other that is least utilizing ionizing radiations and capabilities, magnetic resonance has become the most widely used and acceptable procedure used for detection and treatment of vascular abnormalities (Sun, Wang, Mackey, & Wong, 2009).
Fig.1.1 the negative and positive feedbacks related with PI3k

PI3k-Akt is the intracellular pathway which will produce response to extracellular signals and are involved in metabolism, growth, survival and development of cell. The whole process is mediated by threonine or kinase acting as the substrates involved in drown streaming of process. Major proteins involved in them are PI3k and Akt protein belonging to kinase B family. The discovery is mediated in 1877 by group of scientist’s previously undescribed property in oncogenes. These sequences related to cancer were then studied and named as AKT sequences. This PI3k –Akt has many down streaming effects and must be carefully regulated. These mechanisms are of two types including positive or negative. Negative type at receptor and resulting in the inactivation of AKT protein (fig1.1).

Suppression of PI3k pathway will result in the increase in the senescence and further partial expression of the mutant PI3k-H147R. (Klein et al., 2003). Of all these factor family factor is also the main factor to consider but genetic factors may add to this in development of PROS syndrome(Ylikorkala et al., 2001).

In both experimental as well as a group of patients, single nucleotide polymorphism were identified in a gene. The next step is analysis of these alleles of single nucleotide polymorphism and their
gene-type. An allele is associated with the disease which will be different in case study when compared with control group (Saadane et al., 2019). The result from this experiment is analyzed with great care because many of studies are intercepted by the type of case in both cases, errors associated with phenotyping or small size of sample in some researches (Leys et al., 1996). Several control associated studies were conducted in order to find out variants in many types of genes that will be useful in checking suspect ability of PROS. These techniques give inconsistent results and lack of relationship with syndrome will lead to the condition where there exists a clear relation among the disease and some of the genes (FITZSIMONS et al., 1987).

Despite of all these conditions, a lot of work has been done in couple of years and contributed much to research since then (Cai, Pardali, Sánchez-Duffhues, & ten Dijke, 2012). Mapping, sequencing of variants and genes responsible for vascular diseases have been recognized for PROS by using genomic wide linkage analysis. So, the main reason for this study is to glance at advances made in molecular genetics of PROS since 2003 and implementation of those in treatment and practical applications (Betsholtz, 2004).

1) Objectives and aims:

Hereditary components assume a basic part in the pathogenesis of vascular peculiarities. Huge advances have been made lately in recognizing the hereditary and sub-atomic determinants of an assortment of vascular abnormalities utilizing a sub-atomic hereditary methodology. The main objectives of this research paper include;

- To discuss standard classifications and terminologies required for accurate diagnosis and selection of treatment.
- To find out why treatment of these vascular abnormalities is multi-disciplinary or multi-modal process.
- To develop new therapeutic approaches which is novel and disease specific leading to identification of disease specific genes mutated along the dysfunctional proteins.
- To identify genetic and molecular abnormalities using molecular genetic approaches leading to identification of genes and underlying mechanism involved in vasculogenesis, PROS and angiogenesis.
- In order to develop tissue targeted therapy on molecular basis of these lesions.
2) Methodology:

2.1. Experimental design:

The experimental design consists of rat model where the two strains of mice i.e. Plk3CA and Tie2Cre will be crossed in order to investigate the effect of PIK3CA activating mutation in vivo. Cre expression will be totally limited to endothelial compartment. The promoter will not be specific to ECs but will be also expressed during early phase of growth. Cre-mediated deletion of lox-p transcription site will result in the expression of mutant alleles. As a result, the produced progeny will not contain the combination of both of the strains and lethality will observe to occur before day 10 of the embryo formation. The mutant strains at the day 9 will be smaller in size and have delayed heart beat than that of wild type. In this way we will be able to obtain the conditional expression of PIK3Ca after injecting tamoxifen. In this way we will be able to decide the mice into two subcategories i.e., syndrome and non-syndrome. Syndrome will be consisting of organisms or mice which will exhibit some symptoms of malformations and in turn will affect other parts of body. While non–syndromic will be that which show no signs or symptoms at all.

2.2. PIK3CA-activating mutations induce both cell senescence and proliferation in EC

In order to find out the effect of Plk3CA mutation in endothelium, a mutant HI047R will be expressed in human umbilical cord by retroviral infection process. We will also express another mutation referred as PIK3CA-E545k. As control group we will use HUVECs infected with an empty retroviral vector alone or one expressing wild-type human PIK3CA. Among the two; those who will be expressing PIK3CA-HI407R and PIK3CA-EC45k will show signs of morphological abnormalities in somatic cells while the wild type will not exhibit any symptoms of malformation which mean that they are asymptomatic. Similarly, large surface area will also be observed for the mutant type as compared to wild type as we will perform cytometric analysis. So, in turn these mutants will show various aspects associated with that of malfunction or senescence such as lesion, presence of multiple nuclei and increased cell sizes. So, expression of beta glycosidase will be evaluated as it is better and suitable biomarker for measurement of senescence in cells. For the decoupling of the increased cell surface from that of proliferation rate by EdU incorporation assay. Mutants will show more replication as compared to non-symptomatic and hence chances of proliferation among larger cells will be more giving the hint that positive senescence are far majority non-proliferating.
2.3. Angiogenesis sprouting assay:

The assay is used for the investigation of the relationship among physiological changes resulted from mutations in PIKC3 pathway in physiological endothelial processes. In this assay only normal and those spheroid cells stimulated by PIk3CA will exhibit protrusions when stimulated by vascular endothelial growth factor. But in some cases, the cells containing the active form of PIK3CA will exhibit sprouts like structures even in absence of vascular endothelial growth factor. But there will be very less difference in the migratory capabilities of both symptomatic and non-symptomatic as analyzed by the chemo taxis assay.

2.4. Induction of vascular malfunctions by localized expression of PIK3CA-H1047R:

The formation of lethal phenotype PIK3CAH1047R- in both adult as well as during the development will result in the construction of model which will be based on partial expression of the mutant. For this reason, we will use a strategy where posterior leg of mice mutant will be injected with tamoxifen containing OH at three prime ends. Even a single shot will be enough for the induction of morphological signs such as bleeding and Cassel abnormalities after one week. Histological analysis will be performed in order to find out enlarged vessels and increased vessel density with lesions and inflammatory cells. Larger vessels will appear dilated resulting in hemangioma like condition. Moreover, increased production of cytokines will result in the formation of mutant genotype. Similarly, tamoxifen related biomarker associated mice will show increase in beta glycosidase activity. And senescence associated markers will also accumulate in pathological tissues.

2.5. Analysis/Diagnosis

2.5.1. Subject of study:

The total number of individuals who will be chosen for testing will be 165 which will be assumed to have the disease (PROS) syndrome. The data will be collected from laboratory during the following year. Referred doctors or physicians will provide all the information for testing as well as clinical information related to brain, overgrowth, malfunctions, epidermal Navis and acral anomalies. The minimum criteria for testing will include congenital, sporadic, and segmental overgrowth of adipose, muscle, skeletal, and/or cerebral tissue combined with at least one other key feature suggestive of PROS. And hence the patients will be decided on the basis of overgrowth
of brain or any other symptoms while the other group which will be considered will be asymptomatic.

2.5.2. Collection of samples:

PIK3CA related PROS syndrome require the collection of tissues from the site of infection which may be skin, lesion or overgrown part. As previous researches have cleared the fact that the syndrome cannot be detected in the blood of individual so sample from fresh biopsy of effected individual is grabbed. Sample DNA will be collected from the cheeks, saliva, fibroblasts and surgical specimens by using pure gene cell and tissue extraction kits. Integrity and quantity will be measured by mass spectrometry, flourpmetry and gel electrophoresis.

2.5.3. Sequencing of PIK3CA

All PIK3CA codons of mutation for individual will be screened and custom intron samples will be employed for amplification of region of interest. We will pool, quantify and purify each infected individual using polymerase reaction for each of infected mice. Then the DNA libraries will be ‘repaired involving fragmentation and tagging by transposition. Sequencing will be performed using paired end sequence using 300 cycle reagent kit.

2.5.4. Analysis:

Analysis involving the basement of quality will be performed using Fast QC and adapters and low-quality bases are removed by trimmometric. Burrows wheeler aligner will be employed for alignment. While reanalysis will be performed by using genome analysis tool GATK which will give quality control and coverage matrix. We will identify candidate single-nucleotide variants and small insertions/deletions by recording all sites of PIK3CA coding exons and splice junctions with at least four reads not matching the reference sequence and by using a stringent base quality threshold of 30 and a mapping quality threshold of 20. We will annotate variants with settles annotation. And we will focus on protein-altering and splice-site changes present at a frequency less than 0.1% in the Exim Aggregation Consortium.
2.6. Candidate gene association studies:

Out of all the genes associated with PROS studies, only few have been reported to show the potential for vascular diseases. These genes include beta–fibrinogen, Apo lipoprotein b, endothelial nitric oxide synthase, methylene reeducates, alpha adducting, interleukin six and glutathione s transferase. However, these is no such report which will provide an association in between the variants of genes and peripheral disease. Previously 135 single nucleotide polymorphisms were identified at 111 genes which were either positional or biological genes having ABI 1046 which belong to hypersensitive regions. The contribution of each of SNP and SNP with covariate and overall relation with genetic factors will be further assessed in this study. Significant associations will be corrected by multiple testing and will be replicated by four cross validations.

The following associations will be considered significant,

- 2 single nucleotide polymers having main effect in PI3KCA.
- PI3kCA interaction for PROS and 29 H1047R polymer interactions associated with it.
- Basic hereditary weakness variations don't completely clarify the heritability of complex illnesses and the degree to which uncommon variations add to disease powerlessness isn't known. The basic infection uncommon variation idea has been delineated by a few reports, including the relationship of exceptional PCS-K9 and PIK3CA variations with vascular disease susceptibility and of uncommon other variations with macular degeneration. Association investigations of uncommon variations in quality coding areas are a coherent following stage to supplement genome-wide examination of regular variations. New genotyping clusters permit testing the relationship of uncommon (characterized as minor allele recurrence less than 2% utilitarian variations with characteristics of intrigue. Such a methodology has been fruitful in distinguishing uncommon hereditary variations related with complex qualities, for example, insulin resistance.

This type of resource and approach will be advantageous for meta-analysis of single nucleotide polymers equal to or less than two thousand but unable to identify the variants involved in the ABI procedure.
2.8. Limitations:

Up to now the genetic data available and methods available are less successful for the peripheral arterial diseases as compared to cardio vascular disease based on many reasons including:

- Strong contribution of environmental factors to PROS diseases.
- Genome association analysis could address phenotypic homogeneity.
- Genetic heterogeneity may also arise in case of finding out variants in both control and experimental group study.
- Large sample sizes are required for uncovering of variants having small sizes, and identification has resulted in genetic consortia for common vascular diseases.
- Gene–gene and environmental interaction have to be considered which will require large sample sizes and measurement of environmental factors.
- Susceptibility of peripheral disease is further influenced by rare variants, large sample size will be required in this case.
- Additional studies are also required in order to find out relation between gene variants and PROS.

The field of complex sickness hereditary qualities has progressed impressively over the most recent quite a long while, principally as a result of gathering of huge case–control associates and accessibility of more up to date genomic advances. In this part, we will feature how these advances may be utilized to expand our comprehension of the hereditary premise of peripheral arterial disease and where conceivable give instances of early illustrative investigations.

3) Statistical considerations:

Upton now many associations have been found between the genes and their relationship to specific disease. But these studies have explained a small variability in traits of disease. Common alleles produce small genetic signals which require larger samples to analyze.

With this development in proof has come an expanding need to gather and sum up the confirmations so as to recognize genuine hereditary relationship among the enormous volume of bogus positives and references in that). Besides, replication of discoveries in free informational collections is currently generally viewed as an essential for persuading proof regarding affiliation.
This is the reason meta-investigation has become an always mainstream approach for the approval of hereditary loci inclining for basic sickness and phenotypes.

Met analysis involve the statistical analysis of studies relate to multiple I dependent value with the objective to produce overall estimators including p value, estimators etc. Most of the genetic variants which have been published so far in analyses were based on GWAS and many have been published so far. Most of Meta analysts have size in their discovery phase including 11000 participants. This has led to increase in the already available data till now and also ensured the risks associated with increase in loci as compared to original samples.

Other one is fisher’s procedure which is totally based on combination of P values. The null hypothesis that is sum of all the data is null as compared to non-null hypothesis truly based on experimental conditions having some values for at least one data set. It can be used in the number of the number of molecular diagnosis and number of mutations as a result of pl3KCA activity aspired with oncogenic activity in different phenotypes.

Closely relate p values are truly based on Z values that is it takes notice on direction of effect rather it is easy to introduce weight for each of the study. So the most optimized and 100% effective approach is to use meta-analysis which is used for GWAS data resulting in discovery of the data which will be associated with single nucleotide polymers

Fixed impacts meta-examination accept that the genuine impact of each danger allele is the equivalent in every informational index. The opposite change weighting is the most utilized model for fixed impacts meta-examination, in which each investigation is weighted by the reverse of its squared standard blunder.

Cochran-Mantel-Hansel approach is a further mainstream utilized technique in hereditary qualities which gives comparative outcomes to the backwards difference weighting strategy. A notable assessor of the between-study change for the arbitrary impact approach is the DerSimonian and Laird assessor. In any case, this strategy may be less powerful regarding uncommon variations. Albeit irregular impact models are not embraced in revelation endeavors, they are reasonable when the objective is to gauge the normal impact size of the researched variation and its vulnerability through various populaces, for instance, with respect to prescient purposes.
In diagram of measurable and computational strategies zeroed in on succession examination and complex illnesses has been introduced. Among the various strategies talked about in this research, Bayesian procedures appear to be encouraging regarding execution in certain fields, for instance, complex infections. Since these strategies by and large require an exceptional computational weight, their application has not been well known previously. In this manner, the improvement of new high performing figuring stages makes conceivable, in the following future, a huge utilization of Bayesian strategies so as to adapt to natural issues and specifically with complex illness assignments. Albeit some organic issues have been comprehended, new ones, much more intricate, emerge speaking to, thusly, novel difficulties for either natural or factual and computational techniques.

Statistical testing is performed using Mann-Whitney to assess difference in mutant allele fraction among different types of tissues.

4) Ethical concerns associated with genetic mapping:

The population being studied is analyzed on the base of their age, people of age 50 were placed in specific grouping and other age having 60 or above will be placed in separate groups. Similarly gender based classification will also be done i.e., will be divided on the basis of gender female or male. Similarly, the inclusion and exclusion criteria will involve the person having specific diseases i.e., any sort of abnormality, diseased or not, children of age above ten will be involved. However pregnant women will not be involved. In the same way prisoners having specific socially deprived state will not be involved. The data will be stored in national committee for detection of specific disease which will maintain its confidentiality. Subjects will be identified on the basis of testing which will get further credentials as including disease history, ages and comparison with control and experimental groups. The study will include medical record in hardcopy also include medical workers which include physicians who will have open access to record. The research involves credentials such as

- Height
- Weight,
- BMI
- Disease history
- Symptomatic or asymptomatic
The population will be identified on basis of already defined criteria which will include two things;

- Age
- Risk factors
- Congenital, sporadic, and segmental overgrowth of adipose, muscle, skeletal, and/or cerebral tissue combined with at least one other key feature suggestive of PROS.

Data will be detected on basis of PROS. ABI technique will be used which is based on ultrasound designed beam. Meta-analysis system will be used for studying genes associated with disease and to find out single nucleotide polymorphisms.

**Table no.01. Types of biological molecules along with the tumor type and risk factors due to PKI3CA suppression or mutation.**

| Molecule | Alternation                                      | frequency      | Tumor type                                               |
|----------|--------------------------------------------------|----------------|----------------------------------------------------------|
|          | Mutations (somatic)                              | Greater than 50%| Glioma, melanoma, prostate cancer                        |
|          |                                                  |                | Endometrial cancer, endometrioid ovarian cancer           |
|          |                                                  |                | Variable in sporadic breast cancers (2–30%)              |
| PTEN     | Decreased expression                             | Greater than 50%| Breast, melanoma, prostate                               |
|          | Methylation                                      |                | Microsatellite instability-high                          |
|          | Loss of heterozygosis                            |                | colorectal cancer                                         |
|          |                                                  |                | Endometrial cancer                                        |
|          |                                                  |                | Leukemia                                                 |
|          | Germ line mutations                              | 80% of Cowden’s disease | High risk of breast, thyroid and |
|          |                                                  |                |                                                          |
| Gene   | Mutation Type   | Frequency | Tissue(s)                          |
|--------|-----------------|-----------|-----------------------------------|
| **P85** | Activating mutations | Rare     | Ovary, colon, glioma, lymphoma cell line (CO) Fusion Very rare Lymphoma |
|        | Amplification   | Up to 50% | Rare Ovary, cervix, lung Breast (BRCA1 associated?) |
| **PIK3CA** | Activating mutation | >50% >25% | Bowel Breast |
| **AKT1** | Amplification  | Low       | Gastric |
| **AKT2** | Amplification  | Low       | Ovary (12–25%) Pancreas (20%), breast (rare) Mutation Low Colorectal |
| **AKT3** | Overexpression  | Low       | Hormone-resistant prostate and breast cancer |
| **PDK1** | Mutation       | Low       | Colorectal |
Conclusion or future outcomes:

The genetic mutations into the families and diseases are the prime tools for understanding the molecular basis of vascular anomalies. This will result in the more accurate diagnosis which will lead to an enhanced level prognosis and development of treatments. Moreover, the mutation in PIK3A pathway and the pathogenicity will result in unrevealing key regulators involved in disease (PROS). Current technologies are unable to sort out the complexity underlying the vascular abnormalities. The treatments like gene therapy and other tissue engineering will provide a way to manipulate natural antigenic factors so better understanding of these angiogenic factors will be of key importance in development of these tools. The great deal of phenotypic variability within known gene mutation suggests that we do not fully comprehend exact genetic contribution to syndromes. But further investigation will open the way to identify the molecular basis of these lesions leading to providing an opportunity to design a therapy against them.

References:

1. Betsholtz, C. (2004). Insight into the physiological functions of PDGF through genetic studies in mice. *Cytokine & growth factor reviews, 15*(4), 215-228.
2. Cai, J., Pardali, E., Sánchez-Duffhues, G., & ten Dijke, P. (2012). BMP signaling in vascular diseases. *FEBS letters, 586*(14), 1993-2002.
3. FITZSIMONS, R. B., Gurwin, E., & Bird, A. (1987). Retinal vascular abnormalities in facioscapulohumeral muscular dystrophy: a general association with genetic and therapeutic implications. *Brain, 110*(3), 631-648.
4. Hanson, D. R., & Gottesman, I. I. (2005). Theories of schizophrenia: a genetic-inflammatory-vascular synthesis. *BMC medical genetics, 6*(1), 1-17.
5. Hicklin, D. J., & Ellis, L. M. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *Journal of clinical oncology, 23*(5), 1011-1027.
6. Iida, T., Kishi, S., & Hagimura, N. (1999). ABNORMALITIES IN CENTRAL, SEROUS CHORIORETINOPATHY. *Retina, 19*, 508-512.
7. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., & Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature, 362*(6423), 841-844.
8. Klein, R., Klein, B. E., Moss, S. E., Wong, T. Y., Hubbard, L., Cruickshanks, K. J., & Palta, M. (2003). Retinal vascular abnormalities in persons with type 1 diabetes: the Wisconsin Epidemiologic Study of Diabetic Retinopathy: XVIII. *Ophthalmology, 110*(11), 2118-2125.

9. Leys, A. M., Leys, M. J., Hooymans, J. M., Craandijk, A., Malenfant, M., VAN GERMEERSCH, D., . . . DE LAEY, J.-J. (1996). Myelinated nerve fibers and retinal vascular abnormalities. *Retina, 16*(2), 89-96.

10. Mulliken, J. B., Fishman, S. J., & Burrows, P. E. (2000). Vascular anomalies. *Current problems in surgery, 37*(8), 517-584.

11. Olofsson, B., Pajusola, K., Kaipainen, A., Von Euler, G., Joukov, V., Saksela, O., . . . Eriksson, U. (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proceedings of the National Academy of Sciences, 93*(6), 2576-2581.

12. Paltiel, H. J., Burrows, P. E., Kozakewich, H. P., Zurakowski, D., & Mulliken, J. B. (2000). Soft-tissue vascular anomalies: utility of US for diagnosis. *Radiology, 214*(3), 747-754.

13. Saadane, A., Mast, N., Trichonas, G., Chakraborty, D., Hammer, S., Busik, J. V., . . . Pikuleva, I. A. (2019). Retinal Vascular Abnormalities and Microglia Activation in Mice with Deficiency in Cytochrome P450 46A1–Mediated Cholesterol Removal. *The American journal of pathology, 189*(2), 405-425.

14. Sun, C., Wang, J. J., Mackey, D. A., & Wong, T. Y. (2009). Retinal vascular caliber: systemic, environmental, and genetic associations. *Survey of ophthalmology, 54*(1), 74-95.

15. Wassef, M., Blei, F., Adams, D., Alomari, A., Baselga, E., Berenstein, A., . . . Lopez-Gutierrez, J.-C. (2015). Vascular anomalies classification: recommendations from the International Society for the Study of Vascular Anomalies. *Pediatrics, 136*(1), e203-e214.

16. Webb, R. C., & Bohr, D. F. (1981). Recent advances in the pathogenesis of hypertension: consideration of structural, functional, and metabolic vascular abnormalities resulting in elevated arterial resistance. *American heart journal, 102*(2), 251-264.

17. Ylikorkala, A., Rossi, D. J., Korsisaari, N., Luukko, K., Alitalo, K., Henkemeyer, M., & Mäkelä, T. P. (2001). Vascular abnormalities and deregulation of VEGF in Lkb1-deficient mice. *Science, 293*(5533), 1323-1326.