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VEGF-A et phénotypes intermédiaires des maladies cardio-vasculaires : Une approche de génomique fonctionnelle

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To my wife, Behjat, who is my life

my children, Afarin , Tahamasoud and my new baby "Shahab",

and all those who taught me and behind the scenes particularly my late Father and my Mother, I did not fully appreciate and acknowledge how difficult it is to be a parent until I became one myself.

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LISTE OF PUBLICATIONS OF THESIS

Please keep in mind:

Some of the articles presented in this thesis report are under review for publishing. Therefore, the displayed results must be kept confidential. These scenarios are indicated in gray in the list below.

REVIEWS

Ndiaye NC*, **Azimi Nehzad M***, El Shamieh S*, Stathopoulou MG*, Visvikis-Siest S*. Cardiovascular diseases and genome-wide association studies. Clin Chim Acta, 2011; 18;412(19-20):1697-701.

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ARTICLES

Mohsen Azimi-Nezhad, Daniel Lambert, Catherine Ottone ,Corinne Perrin, Celine Chapel, Gwenaëlle Gaillard, Michèle Pfister, Christine Masson, Eric Tabone, Fotini Betsou, David Meyronet**,** Marie-Noëlle Ungeheuer, and Sophie Visvikis – Siest. Influence of pre-analytical variables on VEFGA gene expression and circulating protein concentrations. Biopreservation and Biobanking,(In press).

Debette S*, Visvikis-Siest S*, Chen MH, Ndiaye NC, Song C, DeStefano AL, Safa R, **Azimi Nezhad M**, Sawyer D, et al. Identification of Cis- and Trans-acting Genetic Variants Explaining up to Half the Heritability of Circulating VEGF levels. Circulation research, 2011;19;109(5):554-63.

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Mohsen Azimi-Nezhad*, Maria G. Stathopoulou***,** Ndeye Coumba Ndiaye, Amélie Bonnefond, Marc Rancier, Abdelsalam Saleh, Sophie Visvikis-Siest**.** Association of vascular endothelial growth factor trans-acting genetic variant with metabolic syndrome. (submitted)

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Maria G. Stathopoulou*, Ndeye Coumba Ndiaye*, Amélie Bonnefond*, **Mohsen Azimi-Nezhad**, Said El Shamieh, Abdelsalam Saleh, Marc Rancier, John Lamont, Peter Fitzgerald, Sudha Seshardi, Sophie Visvikis-Siest. Interrelationships between VEGF and lipids profile. Lipid Res (accepted with modifications)

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M. Azimi-Nezhad, Herbeth, G. Siest, H. Esmaili, S.J. Hosseini, M. Ghayour-Mobarhan, S. Visvikis-Siest. High prevalence of metabolic syndrome in Iran in comparison to France: What Are the Components That Explain This? Metabolic syndrome and related disorders 2012 Jan 27;10(3):181-8. (Epub ahead of print).

Abstract

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that has been linked to cardiovascular diseases (CVDs) and related predisposing statuses such as metabolic syndrome (MetS).The identification of genetic variants that influence the VEGF circulating levels and their associations with MetS and adhesion and inflammation molecules could enable us to have a comprehensive approach of the relationship of this molecule with CVDs. Therefore, we aimed at first to investigate the genetic background of VEGF levels, via a genome wide association analysis, and the pre-analytical and analytical variation factors of VEGF levels measurements before examining the implication of this molecule in inflammation and in MetS. Also, we examined the differences of MetS between Iranian (MASHHAD cohort) and French (STANISLAS cohort) populations.

The main findings of this thesis are: 1) the identification of 4 genetic variants(rs6921438, rs4416670, rs6993770 and rs10738760) that explain 47.6% of circulating VEGF levels, 2) the associations of VEGF and its identified genetic variants with adhesion and inflammation molecules such as ICAM-1, E and L selectins , TNF-α, IL-6 and CRP at protein and transcription levels, 3) the association of VEGF-related polymorphism rs10738760 with MetS, 4) the relationship between VEGF regulatory variant, rs6921438, and LDL-C and HDL-C,5) the proposition of the best conditions for measuring both circulating VEGF (serum being the most stable anticoagulant) and its gene expression by reducing time between blood collection and centrifugation, and by avoiding multiple freeze-thaw cycles,6) a high prevalence of MetS in Iranian women. Our results propose the biological connections between VEGF, inflammation and adhesion molecules, lipids and MetS.

Key words: Vascular endothelial growth factor, functional genomics, cardiovascular diseases, metabolic syndrome

Résumé

Le facteur de croissance de l'endothélium vasculaire (VEGF) est une cytokine multifonctionnelle qui a été liée aux maladies cardiovasculaires (MCV) et à des divers troubles/ facteurs de risque cardiovasculaires tel que le syndrome métabolique (SM). L'identification de variants génétiques qui agissent sur les taux de VEGF circulant et leurs associations avec le SM et les molécules d'adhésion et d'inflammation pourraient permettre la compréhension des liens entre les taux de VEGF et les MCV. Par conséquent nous avons recherché l'origine génétique des taux de VEGF, via une étude d'association pangénomique, et les facteurs de variation pré-analytiques et analytiques influant les taux de VEGF mesurés avant d'étudier les implications de cette molécule dans l'inflammation et le SM. Nous avons également examiné le profil du SM dans des populations françaises (cohorte STANISLAS) et iraniennes (cohorte MASHHAD). Les principaux résultats de cette thèse sont : 1) l'identification de variants génétiques rs6921438, rs4416670, rs6993770 et rs10738760 expliquant 47.6% des taux de VEGF circulant, 2) l'association du VEGF et des variants génétiques identifiés avec des molécules d'adhésion et d'inflammation telles que ICAM-1,sélectines E et L,TNF-α, IL-6 et CRP au niveau protéique et transcriptomique, 3) l'association du rs10738760 avec le SM, 4) la relation entre le rs6921438 et le HDL-C et le LDL-C, 5) la détermination optimale à la fois des taux de VEGF (le sérum serait plus stable que le plasma) et de son expression génétique en proposant une durée minimale entre le recueil du sang et sa centrifugation, et en évitant des cycles de gel/dégel répétés. 6) la prévalence élevée du SM chez les femmes iraniennes. Nos résultats proposent des liens biologiques entre le VEGF et les molécules d'inflammation et l'adhésion, les lipides et le SM.

Mots clés: Facteur de croissance des cellules endothéliales vasculaires, génomique fonctionnelle, maladies cardiovasculaires, syndrome métabolique

List of abbreviations

ACD	Acid citrate dextrose
AHA	American heart association
Akt	Protein kinase B (see AKT)
Ap-1	Activating protein-1
Apo E	Apolipoprotein E
ASCVD	Atherosclerotic cardiovascular disease
ATP III	Adult treatment panel III
AVC	Accident vasculaire cérébral
BMI	Body max index
β2m	Beta 2 microglubulin
CAM	Cellular adhesion molecules
CMP	Centre for preventive medicine
CRP	C- reactive protein
CRB	Centre of biologic resources
CVDs	Cardiovascular diseases
DNA	Deoxy nucleic acid
EDTA	Ethylene diamine tetra acetic acid
E-selectin	Endothelial cell selectin
FGF	Fibroblast growth factor
FHS	Framingham heart study
GWAS	Genome wide association study
HIF	Hypoxia inducible factor
HDL-C	High density lipoprotein cholesterol
HbA1c	Hemoglobin A1C
ICAM	Inter cellular adhesion molecule
ICAReB	Investigation clinic and access to the biologic resources
IDF	International diabetes federation
IL-6	Interleukin- 6
IL-8	Interleukin-8
IGF	Insulin-like growth factor
IGFBP-3	Insulin-like growth factor-binding protein 3
IRS	Insulin receptor substrate
Jak/Stat	Janus kinase/ Signal Transducer and Activator of Transcription
Kv	Potassium voltage-gated channel
KCNV2	Potassium voltage-gated channel subfamily V, member 2
LDL-C	Low density lipoprotein cholesterol
LRP12	Low density lipoprotein receptor-related protein 12
L-selectin	Leukocyte selectin
MAPK	Mitogen-activated protein kinase
MCV	Maladies cardiovasculairs

MetS	Metabolic syndrome
mRNA	messenger ribonucleic acid
MRPL14	Mitochondrial ribosomal protein L14
MMP-9	Matrix metaloproteinase -9
NCEP ATP III	National cholesterol education program adult treatment panel III
NHLBI	National heart, lung and blood institute
NO	Nitric oxide
NF-κB	Nuclear factor kappa B
PAI-1	Plasminogen activator inhibitor type 1
PBMCs	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
PlGF	Placental growth factor
PI3K	Phosphatidyle inositol three kinase
PIVUS	Prospective investigation of the vasculature in Uppsala seniors
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein gene
P-selectin	Platelet selectin
SC	STANISLAS cohort
sICAM	Soluble intercellular adhesion molecule
SM	Syndrome métabolique
SNP	Single nucleotide polymorphism
STANISLAS	Suivi Temporaire Annuel Non Invasif de la santé des Lorrains Assurés sociaux
Sp1	Specific protein 1
sVCAM	Soluble vascular cell adhesion molecule
OMS	Organisation mondiale de la santé
TC	Total cholesterol
TG	Triglyceride
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
T2D	Type 2 diabetes
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDLR	Very low density lipoprotein receptor
WHO	World health organization
ZFPM2	Zinc finger protein multi-type 2

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PREFACE

Cardiovascular diseases (CVDs) represent the leading cause of death worldwide, although the mortality due to this cause is falling gradually due to advances in diagnosis and therapy. According to World Health Organization (WHO) data, in 2008, the mortality rate due to CVDs was 214-455 per 100,000 deaths, being lower in developed countries.

In the European Community, CVDs are the leading cause of death in adulthood as well. In 2005 the death rate from circulatory system diseases was 241.2 per 100,000 (295.4 in males, 196 in females). This rate decreased to 226.1 (273 and 183 respectively) the following year thanks to constructed preventive and treatment systems. Detection of apparently healthy people who are at increased risk of developing a disease, particularly CVDs, is considered a high priority commitment for health systems.

The metabolic syndrome (MetS) is a cluster of interrelated risk factors that are associated with impaired angiogenesis and appear to directly promote the development of T2D and CVDs. The risk for CVDs and T2D is doubled and fivefold increased respectively in the presence of MetS. Individuals with MetS present elevated insulin levels as well as vascular endothelial growth factor (VEGF) but, interestingly, an impairment in neovascularisation/angiogenesis. Thus, the explanation for this paradox (elevated VEGF levels and angiogenesis impairment) could lead consequently to possible new preventive/therapeutic strategies against switching from the pre-pathological/pre-diabetes status to established disease.

The laboratory of “Cardiovascular Genetics” EA 4373, provide some fundamental infrastructure to conduct a study in order to investigate the possible genetic and environmental factors as well as gene-environment interactions, which may influence the MetS. For this purpose, the STANISLAS Cohort (SC) study or STANISLAS Family Study (SFS) , which includes 1006 families, has been set up with the main aim of seeking out gene-gene and gene-environment interactions in the field of CVD. This family cohort was recruited

between 1993–1995 (first visit) at the Center for Preventive Medicine (CMP) of Vandoeuvre-lés-Nancy during a periodical health assessment. The families were supposed healthy and free from any declared acute and/or chronic disease in order to be able to assess the effects of genetics on the variability of the intermediate phenotypes in physiological conditions without the influence of any medical treatment or disease. The longitudinal nature of this study should enable the observation of the evolution of intermediate phenotypes in relation to genetic factors during the monitoring stages. The second visit took place between 1998 and 2000 with a participation rate of 75%. During these two visits, serum, plasma, DNA and also during the third visit between 2004 and 2005 peripheral blood mononuclear cells samples were collected. Additionally, a database was created, combining data from the health examination and data specific to the SC (clinical, biological and genetic data).

Our colleagues in this lab previously have identified some biological variables that influence VEGF levels and they have also assessed the heritability of VEGF. Therefore, we were motivated to determine the genetic factors which may influence the circulating levels of VEGF in collaboration with Framingham Heart Study and the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study via a genome wide association study (GWAS). After identification of the explanatory genetic variants of VEGF levels by this GWAS we conducted a research in order to investigate the association of identified polymorphisms and VEGF levels with MetS and its related components. Simultaneously, we examined the possible differences of MetS components patterns between Iranian and French (STANISLAS cohort study) populations during a common study with Mashhad University of Medical Sciences. In parallel, thanks to availability of several biochemical and medical examinations data related to CVDs which are available in our biobank /Biologic Resources Center (CRB), we have assessed the relationships of identified polymorphisms and VEGF levels (in circulating and transcriptional levels) with the most related inflammatory and

adhesion molecules to CVDs. Obviously, the improvement of our insights on biological and pre-analytical variations which affect the VEGF measuring was the prerequisite of all designed researches. For this reason, we performed a common project with Pasteur institute in Paris and Lyon University.

CHAPTER 1

HYPOTHESIS AND OBJECTIVES

As the burden of CVDs is rising throughout the world, it is of increasing importance to be engaged in a dynamic prevention process and to investigate the possible genetic and environmental risk factors, which are implicated in these multifactorial diseases worldwide. Preventive care and treatment of individuals with established CVDs, early screening of symptom free patients, detection of apparently healthy people who are at increased risk of developing the disease are considered as high priority issues for public health.

The metabolic syndrome (MetS) is a constellation of interrelated risk factors of metabolic origin—*metabolic risk factors*—that are associated with impaired angiogenesis and appear to directly promote the development of CVDs. The risk for CVDs and T2D is doubled and fivefold increased respectively in the presence of MetS. The mortality risk in French men and women with MetS is reported to be 1.82 and 1.80 respectively. The predominant underlying risk factors for MetS appear to be abdominal obesity and insulin resistance; other associated conditions can be physical inactivity, aging, low grade inflammation and hormonal imbalance.

VEGF is a multifunctional cytokine that shows distinguished functions in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis.

VEGF has been linked with a number of vascular pathologies including CVDs (ischemic heart disease, heart failure, and stroke). Moreover, elevated circulating VEGF levels have been also observed in various cardiovascular related disorders/ risk factors including MetS, T2D, hypertension, pre-eclampsia, chronic kidney disease and polycystic ovarian syndrome as well as other disorders such as cognitive decline dementia, reproductive, immune-inflammatory disorders and in several types of cancer. VEGF also has a key role during the vascularization of the human ovarian follicle and corpus luteum, in placentation and embryonic period, and in bone and wound healing.

What could be the cause of this wide diversity of VEGF functions throughout life? The first hypothesis was arisen to answer this question which therefore, the existence of common molecular pathway(s) interplaying between VEGF and inflammatory/ cellular adhesion molecules could probably explain this broad range of VEGF functions.

It is noteworthy to mention that in the presence of obesity, the main underlying factors of MetS/CVDs, elevated levels of VEGF(the main angiogenic factor), have been observed. On the other side, there is an impairment of angiogenesis/neovascularization state in MetS. VEGF plays an important role in fat mass development via induced angiogenesis. Capillary endothelial cells, which are the main VEGF-expressing cells, communicate with adipocytes through paracrine signaling pathways, extracellular components, and direct cell-cell interactions and they mediate visceral fat angiogenesis and finally fat mass development. What is the reality behind this paradox of increasing VEGF levels and angiogenesis impairment in MetS? VEGF could be one the links between obesity, inflammation and MetS or play a protective role in front of T2D/ CVDs development in individuals with MetS? However, it seems that the implication of VEGF on MetS and its related components is not extensively studied. Both VEGF and MetS have significant genetic background, nevertheless, the identification of variants that explain a significant proportion of their heritability is limited. The assessment of possible common genetic factors between VEGF and MetS as well as with its related components could be interesting and it would lead to a possible answer for the previously mentioned questions and for the following issues:

Therefore, we aimed 1) to investigate the genetic background of VEGF levels and 2) to define the link between VEGF levels/ VEGF-related SNPs and inflammation and adhesion molecules 3) to investigate the association of VEGF levels and VEGF-related SNPs with MetS and its related components. In order to present a holistic approach, pre-analytical and

analytical variation factors and thus the accuracy of laboratory analysis of VEGF was also among the purpose of our work.

Main objectives:

1) Plasma or serum? Which type of anticoagulant is recommended for VEGF quantification?

2) Determination of pre-analytical variables influencing the circulating VEGF levels and its expression by peripheral blood mononuclear cells as well as its expression in non tumor tissues.

3) Identification of genetic variants associated with circulating VEGF in order to determine the genetic heritability score in healthy populations.

4) Investigation of the potential associations of VEGF levels and related SNPs with adhesion and inflammatory molecules

5) Investigation of the relationships of VEGF levels and related SNPs with MetS and related components.

6) Elucidation of differences in the prevalence of MetS between Iran and France and explanation of the influencing biological factors in the two populations.

CHAPTER 2

INTRODUCTION

2.1. Cardiovascular diseases

Cardiovascular disease (CVDs) constitutes the leading cause of death worldwide and accounts for as much as 30% of global mortality (1-3). It is also the leading cause of hospitalization and of >1.5 million deaths in the European Union, as well as the main cause of years of life lost from early death (4-6). In 2008, according to the data of "Centre d'Epidemiologie sur les causes médicales de Décès" CépiDC-Inserm, (www.cepidc.vesinet.inserm.fr) approximately 150.000 deaths have been registered related to CVDs in France.

It is now well established that the pathogenesis of CVDs begins in uterus and in early childhood (7). The in utero pathophysiologic derangements associated with the risk of CVDs are believed to involve alterations in vascular function that implicate nitric oxide synthesis and endothelial function (8). In adolescence, endothelial dysfunction has been increasingly observed in the setting of personal and family history of CVDs risk factors, especially obesity, hypertension, and T2D mellitus (8).

2.1.1. Risk factors of CVDs

A risk factor of CVDs (CVD) encompasses any measurable trait that may be associated to an increased probability of developing a future CVDs (9). From the viewpoint of accessibility of scientific evidence it is more useful to distinguish principal or main risk factors, predisposing factors and potential risk factors (10).

2.1.1.1 Principal risk factors of CVDs

The principal risk factors represent a group with significance proven in several prospective cohort studies (11). Such factors include elevated blood pressure, increased concentrations of total and LDL cholesterol, decreased serum levels of HDL cholesterol, diabetes, cigarette smoking, male gender and aging (12).

2.1.1.2 Predisposing risk factors of CVDs

The predisposing risk factors are responsible for an increased frequency of principal risk factors (13). Predisposing risk factors are strongly linked with principal risk factors. Obesity, sedentary life style, positive inherited risk factors and an atherogenic diet are included to predisposing risk factors. Genetic, psychosocial and socioeconomic factors were also included in the group of predisposing risk factors (10).

2.1.1.3. Potential risk factors of CVDs

Potential risk factors, also called "the novel cardiovascular risk factors" represent the largest group, although these may have the least significance. Statistical analyses link potential risk factors with the cardiovascular risk and it remains unclear whether they actually represent independent risk factors or they reflect only the developing disease. Their significance is additionally restricted by difficulties of measurement. (14). Among the novel biochemical risk factors for circulatory disease, the best recognized include the increased concentration of triglycerides, the presence of small, dense LDL, the elevated concentration of lipoprotein (a) (Lpa), the elevated concentration of homocysteine, the elevated concentrations of fibrinogen and other clotting proteins, the increased concentrations of endothelial dysfunction markers and the elevated levels of proinflammatory factors (12). The most widely described proinflammatory markers are: C-reactive protein (CRP), interleukins 6 and 18 (IL-6, IL-18), tumor necrosis factor-α (TNF-α), metalloproteinase-9 (MMP-9) and myeloperoxidase (15-17). The most significant endothelial dysfunction markers include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin , nitric oxide (NO), endothelin-1 and endothelin-3, and asymmetric dimethylarginine (ADMA) (18). Additionally, among clotting proteins, apart from fibrinogen, new risk factors for CVDs have been identified: clotting factor VII, von Willebrand's factor (vWF),

plasminogen activator inhibitor type 1 (PAI-1), tissue-type plasminogen activator (tPA) and D-dimers (19-21). A separate subgroup of potential risk factors involves the vascular factors, which can be estimated using modern imaging methods. Among them, the highest significance is linked to intima-media thickness (IMT) of the carotid artery, calcium score of coronary arteries and indices of vascular stiffness, i.e. brachial artery flow-mediated dilation (FMD). Moreover, tachycardia and the incidence of influenza were recognized as new risk factors for circulatory diseases. The group of risk factors includes also environmental factors, such as air pollution (22;23).

2.1.2. Pathophysiology of CVDs

The vascular endothelium is a physical barrier between the blood and the vessel wall structures. It is also the source of enzymes that activate and deactivate cardiovascular hormones and can produce relaxing and contracting factors, growth factors, growth inhibitors and inflammatory mediators (24). These endothelium- derived factors contribute to the finely tuned control of circulation, homeostasis and inflammation by endocrine, autocrine and paracrine activity (25). Activation of the vascular endothelium by cardiovascular risk factors such as high levels of LDL, high blood pressure, elevated levels of plasma glucose and cigarette smoke exposure cause an impairment in endothelial function through different pathways. The figure 1 shows a number of important mechanisms in the pathogenesis of CVDs.

Figure 1: Cardiovascular risk factors influences on endothelial and vascular smooth muscle cells

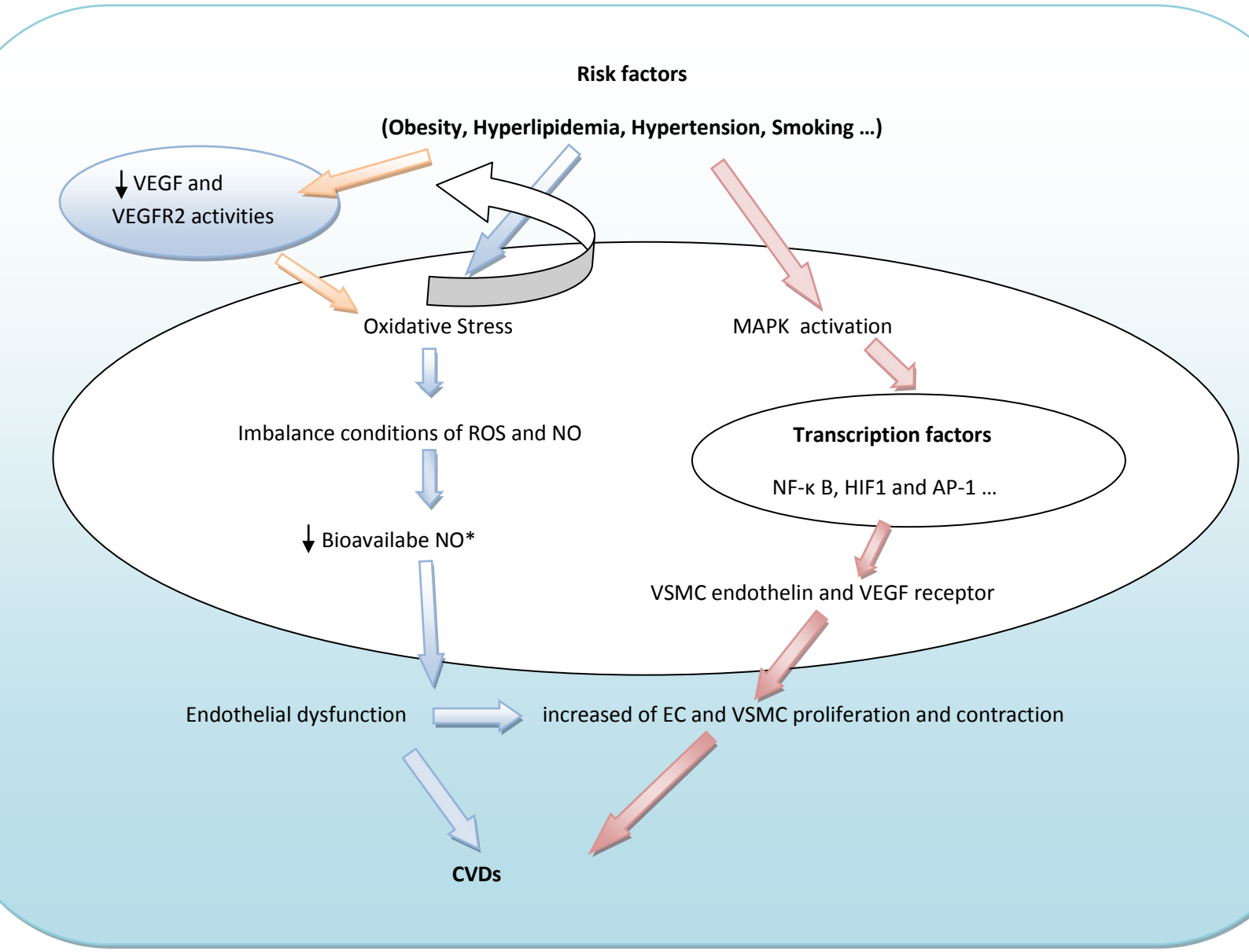


Figure 1: Cardiovascular risk factors (Hyperlipidemia, hypertension, cigarette smoke, etc) may induce vascular smooth muscle cells (VSMC) endothelin receptor upregulation via activation of mitogen-activated protein kinase MAPK-mediated NF-κB signal pathways. The endothelin receptor upregulation may lead to increased VSMC proliferation and contraction and, subsequently, cause CVD. Large circle line represents cell membrane and small circle line indicates nuclear membrane. ROS: reactive oxygen species [Adapted from *(26-28)*].

2.1.3. Systems biology and the network of gene-environment interactions in CVDs

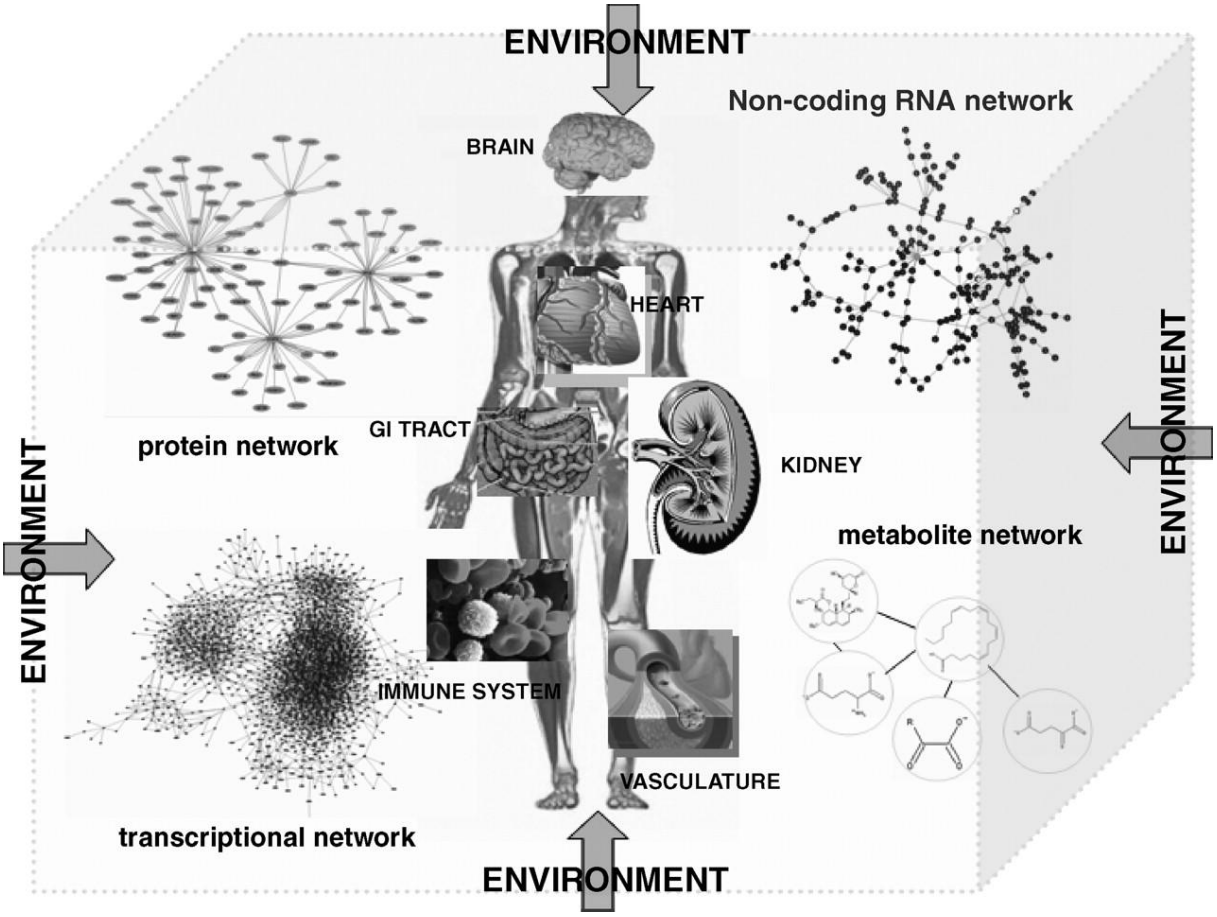
With the ending of the sequencing of genomes from multiple species, the challenge in the life and biomedical sciences now is to investigate and describe the biological function of individual genes, pathways, and, more generally, biological networks that drive complex phenotypes, including common human diseases. The identification of single genes for common diseases has been widely accelerated over the past decades. With access to the complete genome sequence for a diversity of species, large-scale haplotype maps, technologies capable of screening DNA polymorphisms and gene activity on unprecedented scale, and the availability of well-characterized human cohorts, the identification of genes explaining an appreciable risk for a some of common human diseases has been made feasible (29). Eminent examples are TCF7L2, a major disease gene for common forms of T2D (30;31); INSIG2, a major obesity gene potentially explaining 4% of lifetime body mass index (BMI) in the human population (32) and ALOX5, a gene identified in human and mouse populations that predisposes to a number of disease-related traits, including atherosclerosis (33), hyperlipidemia-dependent aortic aneurysm, and obesity and bone phenotypes (29). Although the mentioned examples represent only a few number of the investigations that have been made during the recent years, they explain how leveraging large-scale, high-throughput genetic and functional genomic technologies, in addition to well-characterized animal and human populations, can guide directly to the identification of key drivers of disease.

However, despite the identification of a number of novel disease-predisposing genes, progress in uncovering the mechanisms by which these genes lead to disease has been far slower. Even in cases in which genes validated as causal for disease through well-understood pathways, it is often unclear whether the genes' connection with a disease involves the known pathways,

whether these “known” pathways are as general as they were believed, or whether the disease-associated genes act through multiple pathways, some of which are yet to be defined (29).

Although the physiology of diseases such as atherosclerosis is beginning to be better understood, the vast networks of molecular interactions within the cells and tissues related to the disease have not yet been fully exploited. Figure 2 shows that there is a diversity of molecular networks functioning in any given tissue, including genomics networks, networks of coding and noncoding RNA, protein interaction networks, protein state networks, signaling networks, and networks of metabolites. Further, these networks are not acting independently within each cell, but they interact with one another to form complex, giant molecular networks within and between cells that regulate the activity of the different tissues, as well as the signaling between tissues. Variations in DNA and environment lead to modifications in these molecular networks, which, in turn, induce complicated physiological processes that can be manifested as disease (29).

Figure 2: Complex interplay among tissues, molecular networks, and environment that leads to disease



Atherosclerosis and hypertension encompass a diversity of different disease subgroups involving multiple organs and tissues. Operating within each tissue (and each cell within a given tissue) are a number of molecular networks that finally lead the onset of disease. Internal and external environmental conditions as well as genetic background influence these networks. Variations in the connectivity structure of these networks are induced by variations in the genetic background and environmental conditions, where these variations in turn lead to phenotypic variations, including disease. System biology follows the studying of molecular networks in all relevant tissues and associating them with clinically relevant phenotype data to identify the networks driving disease. By having a holistic approach, it may be possible to better understand the complex interplay among tissues, molecular networks, and environment that leads to disease [Adapted from Schadt et al (29)].

2.2. Metabolic syndrome (MetS)

2.2.1. MetS historical mirror

The recognition of the MetS (MetS) can be dated to the 1920s when Kylin recognized a triad of gout, hypertension, and hyperglycemia in his patients. Since then, studies have correlated complications of obesity to body fat, hypertriglyceridemia, hyperinsulinemia and hypertension. Reaven's Banting lecture in 1988 defined syndrome X as metabolic complications of insulin resistance among obese and non-obese patients (34).

MetS is also known as MetS X, cardioMetS, syndrome X, insulin resistance syndrome, Reaven's syndrome (named for Gerald Reaven), and CHAOS (in Australia). (http://en.wikipedia.org/wiki/Metabolic_syndrome).

In 1988, Gerald M. Reaven have proposed insulin resistance as the underlying factor and named the constellation of abnormalities Syndrome X. Reaven did not include abdominal obesity, which has also been hypothesized as the underlying factor, as part of the condition (35).

2.2.2. MetS increases the risk of atherosclerosis and T2D

MetS refers to a cluster of atherosclerotic risk factors that are associated with impaired angiogenesis, and it is narrowly associated with insulin resistance (36). The risk for atherosclerotic CVDs and T2D accompanying the MetS are approximately doubled and fivefold respectively compared with an absence of the syndrome (37). The mortality risk is reported to be 1.82 and 1.80 in French men and women respectively with MetS compared to non MetS individuals (36).

2.2.3. MetS risk factors

The most widely recognized metabolic risk factors include the atherogenic dyslipidemia, the elevated blood pressure and the elevated plasma glucose (36). Individuals with these characteristics commonly manifest the prothrombotic and proinflammatory states.

Atherogenic dyslipidemia consists of an aggregation of lipoprotein abnormalities including elevated serum triglyceride and apolipoprotein B (apoB), increased small LDL particles, and a reduced level of HDL cholesterol (HDL-C)(38)

The predominant underlying risk factors for the syndrome appear to be abdominal obesity and insulin resistance; other associated conditions can be physical inactivity, aging, and hormonal imbalance (39). An atherogenic diet (eg, a diet rich in saturated fat and cholesterol) can enhance risk for developing CVDs in people with the syndrome, although this diet is not listed specifically as an underlying risk factor for the condition (39).

2.2.4. Proposed MetS's pathophysiologies

One theory holds that insulin resistance is the essential cause of the MetS (40).Excess upper body fat can accumulate either intra peritoneally or subcutaneously. Many investigators claim that excess visceral fat is more strongly associated with insulin resistance than any other adipose tissue compartment; other researchers found that excess subcutaneous abdominal (or truncal) fat also carries a significant association with insulin resistance (39) Some data suggest that hyperleptinemia rather than hyperinsulinemia may play an important role in the genesis of the MetS (41). In contrast, others believe that MetS is associated with insulin resistance but it is not a consequence of insulin resistance alone, nor is it a direct consequence of the lack of insulin action. This is most documented in patients with insulin receptor mutations or autoimmune antibodies to the insulin receptor; they may have 100 times or greater increasing of circulating insulin or need similarly high doses of exogenous insulin to control diabetes. These patients show a distinct syndrome with acanthosis nigricans and a high risk of diabetes, but typically have no cardiovascular risk factors such as obesity, hypertension, or atherogenic dyslipidemia(42) Furthermore, individuals with type 1 diabetes do not present the same atherogenic lipoprotein phenotype typical of patients with MetS or T2D. Lean type 1 diabetic patients do not characteristically have insulin resistance (43). If

MetS does not result purely from the lack of insulin, then how might insulin resistance generate other features of the syndrome? Proposed mechanisms are centered on three themes: effects of mild to moderate hyperglycemia, effects of compensatory hyperinsulinemia, and effects of unbalanced pathways of insulin action. Hyperglycemia, largely postprandial and below diabetic levels, may lead to a variety of effects usually associated with diabetes. For example, moderate hyperglycemia might be postulated to cause accelerated atherogenesis via advanced glycosylated end products or via enhanced collagen formation. A more important mechanism may be compensatory hyperinsulinemia. The maintenance of normal postprandial glucose homeostasis requires that the pancreatic beta cells release a normal level of insulin in response to the hyperglycemic challenge and that the resultant hyperinsulinemia (1) stimulates glucose uptake by tissues like muscles, (2) suppresses endogenous glucose production (>80% of which is derived from the liver). In insulin-resistant conditions, the ability of insulin to augment glucose uptake and inhibit hepatic glucose production is impaired. The resultant hyperglycemia presents a stimulus to the beta cells, which secrete large amounts of insulin after meals. Initially, attention was directed to the concept that certain organs and tissues can have lesser degrees of insulin resistance than skeletal muscle and liver. For example, the high insulin concentration required to produce normal glucose uptake in skeletal muscle may overstimulate cells of the arterial wall. Now, this concept has been developed to include the idea that not only different cell types, but also different metabolic pathways within the same cell, may differ in their responsiveness to insulin. The pathways of insulin action are thrown off balance (42).

2.2.4.1. Explanatory molecular pathways disturbance underlying of MetS

Binding of insulin to the insulin receptor leads to activation of its tyrosine kinase activity and autophosphorylation of specific tyrosine residues of the receptor (Figure 1). In turn, the activated insulin receptor phosphorylates tyrosine residues on substrate proteins, initiating the

intracellular signaling cascade. The two main pathways for insulin signaling are the phosphatidylinositol 3-kinase (PI-3K) and the mitogen-activated protein (MAP) kinase pathways. Insulin produces most of its metabolic actions through the PI 3-kinase pathway. In contrast, inhibition of ERK activation does not reduce insulin-stimulated glucose transport or glycogen synthesis (44). In MetS and T2D mellitus, the pathways leading to activation of PI-3K are blocked, possibly through serine phosphorylation of the insulin receptor and/or IRS proteins, whereas the MAP kinase pathway remains open and may even be hypersensitive (44).

The PI-3K signaling pathway also increases nitric oxide, which is a potent inhibitor of vascular smooth muscle cell growth. Thus, impairment in the PI-3K pathway could contribute to vascular endothelial dysfunction. Conversely, smooth muscle cell growth and proliferation are stimulated by activation of the ERK-MAPK pathway, which maintains normal sensitivity to insulin even in insulin-resistant conditions. The overall effect may be to enhance atherogenesis (43).

2.2.5. Endothelial dysfunction and MetS

Tooke (45) has proposed that vascular changes, possibly related to insulin resistance, occur prior to the emergence of T2D and increase vascular resistance at the arteriolar level. All of the individual components of the MetS appear to have an adverse effect on the endothelium and its capacity to vasodilate or they may be caused by endothelial dysfunction, for example, hypertension (45;46). Thus, endothelial dysfunction may be an antecedent for both T2D and the MetS. Indeed, the risk and the actual development of endothelial dysfunction may commence many years before glucose intolerance manifests. Endothelial dysfunction, a vascular condition characterized mainly by impaired nitric oxide dependent vasodilatation, has recently been proposed as the underlying mechanism that links together the components of the MetS (44). The presence of endothelial dysfunction has been reported in both Type 1

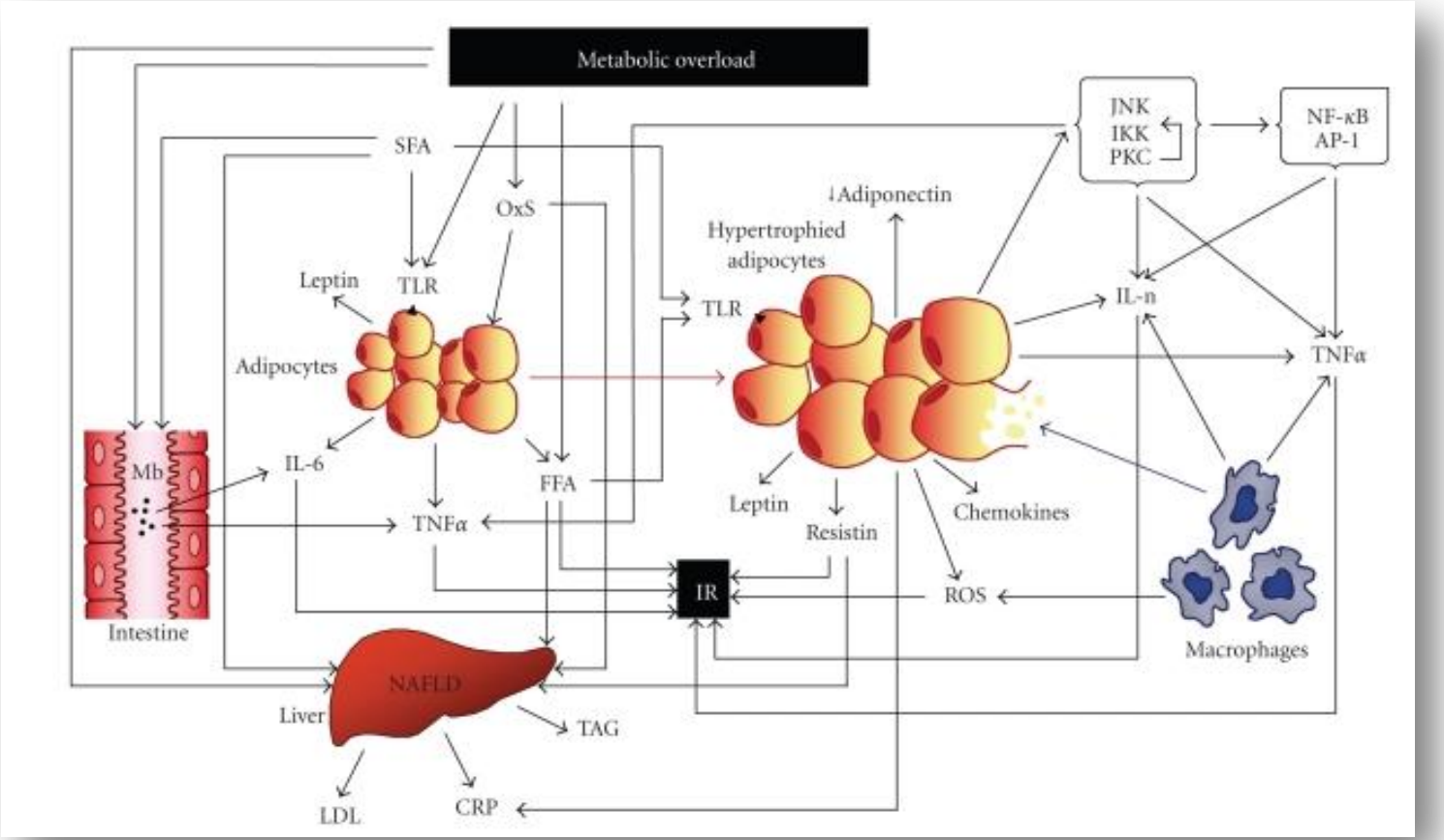
and T2D and in obese, insulin-resistant subjects without diabetes (46). Further investigations have demonstrated that endothelial dysfunction is a feature of hypercholesterolemia; cholesterol levels at the upper range of normal, and essential hypertension (47;48).

2.2.6. Inflammation role in the pathophysiology of MetS

The inflammatory state that accompanies the MetS is a quite peculiar manifestation, as MetS is not accompanied by infection or sign of autoimmunity and no massive tissue injury seems to have taken place. Furthermore, the dimension of the inflammatory activation is not extended and it is often called "low-grade" chronic inflammation (49). Other researchers have attempted to name this inflammatory state as "metaflammation", meaning metabolically triggered inflammation, or "parainflammation" as a term to define an intermediate state between basal and inflammatory states (50;51).

In the existence of obesity, adipose tissue produces cytokines in excess, whereas output of adiponectin is diminished; these responses appear to elevate the connection between obesity and inflammation (Figure 3). It should be noted that insulin-resistant individuals show a low-grade inflammation evidence even without an increase of total body fat (52). It has been described that elevation of CRP, fibrinogen, IL6, vascular endothelial growth factor, soluble intercellular cell adhesion molecule-1, soluble vascular cell adhesion molecule-1, and plasminogen activator inhibitor-1 increase with the number of components of the MetS (53-56).

Figure 3: Overview of the complex interplay between obesity-inflammation-MetS



Metabolic overload impacts on adipose tissue, leading to organelle stress with production of ROS and adipokines, as well as activation of kinases that potentiate the transcription of inflammatory genes and interfere with insulin signaling. Hyperthrophy facilitates rupture of adipocytes that attract and activate macrophages that markedly reinforce the inflammatory process through further production of ROS and inflammatory cytokines. Production of adiponectin, an anti-inflammatory cytokine, is reduced. Increase of FFA concentration, namely, SFA, coming both from feeding and adipose tissue overflow, accumulates in the liver, among other organs. Fat accumulation in the

liver leads to overproduction of LDLs and, together with IL-6, of CRP. NAFLD is a frequent consequence of these metabolic dysregulations, and all this impacts on insulin sensitivity. SFA activates TOLL-like receptors in adipocytes, contributing to the activation of the inflammatory response. Fat has also effects on intestinal permeability and on the microbiota, with systemic inflammatory consequences. Most excess metabolites and cytokines produced throughout these processes converge on insulin resistance, a central characteristic of the MetS. AP-1: activator protein-1; CRP: C-reactive protein; FFA: free (nonesterified) fatty acids; IL-n: interleukins; IKK: inhibitor of NF-κB kinase; IL-6: interleukin-6; Int: intestine; IR: insulin resistance; JNK: c-Jun N-terminal kinase; LDL: low density lipoprotein; M: microbiota; NAFLD: nonalcoholic fatty liver disease; NF-κB: nuclear factor κB; OxS: oxidative stress; ROS: reactive oxygen species; PKC: protein kinase C; SFA: saturated fatty acids; TAG: triacylglycerols; TLR: TOLL-like receptors; TNFalpha: tumor necrosis factor alpha. [Adapted from H. Monteiro et al *(57)]*

2.2.7. Genetic and ethnic variations in MetS

There is a genetic basis for all of the key components of the MetS (46). Also, the heterogeneity of these components including obesity, T2D, dyslipidemia, and hypertension alone means that there is a number of potential genetic defects in insulin secretion, in the regulation of lipid and carbohydrate metabolism (such as transcription factors), and/or in carbohydrate metabolism in the liver. The genetic basis of the MetS may be composed by the multiplicative effects or interaction of polymorphisms in a number of minor genes rather than from a single major regulatory defect (46).

Also, notable individual and ethnic variation exists in the clinical pattern of metabolic risk factors in obese/insulin-resistant subjects (58;59).

It is likely that the presence of each metabolic risk factor is partially under its own genetic control, which influences the response to different environmental exposures. For example, a variety of polymorphisms in genes influencing lipoprotein metabolism are associated with lipid disorders in obese individuals (60;61). Similarly, a genetic predisposition to defective insulin secretion when combined with insulin resistance can increase the plasma glucose levels (61). Currently, SNPs on the adiponectin gene have been widely studied in relation with MetS (62). In addition, the perilipin gene is appearing as a potential main player for obesity and other metabolic traits. Moreover, the later has also been indicated to regulate the response of MetS traits to diet modifications (62).

A recent genome-wide association study has demonstrated an association between some polymorphisms close to PMAIP1 gene(phorbol-12-myristate-13-acetate-induced protein) and MetS in Indian Asians and in Europeans (63).

2.2.8. Genetic heritability of MetS related components

The genetic heritability (usually named broad-sense genetic) is defined as the proportion of the total phenotype variance that is due to genetic effects, mainly due to a large number of genes, each with a small, linear, and additive effect. It has been suggested that genes influencing insulin resistance have pleiotropic effects on MetS-related traits (64). All MetS traits are strongly influenced by genetic factors. As heritability of the same trait may vary quite widely across populations, epidemiologic data show a wide difference between European (h^2 between 0.07-0.44) and Asian populations (h^2 between 0.27-0.53) (64;65). As heritability estimate are specific for the particular population studied they reflect both the diversity of the population and the diversity of the environment. However, the clusters of "protective lipids" such as HDL-C (with 70% heritability rate) and Apolipoprotein A1 as well as "risk lipids" for example total cholesterol, apolipoprotein E and apolipoprotein B are in the top of the heritable components list of MetS in several studies. Inflammatory markers are also included in the list of heritable factors for MetS (64;65).

2.2.9. Diagnostic criteria of MetS

Several expert groups have attempted to set some simple diagnostic criteria to be used in clinical practice to identify patients who manifest the multiple components of the MetS. These criteria have varied somewhat in specific elements, but in general they include a combination of both underlying and metabolic risk factors.

Diagnostic criteria of MetS have ameliorated over the past decade (66). The suggested measurements for detection have been conditioned in part by views of the pathogenesis of the syndrome. For instance, in 1998, the World Health Organization (WHO) task force on diabetes announced that insulin resistance is the dominant cause of the MetS (67). By these criteria, clinical measurements of insulin resistance were required for the diagnosis. But with

growing evidence for the critical role for abdominal obesity, it obtained a more important position among diagnostic criteria. The latter led to the National Cholesterol Education Program (NCEP) criteria for the MetS in which the need for demonstration of insulin resistance was replaced by an increased waist circumference (central obesity) (36). In the recent years, clinical *guidelines* have been widely harmonized. This harmonization is represented in the American Heart Association (AHA)/ National Heart, Lung, and Blood Institute (NHLBI) update of the National Cholesterol Education Program (NCEP) criteria, and the International Diabetes Federation (IDF) recommendations (66). The WHO criteria (67) along with those of the AHA/NHLBI (68) and IDF (69) are summarized in Table 1. Recently, a large number of studies have been carried out to determine the prevalence of the MetS in different populations (Table 2). The majority of epidemiological studies have used NCEP criteria, (36) but there have been several comparisons of NCEP criteria with WHO and IDF recommendations for estimating prevalence.

Table 1: Proposed Criteria for Clinical Diagnosis of the MetS

Clinical measure	IDF(2005)	NCEP(2001)	WHO(1998)	EGIR	AACE	AHA
Insulin resistance	None	None, *but any 3 of the following 5 features*	IGT, IFG, T2DM, or lowered insulin sensitivity* *plus any 2 of the following*	Plasma insulin >75th percentile *plus any 2 of the following*	IGT or IFG *plus any of the following based on clinical judgment*	None, *but any 3 of the following 5 features*
Body weight	Increased WC (population specific) *plus any 2 of the following*	WC ≥102 cm in men or ≥88 cm in women	Men: waist-to-hip ratio >0.90; women: waist-to-hip ratio >0.85 and/or BMI >30 kg/m^2	WC ≥94 cm in men or ≥80 cm in women	BMI ≥25 kg/m^2	WC ≥102 cm in men or ≥88 cm in women
Lipid	TG ≥150 mg/dL or on TG Rx HDL-C <40 mg/dL in men or <50 mg/dL in women or on HDL-C Rx	TG ≥150 mg/dL HDL-C: <40 mg/dL in men or <50 mg/dL in women	TG ≥150 mg/dL and/or HDL-C <35 mg/dL in men or <39 mg/dL in women	TG ≥150 mg/dL and/or HDL-C <39 mg/dL in men or women	TG ≥150 mg/dL and HDL-C <40 mg/dL in men or <50 mg/dL in women	TG ≥150 mg/dL HDL-C: <40 mg/dL in men or <50 mg/dL in women
Blood pressure	≥130 mm Hg systolic or ≥85 mm Hg diastolic or on hypertension Rx	≥130/85 mm Hg	≥140/90 mm Hg	≥140/90 mm Hg or on hypertension Rx	≥130/85 mm Hg	≥130/85 mm Hg or on hypertension Rx
Glucose	≥100 mg/dL (includes diabetes)	>110 mg/dL (includes diabetes)¶	IGT, IFG, or T2DM	IGT or IFG (but not diabetes)	IGT or IFG (but not diabetes)	≥100 mg/dL (includes diabetes)
Other			Microalbuminuria (*urinary average excretion rate≥20μg/min or albumin-creatinine ratio≥20mg/g)*		Other features of insulin resistance§	

IDF: International Diabetes Federation, NCEP: National Cholesterol Education Program, WHO: World Health Organization, EGIR: European Group for studying Insulin Resistance, AACE: American Association of Clinical Endocrinology, AHA: American Heart Association, IGT: Impaired Glucose Tolerance (2-h glucose ≥140 mg/dL), IFG: Impaired Fasting Glucose (110 ≤fasting glucose ≤126 mg/dL), T2DM: Type 2 diabetes Mellitus; WC: Waist Circumference; BMI: Body Mass Index; TG: Triglycerides, HDL-C: High density Lipoprotein Cholesterol, Rx: Recommended treatment.

¶ The 2001 definition identified fasting plasma glucose of ≥110 mg/dL (6.1 mmol/L) as elevated. This was modified in 2004 to be ≥100 mg/dL (5.6 mmol/L), in accordance with the American Diabetes Association's updated definition of IFG. §Includes family history of T2D mellitus, polycystic ovary syndrome, sedentary lifestyle, advancing age, and ethnic groups susceptible to T2D mellitus.

2.2.10. MetS's epidemiology in the world

A relatively high prevalence of the MetS is a worldwide phenomenon (66). The likelihood of an increasing in the MetS prevalence could be predicted because of anticipations of a higher prevalence of obesity in the future (70). A notable issue in the determination of the prevalence of MetS in different parts of the world depends on defining criteria. It has been predicted that up to 80% of the middle aged individuals in eastern Europe may be obese, with the highest prevalence being amongst women (71). Globally, 10% of the school-age children are overweight, with approximately 3% of these being clinically obese. In parts of Europe an additional 1% of the children are becoming overweight each year. Indeed, throughout Asia, Latin America and parts of Africa, obesity has increased 2–3-fold in the past decade (71). Based on current published criteria estimates suggest that the syndrome influences a notable percentage of the middle-aged and elderly populations of most European societies (10-20%) and confers increased risk of T2D (2-8.8-fold) and CVDs (1.5-6-fold), as well as having a prominent effect on morbidity (71).

The prevalence of MetS in France is lower than in North America and in other European countries (72). Prevalence of MetS is not uniform around the France while its prevalence in Lille is higher than in Toulouse (73). In France, the prevalence of MetS varies from 11.7%-26% in men and 7.5%-18.4% in women according to NCEP ATP III and the International Diabetes Federation (IDF) definitions respectively (65;72;73).

Table 2: Prevalence of MetS in different geographic regions of the world

Country	Study year	Population category	Overall prevalence (%)
Brazil (74)	1999-2000	Brazilians	19
Ecuador (74)	2005	Postmenopausal women	41.1
Finland (75)	1988-1993	Finnish men	24
China (76)	2006	Chinese	32.2
France European Cohort (77) PRIME cohort (78) DESIR study (79) EPIMIL study (72)	 1994-1996 1991-1994 1998-2001 2003	 French (40-55y) French (50-59 years) French (30-64years) Military men (20-58 y)	 9 29.1 8-12 9
Greece (85)	2003	Greeks	23.6
India (85)	1995	Asian Indian	41.4
Iran Center (80) East (81) West (82) North (83) South (84)	 1999-2001 2003 2008 2009 2007	Iranians	 33.7 39.9 23.7 31 49.08
Ireland (85)	1998	Irish (50-69 years)	20.7
Latin America (85)	2005	Postmenopausal women	35.1
New Zealand (85)	2002-2003	Maori Pacific Others	32 39 16
Australia (85)	1999-2000	Australians	18.3
Turkey (85)	1997-2001	Turkish	32.2-45
Pakistan (86)	2008	Pakistanians	18-46
England (85)	1992		Men: 17.9 Women: 14.3(EGIR definition)
Spain (85)	1996	Spanish	Men = 16.0 women = 15.4 (EGIR definition)
USA (87-89)	1988-1994	Overall African American Mexican American Whites Others	23.7 21.6 31.9 23.8 20.3
USA(87)	1999-2002	Overall	34.6

2.3. Vasculogenesis and angiogenesis

The formation of the vascular system is a prerequisite for vertebrate embryogenesis and involves two fundamental processes: vasculogenesis, defined as the differentiation of endothelial cell progenitors and their assembly into the primary capillary plexus, and angiogenesis, the sprouting of new capillaries from pre-existing vessels (90).

2.3.1. Vascular system in embryonic period

Induction by fibroblast growth factors of mesoderm during gastrulation leads to blood-forming tissue, including angioblasts and hemopoietic cells, that together they constitute the blood islands of the yolk sac. The differentiation of angioblasts from mesoderm and the formation of primitive blood vessels from angioblasts at or near the site of their origin are the two distinct steps during the onset of vascularization that are defined as vasculogenesis. Although vasculogenesis occurs mainly during fetal development, recruitment of angioblasts from bone marrow and peripheral blood in response to ischemic insult has been described in adults (91;92).

2.3.1.1. Vasculogenesis steps and angiogenesis forms

Vasculogenesis consists of three major steps: induction of hemangioblasts and angioblasts (mediated mainly through fibroblast growth factor (FGF)), assembly of primordial vessels (mediated mainly by vascular endothelial growth factor/vascular endothelial growth factor receptor system, VEGF/VEGFR) and transition from vasculogenesis to angiogenesis (93). Angiogenesis represents the development of new vessels from pre-existing vessels. Two forms of angiogenesis have been described: sprouting and non-sprouting angiogenesis or intussusceptive microvascular growth (intussusception) (94). The sprouting process is based on endothelial cell migration, proliferation and tube formation. Intussusception divides existing vessel lumens by formation and insertion of tissue folds and columns of interstitial tissue into the vessel lumen (94). Physiologic angiogenesis plays an important role in wound

and fracture healing, endometrial growth, embryo implantation and placentation. In contrast, pathologic angiogenesis underlies pathophysiology of the following conditions: tumor growth and metastasis, rheumatoid arthritis, retinopathies, chronic inflammation and psoriasis (95). Therapeutic angiogenesis, defined as the use of biological agents or bioactive materials to stimulate the growth of new blood vessels, was developed for the treatment of ischemic heart disease, cerebrovascular disease and delayed wound healing (96)

2.3.2. Angiogenesis regulatory factors

Several regulatory factors (Table 3) play a role in angiogenesis. Among them, during angiogenesis, VEGF interacts with several other angiogenic factors and plays an important role in cell proliferation, differentiation, migration, cell survival, nitric oxide (NO) production and release of other growth factors (97). VEGF is a highly conserved, disulfide-bonded and heparin-binding homodimeric glycoprotein of 34 to 45 kDa (90).

Table 3: Regulatory factors of angiogenesis

Protein
a) Angiogenic Factors
FGF-β
FGF-α
Angiogenin
Transforming growth factor-α
Transforming growth factor-β
Tumor necrosis factor-α
Vascular endothelial growth factor (VPF/VEGF)
Platelet-derived endothelial growth factor
Granulocyte colony-stimulating factor
Placental growth factor
Interleukin-8
Hepatocyte growth factor
Proliferin
Angiopoietin-1
Leptin
hCG human chorionic gonadotropin
Estrogens
b) Angiostatic factors (natural)
Angiostatin
Thrompospodin
Endostatin
Tumor necrosis factor-α
Prolactin
Thromboxane A2
c) Angiostatic factors (therapeutic)
Thalidomide
Steroids

FGF-β = basic fibroblast growth factor; FGF-α = acidic FGF; VPF = vascular permeability factor

[Adapted from (90, 97,103)].

2.3.3. Vascular endothelial growth factor (VEGF)

2.3.3.1 VEGF brief history

Vascular endothelial growth factor (VEGF) was first characterized as vascular permeability factor (VPF) by Senger et al. (98) in 1983. They reported that this protein promotes extravasation of proteins from tumor-associated blood vessels. VEGF was characterized in 1989 when two groups independently identified a heparin-binding protein acting as a mitogen specific for endothelial cells. Subsequently, it was revealed that VPF and VEGF are the same protein encoded by a single gene (99;100). VEGF is a potent mitogen in embryonic and somatic angiogenesis with a unique specificity for vascular endothelial cells (101;102). The central role of VEGF in embryonic angiogenesis was illustrated in heterozygote knock-out mice suffering from fatal deficiencies in vascularization (102).

2.3.3.2. VEGF family members, receptors and mode of action

There are five VEGF variants including VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor; all described in mammals, as well as VEGF-E (found in Parapoxviridae), VEGF-F (also called svVEG-F for snake venom VEGF found in viper venom), each with structurally similar proteins involved in the regulation and differentiation of the vascular system, particularly in the blood and lymph vessels (90;97;101;102). Table 4 shows the similarities and differences of the human VEGF members' family.

Among these subtypes, VEGF-A has a main role in mediating angiogenic effects (90;101). VEGF-A binds to and activates two corresponding receptors on the cell membrane of endothelial cells, namely VEGF receptor 1(also VEGFR-1 of Flt-1) and VEGF receptor-2(also VEGFR-2 or FlK-1 or KDR). These receptors regulate physiologic and pathologic angiogenesis (90). VEGFR-2 is mainly associated with pathological angiogenesis, such as vascular network formation in tumors and diabetic retinopathy. VEGFR-1, however, has a

dual role; in embryo it has a negative effect on angiogenesis via isolation of VEGF-A, while in adults it has a main influence on monocytes and endothelial cells that stimulate angiogenesis (90;93;101).

Table 4: The chromosomal localization, the similarities of and splice variants of different VEGF family members (90;103)

Gene	Sequence homology	Number of exons	Chromosomal localization	Splice variants
VEGF		8	6p23.1	121,145,165*,183*,189*,206*
VEGF-B	45% homology with VEGF-A	7	11q13	183,189,206
VEGF-C	30% homology with VEGF-A_{165}	7	4q34	-
VEGF-D	61% homology with VEGF-C; 31% with VEGF-A_{165}	7	Xp22.31	-
PlGF	42% homology with VEGF-A		14q24	131,152*,219

***** Splice variants that bind to heparan sulfate proteoglycans

Table 5: Properties of different VEGFs

Ligand	Isoforms	Receptor	Solubility	Source in adults	Biological Activities	Phenotype of knockout Mouce
VEGF-A	VEGF-A_{121}, VEGF-A_{165} VEGF-A_{189}, VEGF-A_{206} (also VEGF-$A_{138/145/162/165b}$ have been described)	VEGFR-1 and R-2, $VEGF_{165}$ binds to neuroplin-1 and -2, $VEGF_{145}$ neuroplin-2	VEGF121 soluble, longer forms bind to heparin sulfates with increasing affinity	Almost all vascularized tissues, especially fenestrated and sinusoidal endothelium, up-regulated by ischemia (via HIF-1α)	Vasculogenesis, angiogenesis, vascular homeostasis, vascular permeability and recruitment of bone marrow-derived cells	Loss of even single VEGF allele leads to embryonic lethality due to impaired vasculogenesis and angiogenesis
PlGF	$PlGF_{131}$ (PlGF-1), $PlGF_{152}$ (PlGF-2), $PlGF_{203}$ (PlGF-3)	VEGFR-1, $PLGF_{152}$ binds to neuroplin-1 and -2	PlGF131 and PlGF203 soluble, PlGF152 binds to heparin sulfate	Placenta, thyroid, lung, and goiter	Angiogenesis, monocyte migration, recruitment of bone marrow-derived cells, up-regulation of VEGF-A	Almost-normal phenotype and fertile with minor defects in vascular growth in pathological conditions
VEGF-B	VEGF-B_{167} and VEGF-B_{186}	VEGF-1 and neuroplin-1	VEGF-B167 binds to heparin sulfates, VEGF186 soluble	Heart, skeletal muscle, and vascular smooth muscle cells	Angiogenesis, recruitment of bone marrow-derived cells	Almost-normal phenotype with minor possible defects: reduced heart size, prolonged PQ-time, impaired recovery from ischemia
VEGF-C (VEGF-2)	Unprocessed and proteolytically processed mature forms	VEGF-2, R-3, and neuroplin-2, processing increase receptor affinity	Soluble	Neuroendocrine organs, lung, heart, kidney, and vascular smooth muscle cells	Development of lymphatics and lymphangiogenesis, angiogenesis	Lethal because of impaired development of lymphatics
VEGF-D	Unprocessed and proteolytically processed mature forms	VEGF-2 and VEGF-3, processing increases receptor affinity	Soluble	Neuroendocrine organs, lung, heart, skeletal muscle, intestine, and vascular smooth muscle cells	Lymphangiogenesis and angiogenesis	Normal
VEGF-E	---	VEGF-2 and neuroplin-1	Soluble	Virus-derived	Angiogenesis	---
VEGF-F	---	VEGFR-2	Binds to heparan sulfates	Snake venom	Angiogenesis and vascular permeability	---

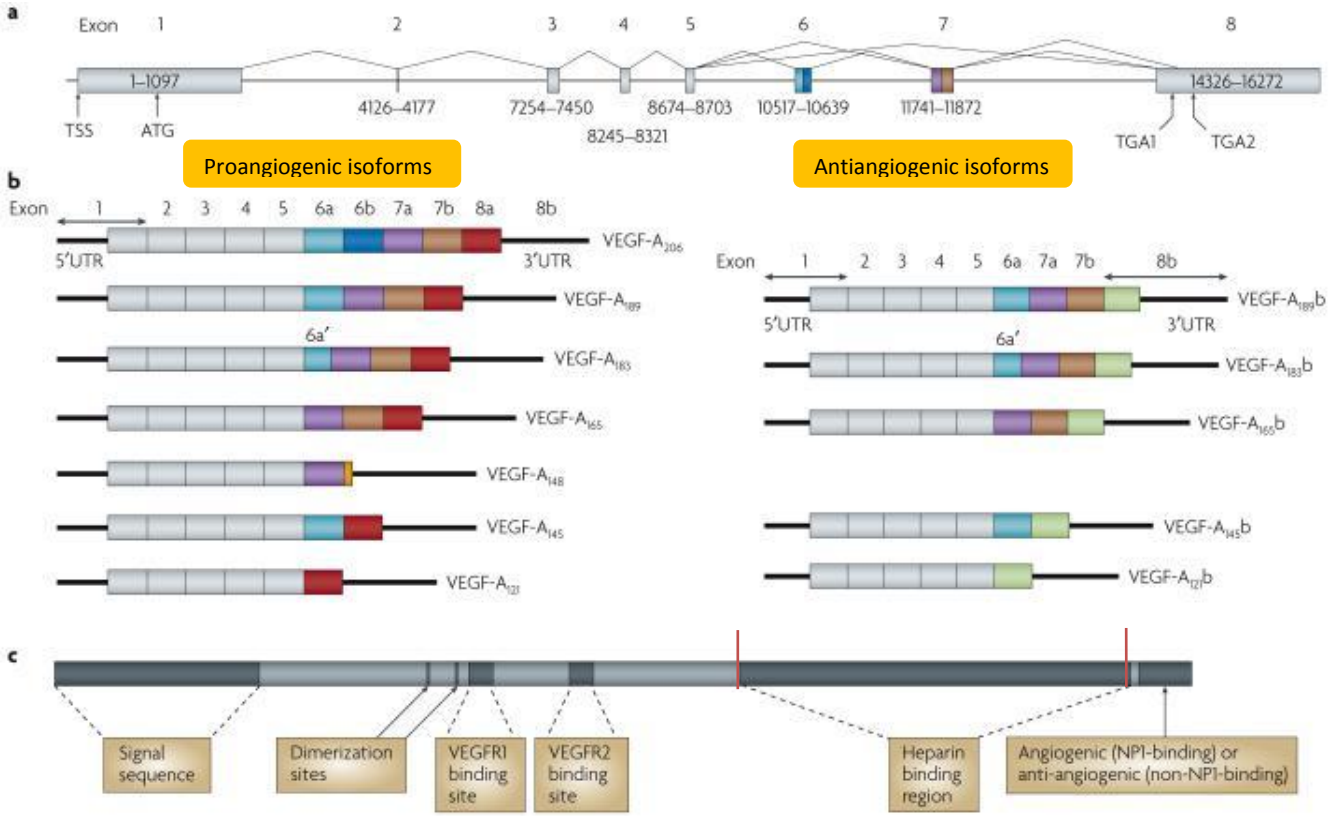
HIF = hypoxia-inducible factor; PlGF = placental growth factor; VEGF = vascular endothelial growth factor [Adapted from (232)].

2.3.3.3.VEGFA gene, related isoforms and proteins

The human VEGF-A gene is organized in eight exons, separated by seven introns and is located in chromosome 6p21.3. The coding region spans approximately 14 kb. Alternative exon splicing results in the generation of four different isoforms, having 121, 165, 189, and 206 amino acids, respectively, after signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). $VEGF_{165}$, the predominant isoform, lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, including $VEGF_{145}$, $VEGF_{183}$, as well as $VEGF_{121b}$, $VEGF_{145b}$,$VEGF_{165b}$, $VEGF_{189b}$ the variants reported to have paradoxically an inhibitory effect on VEGF induced mitogenesis (90). Figure 4 shows more details of VEGF gene, its isoforms and protein.

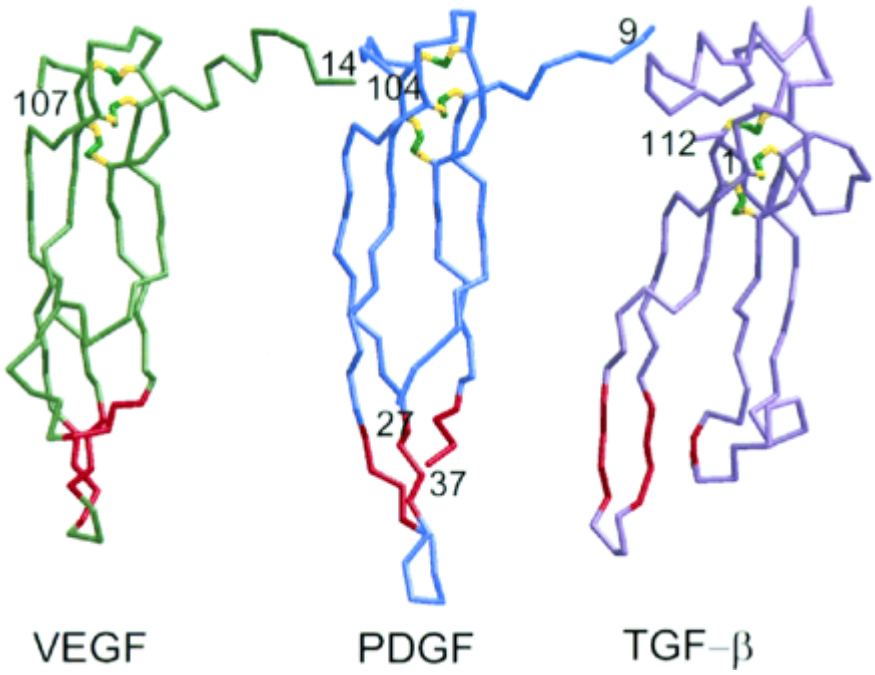
Solution of the crystal structure of VEGF has shown that VEGF forms an antiparallel homodimer covalently linked by two disulfide bridges (90;104)(Figure 5). This mode of dimerization is similar to that of the PDGF monomers. $VEGF_{121}$ is an acidic polypeptide that fails to bind to heparin. $VEGF_{189}$ and $VEGF_{206}$ are highly basic and bind to heparin with high affinity. $VEGF_{121}$ is a freely diffusible protein. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix. $VEGF_{165}$ has intermediate properties, because it is secreted, but a significant fraction remains bound to the cell surface and extracellular matrix. Several researches suggest that $VEGF_{165}$ has optimal characteristics of bioavailability and biological potency (90;101;104).

Figure 4: **Protein and mRNA products of human vascular endothelial growth factor A (VEGF-A)(105)**



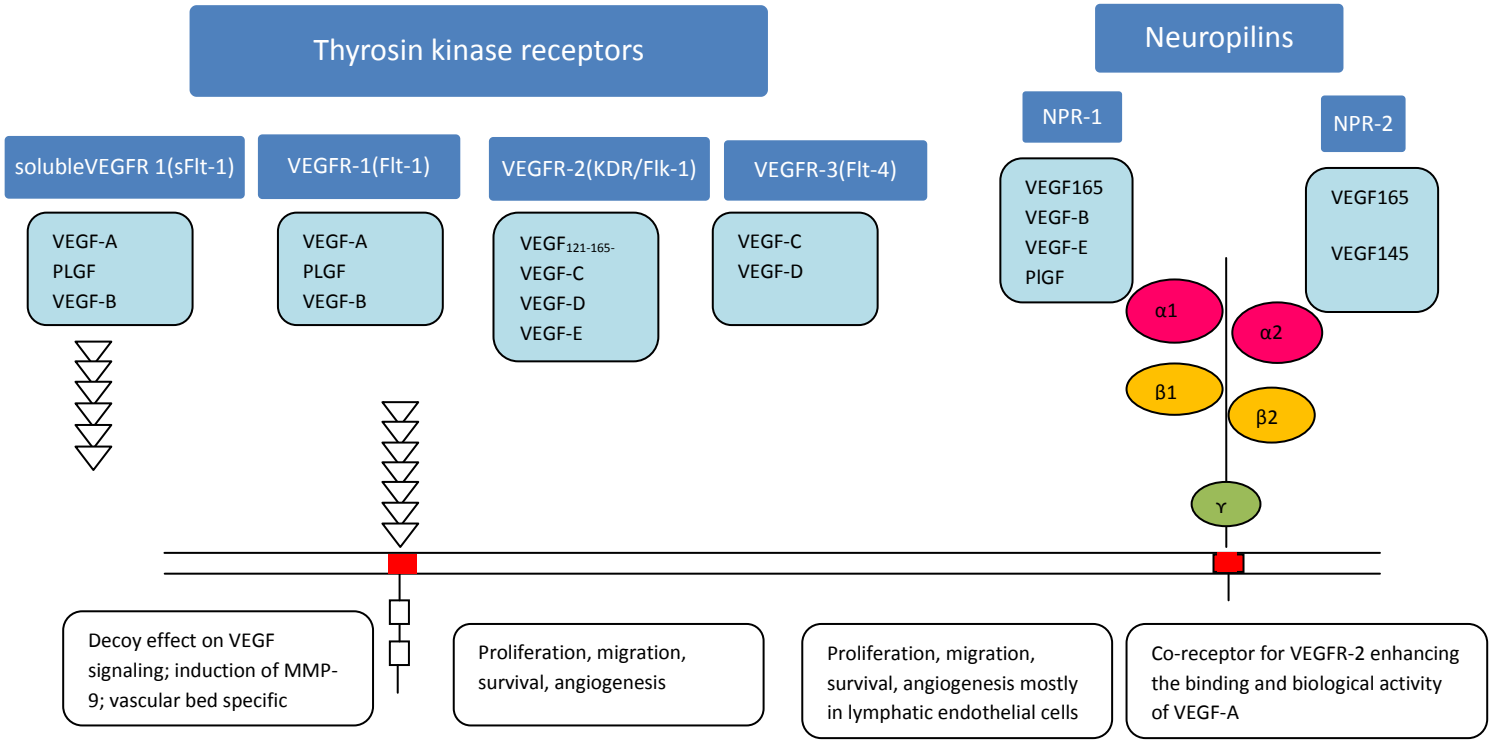
a | Gene structure of human *VEGF-A*. *VEGF-A* spans 16,272 bp of chromosome 6p12 and consists of eight exons. Alternate 5′ and 3′ splice site selection in exons 6, 7 and 8 generate multiple isoforms. Exons 6 and 7 encode heparin-binding domains. The transcriptional start site (TSS) and translational start site (ATG) in exon 1 are indicated. Alternative stop codons within exon 8 are also indicated (TGA1 and TGA2). **b** | Alternative splicing can occur either at the 5′ donor splice site (for example, *VEGF-A*$_{189}$ versus *VEGF-A*$_{206}$) or the 3′ acceptor splice site (for example, *VEGF-A*$_{189}$ versus *VEGF-A*$_{165}$). Two mRNA isoform families are generated. The pro-angiogenic isoforms (*VEGF-A*$_{xxx}$, left) are generated by proximal splice site (PSS) selection in exon 8 and the anti-angiogenic family (*VEGF-A*$_{xxx}$*b*, right) from exon 8 distal splice site (DSS) choice. Thus, *VEGF-A*$_{165}$, formed by PSS selection in exon 8, has *VEGF-A*$_{165}$*b* as its DSS sister isoform, the DSS-selected mRNA encoding a protein of exactly the same length. Exon 6a' occurs in *VEGF-A*$_{183}$ as a result of a conserved alternative splicing donor site in exon 6a and is 18 bp shorter than full-length exon 6a. *VEGF-A*$_{148}$ is a truncated isoform splicing from exon 7a into exon 8a out of frame and resulting in a premature stop codon. *VEGF-A*$_{206}$*b* has not yet been identified. **c** | Protein structure of VEGF-A containing the dimerization sites and binding sites for heparin, VEGF-A receptor 1 (VEGFR1; encoded by exon 3) and VEGFR2 (encoded by exon 4), which are present in all isoforms. The six amino acids at the extreme carboxyl terminus of the protein can be either pro-angiogenic (CDKPRR, encoded by exon 8a) or anti-angiogenic (SLTRKD, encoded by exon 8b). The epitopes recognized by most commercial antibodies are in the region of the VEGF-A receptor-binding domains, present in VEGF-A isoforms of both families. UTR, untranslated region [Adapted from (105)].

Figure 5: Comparison of the monomer of VEGF with PDGF and TGF-β.



Comparison of the monomer of VEGF (*Left*) with PDGF (*Center*) and TGF-β (*Right*). PDGF and TGF-β were aligned with VEGF using 71 and 45 Cα positions, respectively, in the cystine knot region of the molecules. The short three-stranded sheet at the opposite end of the molecules from the cystine knot is shown in red. The bend in the middle of the four-stranded sheet of TGF-β has the effect of twisting this sheet compared with the orientation observed in VEGF and PDGF. In the VEGF dimer, residues from this short sheet and the N-terminal helix from the other monomer that packs against it contain the functional determinants for KDR binding. VEGF: vascular endothelial growth factor, PDGF: Platelet derived growth factor, TGF: Transforming growth factor[Adapted from (102)].

Figure 6: The members of VEGF family , their related receptors and the outcome of their interactions



The cell surface receptors for VEGF. Known examples of each receptor type are indicated and identified ligands listed below. Where particular ligand isoforms are not specified, the receptor is believed to bind all identified variants. VEGF tyrosine kinase receptors consist of seven extracellular Ig-like domains (numbered), a transmembrane region and an intracellular tyrosine kinase domain interrupted by a kinase-insert sequence. The neuropilins are isoform-specific receptors for certain VEGF family members. NRP1 acts as a co-receptor for VEGFR-2, enhancing the binding and biological activity of VEGF165. The α1-α2 region has homology to components of the complement system; β1-β2 shares homology with coagulation factors V and VIII. [Adapted from (90, 93, 101, 103)].

2.3.3.4 VEGF heritability

Heritability of growth factors has been largely studied because of their physiological and pathological importance, for example, in skeletal system aging. In apparently healthy and ethnically homogeneous white families a significant heritability component has been reported for IGF-I, IGFBP-3 and TGF-beta1 46.7%, 57.8%, and 34.0%, respectively (106).According to the familial correlation study there were familial factors influencing plasma VEGF concentration, the most parsimonious model being the one which did not take into account parents' and children's sex (107). Negligible spouse correlation suggested little influence of shared household environment on this trait. Evidence indicates that heritability of circulating VEGF levels is very high, ranging between 60% and 80%(107).

2.3.3.5. Regulation of VEGF expression

2.3.3.5.1. VEGF expressing cells and tissues

VEGF is secreted under normal conditions by endothelial cells, platelets and vascular smooth muscle cells, macrophages, monocytes, lung epithelial cells, kidney epithelial cells (podocytes), follicular cell in the pituitary, corpus luteum cells, adipose derived stem cells adipose stromal cells and in abnormal conditions by all mentioned cells and omentum adipocytes, retinal pigment epithelial cell, synovial cells and by several tumor cells in vivo and in vitro. Regulation of VEGF levels has been reported to occur at the gene transcriptional, translational, and posttranslation levels. Transcriptional regulation of VEGF expression has been studied extensively, because the effect of most genetic and epigenetic factors on VEGF expression is mediated by the regulation of the VEGF gene transcription. VEGF mRNA is expressed in several normal human tissues including lung, kidney, liver, gastric mucosa and at a lower level in breast tissue (108). Several evidences indicate that peripheral blood mononuclear cells, circulating and tissue macrophages (peritoneal macrophage), monocytes, fibroblasts cell line CCD 18, mast cells and adipocytes express the VEGF gene as well (108).

2.3.3.5.2. VEGF expression influencing factor

VEGF expression can be extremely enhanced by numerous microenvironmental factors such as hypoxia, acidosis, oxidative stress and disregulated production of several growth factors and cytokines such as insulin growth factor-1, hepatocyte growth factor, fibroblast growth factor, epidermal growth factor, vascular endothelial growth factor, platelet drivating growth factor (Table 6) (109). Many other stimuli can modulate VEGF expression including iron chelators, glucose deficiency, prostaglandins, serum starvation, ionizing radiation and ultraviolet B radiation. It should be mentioned that VEGF expression induced by these signals may result in a function not as an angiogeneic factor but rather as a surviving element for preexisting vessels and endothelial cells (109).

2.3.3.5.3. VEGF transcription factors

Each of these regulators acts through transcriptional factors. Many transcriptional factors are involved in the up regulation or repression of VEGF gene transcription (110), including: hypoxia inducible factors (HIF1 and HIF2), specific protein 1 (Sp1), activating protein-1 (Ap-1), nuclear factor kappa b (NF-κB) and some of other factors. The way that these factors come together to regulate the cell type-, tissue- and context-specific expression of each of the VEGF gene remains unclear (111).

Table 6 : Influence of growth factors and cytokines on VEGF expression(109)

Cytokines or growth factors	Cell lines	Effect on VEGF level
IL-1α	Blood mononuclear cells, umbilical endothelial cells	Up regulation, up regulation
IL-1β	Smooth muscle cells, synovial cells, Visceral glomerular epithelial cells	Up regulation, up regulation, Up regulation
IL-3	Vascular endothelial cells	Up regulation
IL-4	visceral glomerular epithelial cells, Blood mononuclear cells	Down regulation
IL-6	synovial cells, cervical and pancreatic cancers	Up regulation
IL-10	Blood mononuclear cells, Visceral glomerular epithelial cells	Down regulation
IL-12	Blood mononuclear cells	Up regulation
IL-13	Smooth muscle cells, Visceral glomerular epithelial cells	Up regulation, Down regulation
IL-15	Blood mononuclear cells	Up regulation
IL-18	Blood mononuclear cells	Up regulation
IFN-α	Smooth muscle cells, Blood mononuclear cells	Down regulation
bFGF	Vascular endothelial cells	Up regulation
EGF	Gastric and endometrial stromal and prostate cancers cells	Up regulation
IGF-1	Endothelial cell, mesangial cells	Up regulation
PDGF	Vascular smooth muscle cells, endothelial cells	Up regulation
TGF-β	Vascular smooth muscle cells, endothelial cells	Up regulation
TNF-α	Vascular smooth muscle cells, endothelial cells	Up regulation
Leptin	Endothelial cells, adipocytes	Up regulation of VEGFmRNA

2.3.3.6 VEGF activities

2.3.3.6.1. VEGF functions in angiogenesis and lymphangiogenesis

A well-documented *in vitro* activity of VEGF is the ability to promote growth of vascular endothelial cells derived from arteries, veins, and lymphatics. VEGF promotes angiogenesis in tridimensional *in vitro* models, inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures. Also, VEGF induces sprouting from rat aortic rings embedded in a collagen gel. VEGF also elicits a pronounced angiogenic response in a variety of *in vivo* models including the chick chorioallantoic membrane, the rabbit cornea; the matrigel plug in mice, the primate iris, *etc.* (90;104). VEGF delivery also induces lymphangiogenesis in mice, at least in some circumstances. Ergun *et al.* (112) have proposed that induction of carcinoembryonic antigen-related cell adhesion 1, a membrane glycoprotein expressed in some microvascular endothelial cells, mediates some of the angiogenic effects of VEGF.

VEGF is also a survival factor for endothelial cells, both *in vitro* and *in vivo* (113;114). *In vitro*, VEGF prevents endothelial apoptosis induced by serum starvation. Such activity is mediated by the phosphatidylinositol 3-kinase (PI3 kinase)/Akt pathway (113;114).

Although endothelial cells are the primary targets of VEGF, several studies have reported mitogenic effects also on other cell types, such as retinal pigment epithelial cells (90;101;104), pancreatic duct cells, and Schwann cells. Compernolle *and colleagues* (115) have also shown that VEGF stimulates surfactant production by alveolar type II cells, resulting in a protective effect from respiratory distress syndrome in mice. Other studies have emphasized the potential role of VEGF as a neuronal protective factor, and a haplotype in the VEGF gene promoter associated with reduced VEGF expression has been reported to be is a risk factor for amyotrophic lateral sclerosis(104). VEGF also has several secondary influences

through the induction of a number of active enzymes that have a wide range of actions, such as nitric oxide, plasminogen activators and endothelial cell decay-accelerating factor.

2.3.3.6.2. Influences of VEGF on bone marrow cells and hematopoiesis

The ability of monocyte chemotaxis promotion was the earliest evidence that VEGF can affect blood cells. After that, VEGF was reported to have hematopoietic effects, inducing colony formation by mature subsets of granulocyte-macrophage progenitor cells. Notably, VEGF infusion to adult mice inhibits dendritic cell development, leading to the hypothesis that VEGF facilitates tumor growth by allowing escape of tumors from the host immune system. Also, VEGF increased production of B cells and the generation of immature myeloid cells. Some studies suggest that VEGF controls hematopoietic stem cells survival during hematopoietic repopulation (116)

2.3.3.6.3. Increasing of vascular permeability and hemodynamic effects

Nowadays, VEGF is known also as vascular permeability factor, based on its ability to induce vascular leakage. The permeability-enhancing activity of this molecule underlies dominant roles in inflammation and other pathological conditions. In accordance with a role in the regulation of vascular permeability, VEGF induces endothelial fenestration in some vascular beds and in cultured adrenal endothelial cells (90;104). Different studies have shown a critical role of nitric oxide (NO) in VEGF-induced vascular permeability as well as angiogenesis. Fukumura *et al.*(117) have verified the relative contribution of the NO synthase (NOS) isoforms, inducible NOS and endothelial NOS (eNOS) to these processes

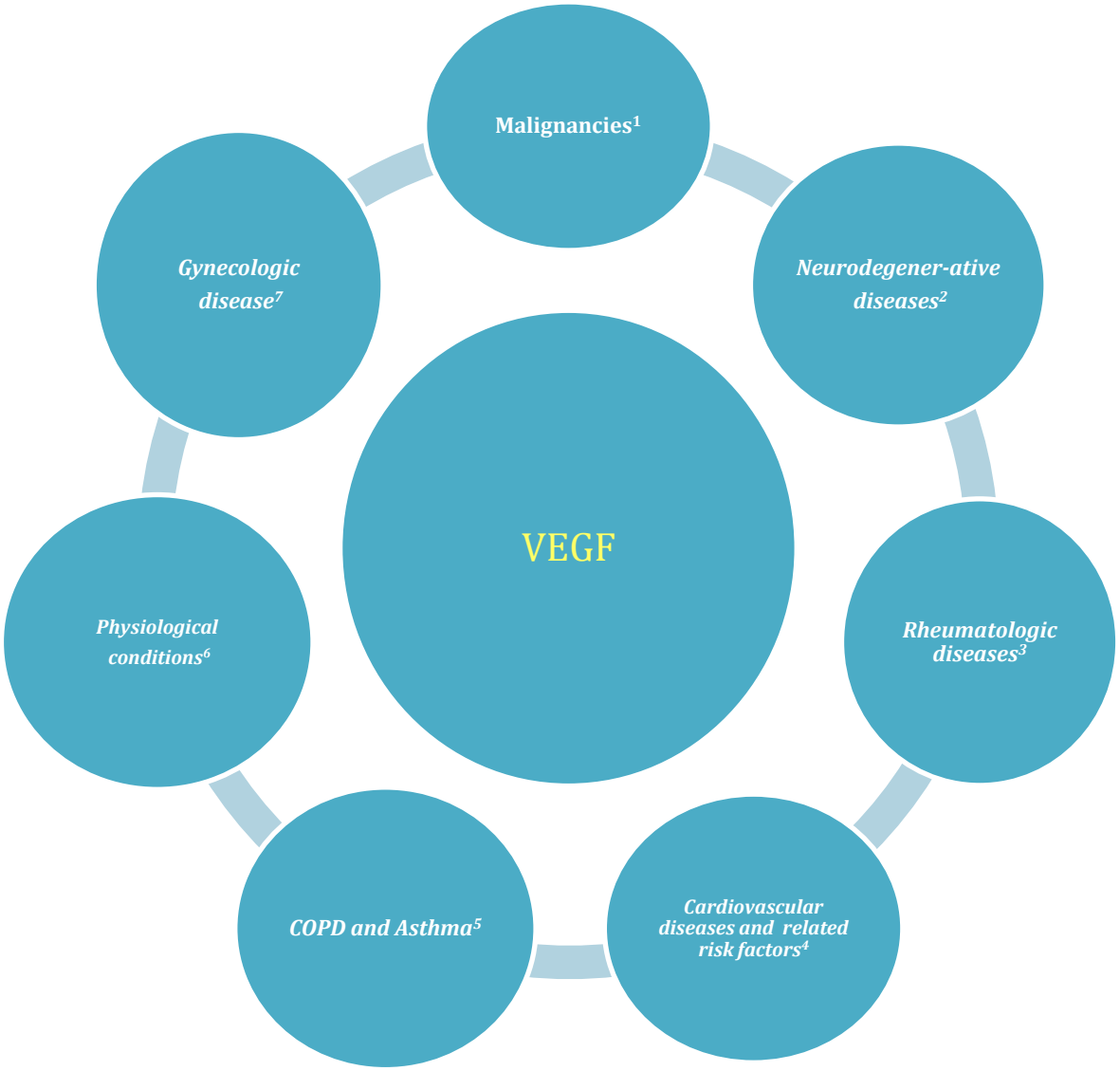
2.3.3.7. Clinical significance of VEGF

2.3.3.7.1. Wide implications of VEGF, from physiological circumstance to pathological conditions

Angiogenesis, or the growth of new blood vessels, is required for any process that results in the accumulation of new tissue as well as many processes involving tissue remodeling. When the regulation of angiogenesis fails, blood vessels are formed excessively or insufficiently. Angiogenesis is thus a characteristic of multiple pathologies including cancer, CVDs, rheumatoid arthritis, psoriasis, macular degeneration, and diabetic retinopathy (Figure 8). Throughout development, VEGF orchestrates the process of angiogenesis by regulating the growth, development, and maintenance of a healthy circulatory system.

VEGF is involved in hematopoietic development and chemo taxis of monocytes, regulation of osteoclast differentiation, stimulation of surfactant production and has neurotrophic and neuroprotective effects on neuronal and gelial cells. Elevated circulating VEGF have been observed in vascular diseases(ischemic heart disease, heart failure and stroke) and in various other disorders including diabetes, cognitive decline and dementia, reproductive (polycystic ovary disease, endometriosis), immunoallergic-inflammatory disease (asthma, rheumatoid arthritis) psoriasis, ophthalmologic disorders (macular degeneration, and diabetic retinopathy), chronic obstructive pulmonary diseases and several neoplastic diseases (118). In point of its physiologic activity view, during pregnancy, VEGF is involved in building the placenta. It is noteworthy to mention its role in response to ovulation. Also it has a dominant role in the wound and bone fracture healing processes. Briefly, we discuss here the role of VEGF in wound healing as one of its physiologic activities as well as some of the relevant clinical aspects of VEGF contribution in CVDs.

Figure 7: VEGF involved in several physiological and pathological conditions.



1) Breast, colorectal, prostate, gastric, esophageal, hepatic, ovarian cancers and hematologic malignancies. 2) Alzheimer, multiple sclerosis, age related macular degeneration. 3) Rheumatoid arthritis, Psoriasis, Behjat disease and systemic sclerosis. 4) Hypertension, obesity, diabetes type 2 (diabetic retinopathy and nephropathy), hyperlipidemia, MetS**.** 5) Chronic obstructive pulmonary diseases ***s***uch as chronic bronchitis and emphysema. 6) Response to ovulation, placentation, embryonic development, wound and bone fracture healing. 7) Pre-eclampsia, endometriosis.

2.3.3.7.2. Wound healing

Skin-wound healing starts immediately after injury and consists of three phases: inflammation, proliferation, and maturation. These phases proceed with complicated but well-organized interaction between various tissues and cells. Wounding can destructs the blood vessels and creates a hypoxic environment because of poor perfusion, and therefore provides an appropriate environment for hypoxia inducible factor alpha stabilization. At the first phase of wounding, induced hypoxia leads to rapid infiltration of inflammatory cells including neutrophils, mast cells, lymphocytes, and macrophages. The formation of granulation tissue, which is necessary for the last phase of wound healing, starts at the wound space approximately 4 days after injury. Numerous new capillaries endow the new stroma with its granular appearance. Macrophages, fibroblasts, and endothelial cells move into the wound space at the same time. Macrophages not only augment inflammatory responses but also secrete vascular endothelial growth factor (VEGF) and FGF, eventually promoting angiogenesis (119;120).

Proangiogenic roles of macrophages in diabetic wounds are similar to those in wounds inflicted by physical injuries. However, severe hypoxia in diabetic wounds often fails to directly trigger effective angiogenesis because of significant cell death under such conditions. As a practical conclusion, the hypoxia inducible factor pathway offers promising therapeutic targets to promote angiogenesis in wounds (119).

2.3.3.7.2. Diabetes and its related complications

Hiroaki Kakizawa et al.(121) have reported that plasma VEGF concentration is higher in diabetic patients who are hospitalized because of poor glycemic control than in healthy subjects. According to them, the increased plasma VEGF concentrations declined along with decreases in fasting plasma glucose and hemoglobin A1c (HbA1c) as a result of treatment. The significant and independent correlation between plasma VEGF concentrations and

HbA1c suggests that chronic hyperglycemia may increase plasma levels of VEGF, and that reduction of high levels of VEGF may be possible by improvement of glycemic control (122). Prolonged hyperglycemia activates the sorbitol pathway and induces intracellular anaerobic conditions and hemodynamic change (123). These conditions may facilitate the production of VEGF that may contribute to diabetic vascular complications and arteriosclerosis. Interestingly, the increasing of VEGF production has a reveres correlation with the reduction of hyperglycemia. Santilli et al (124) have suggested increased serum VEGF levels as a predicting risk factor for developing persistent microalbuminuria in young type 1 diabetic patient. Another research indicates that VEGF mRNA and urinary excretion of VEGF are increased in diabetic nephropathy (125).

Most diabetic patients, especially those with poor glycemic control, develop diabetic retinopathy, which remains the major cause of new-onset blindness among diabetic adults. Diabetic retinopathy is characterized by vascular permeability and increased tissue ischemia and angiogenesis.

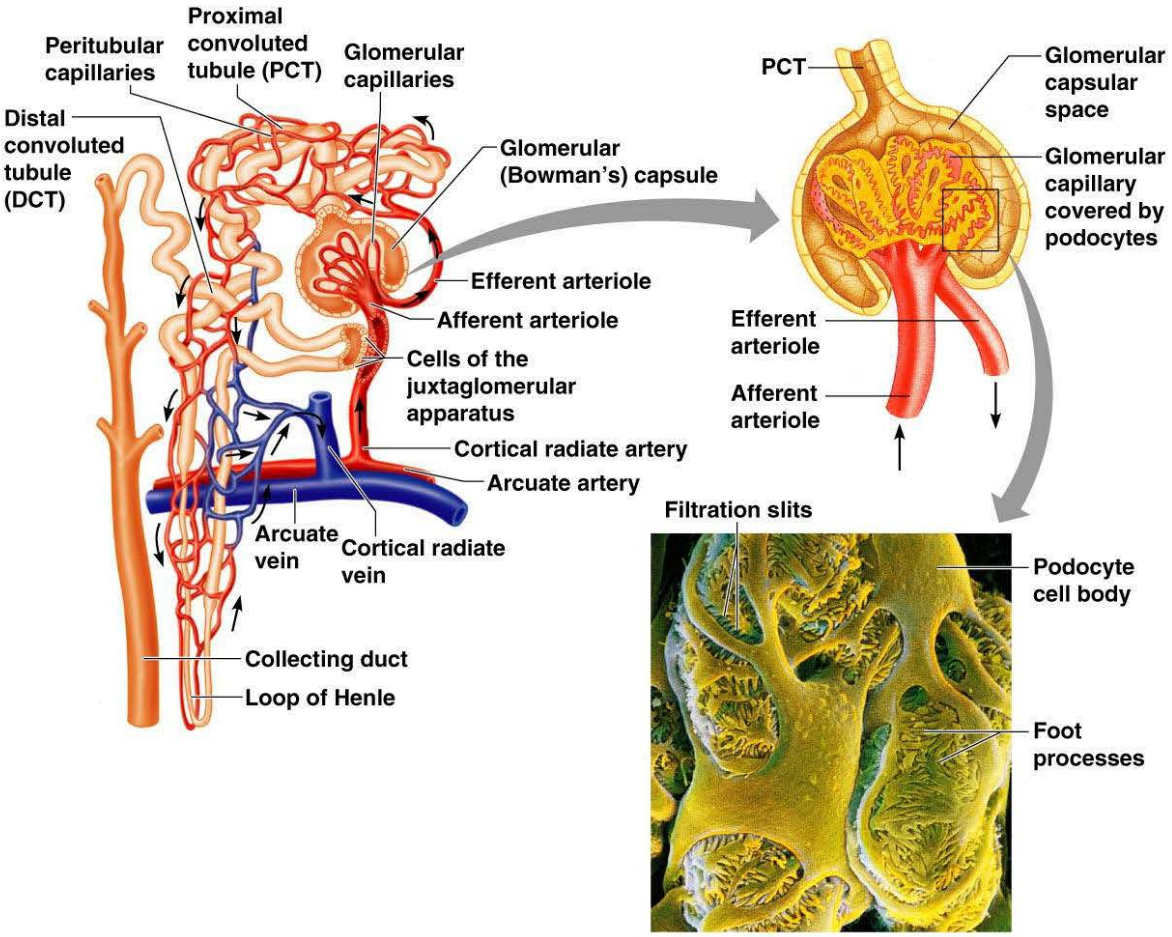
Vascular endothelial growth factor (VEGF) has initially drawn much attention as an important mediator of retinal ischemia– associated intraocular neovasclarization (90;101;104). VEGF is produced from many cell types within the eye and past studies have shown that VEGF levels are markedly elevated in vitreous and aqueous fluids in the eyes of individuals with proliferative diabetic retinopathy (PDR). Recently there has been success in the treatment of diabetic retinopathy as well as other ocular vasculopathies with anti VEGF medication. Bevacizumab (Avastin, Genentech, South San Francisco, CA) is a recombinant, humanized monoclonal anti-VEGF antibody that binds all VEGF isoforms and exerts its neutralizing effect by inhibiting the VEGF–receptor interaction, thus blocking both increased vascular permeability and angiogenesis. The drug is approved by the United States Food and Drug Administration initially for intravenous use for metastatic colorectal cancer. Bevacizumab has

been administered off label for the treatment of neovascular age related macular degeneration and other retinal vascular conditions with encouraging results (90).

2.3.3.7.4. Hypertension

VEGF-A is highly expressed by renal glomerular epithelial cells (podocytes) (figure 9) and plays an important role in the formation of the glomeruli during development (126), but curiously is also highly expressed within the adult glomerulus despite little or no angiogenesis occurring beyond development. VEGF isoform expression in glomeruli is heterogeneous. Individual human glomeruli express one, two, or all three of these ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$) main isoforms at the mRNA level. $VEGF_{189}$ and $VEGF_{165}$ are the most predominantly found in the renal glomerulus. Minor VEGF mRNA splice variants ($VEGF_{206}$, $VEGF_{183}$, $VEGF_{148}$, and $VEGF_{145}$) have also been reported, but they are less well characterized (127).

Figure 8: Podocytes localization in kidney functional unit (nephron)



Cytosolic calcium is an important second messenger in most cells, including endothelial cells, and its concentrations play an important role in VEGF-mediated permeability, mitogenesis, and vasodilatation (127). Foster et al (127) have been reported that VEGF stimulates a reduction in cytosolic calcium concentration under any circumstances in any cells. It is generally accepted that angiotensin II accelerates the increase of cytosolic calcium concentration, because inhibition of the renin-angiotensin system produces benefits in renal survival in both animal models and humans. If the VEGF-induced reduction in cytosolic calcium concentrations is functionally important in vivo, then it may explain the mechanism by which VEGF acts as a cytoprotective agent in such lesions by counteracting the angiotensin II-driven increases in podocyte cytosolic calcium concentrations (127).

It has been suggested that a balance of glomerular VEGF-A may be important in maintaining the normal physiology of the filtration barrier, but that other players are also involved. For example, glomerular VEGF-A expression is increased in many glomerulopathies, such as human crescentic glomerulonephritis and minimal-change nephropathy. It should be mentioned that continued injury of podocytes participates in the progression of chronic renal lesions(127).Exogenous VEGF treatment accelerated glomerular recovery in experimental thrombotic microangiopathy, yet podocyte-specific over expression of VEGF-A led to collapsing glomerulopathy (128). Three common renal related conditions are associated with changes in VEGF expression: hypertension, pre-eclampsia, and diabetic nephropathy.

2.3.3.7.4.1. Essential hypertension

Essential hypertension is an important risk factor for CVDs and stroke, and may be a consequence of an impaired capacity for vascular growth, resulting partly from abnormal regulation of vascular endothelial cell growth factor. Belgore's et al (129) study indicates that plasma VEGF levels are significantly raised in uncomplicated essential hypertensive patients when compared with normotensive controls, and treatment of hypertension significantly

reduced plasma VEGF levels. Interestingly, Tsai et al (130) have been shown that VEGF is significantly higher in hypertensive patients with retinopathy than in hypertensive patients without retinopathy or normotensive subjects. In this direction, Ebinç et al (131) have reported that VEGF may increase glomerular permeability and lead to microalbuminuria in essential hypertension. These studies suggest that VEGF can be a marker for early microvascular damage in hypertension. Additionally, Zorena et al (122) have been shown that systolic blood pressure is an independent factor influencing the concentration of VEGF in children and adolescents with diabetes type 1.

2.3.3.7.4.2. Pre-eclampsia (pregnancy-induced hypertension)

Human placenta is a rich source of angiogenic factors and these may play a crucial role in the regulation of placental vessel formation as well as in maternal vascular adaptation to pregnancy. VEGF expression has been described in villous trophoblast and fetal macrophages within villous stroma (132). An adequate nutrient and substrate supply is essential for the normal intrauterine development of the fetus. Disturbances in uterine blood supply are associated with higher perinatal morbidity and mortality due to preterm delivery, pre-eclampsia and pregnancy disorders such as intrauterine growth restriction (IUGR).

Pre-eclampsia, a pregnancy-specific syndrome characterized by hypertension, proteinuria and edema that usually is diagnosed in late pregnancy. Normal pregnancy is itself characterized by systemic inflammation, oxidative stress and alterations in levels of angiogenic factors and vascular reactivity. This is exacerbated in preeclampsia with an associated breakdown of compensatory mechanisms, eventually leading to placental and vascular dysfunction. The underlying pathology of preeclampsia is thought to be a relatively hypoxic or ischemic placenta. Preeclampsia symptoms disappear shortly after delivery of the placenta. Several studies have confirmed the increased levels of VEGF in pre-eclampsia (133).

Parallel reports have shown that elevated concentrations of VEGF fall to levels similar to those of non-preeclampsia patients within 24 hours of delivery. This suggests that the main source of VEGF production lies within the fetus and placenta(134). Vascular endothelial growth factor (VEGF) and its receptors may play an important role in the altered function of pre-eclampsia.

Physiological concentrations of VEGF are important for podocyte homeostasis and survival, (127;128) thus, according to some documents, the clinical syndrome of pre-eclampsia is caused by impaired vascular endothelial growth factor signaling that disturbs the status of vascular dilatation as well as the symbiosis between the glomerular endothelium and the podocytes (135).

El-salahy et al (133) have been proposed that VEGF may be a clinical biomarker for better diagnosis of pre-eclampsia and also could be useful for the differentiation between mild and severe conditions; furthermore, VEGF may be a potentially useful target for therapeutic intervention in this group of patients.

2.3.3.6.5. Diabetic nephropathy

VEGFA has been reported to affect ureteric bud growth during embryogenesis and hence may affect nephron numbers. In rodent and human kidneys$VEGF_{165}$ was detected predominantly in glomerular podocytes, distal tubules, and collecting ducts, and to a lesser extent in some proximal tubules (136). VEGF mRNA and protein expression were increased in glomerular podocytes, distal tubules and collecting ducts at the onset of diabetes in genetically diabetic rats (137). Bortoloso E et al (138) have demonstrated that in type 2 diabetic patients glomerular qualitative and quantitative changes of VEGF mRNA expression are associated with functional and structural renal changes. Urinary VEGF excretion increases with the progression of diabetic nephropathy and correlated weakly with the levels of serum creatinine, creatinine clearance, microalbuminuria, and proteinuria (139). In biopsies with

mild changes of diabetic nephropathy, VEGF is up regulated in glomerular podocytes and distal tubular cells (139).

In patients with type 2 diabetic nephropathy, glomerular VEGF mRNA expression, observed predominantly in podocytes, is higher than in normal kidneys (139).

The advanced lesions in diabetic nephropathy have associated with an increase of renal VEGF expression (140). According to Waichi Sato and colleagues (141) there is a positive correlation between VEGF expression and macrophage infiltration in glomeruli of diabetic patients. They also found a decrease in VEGF expression in response to insulin that is associated with a reduction of macrophage infiltration in glomerulus. Their evidences have suggested that the accumulation of macrophages might result in part from response to an increased expression of VEGF in podocytes.

Finally, the third proposed mechanism of VEGF activities in diabetic kidney is focused on the influence of Angiotensin II on VEGF. In cultured mesangial cells angiotensin II induces VEGF expression. Also podocytes carry functional angiotensin II (AT1 type) receptors (142). It is previously shown that angiotensin II enhances VEGF-mRNA expression in vascular smooth muscle cells and in human mesangial cells. It is shown that high glucose concentrations alone can induce VEGF-mRNA expression and protein production in vascular smooth muscle cells (142).

2.3.3.7.6. Obesity and metabolic syndrome

Adipose tissue is considered as the largest endocrine gland because it produces free fatty acids, hormones, growth factors, and cytokines such as leptin, adiponectin, resistin, vascular endothelial growth factor (VEGF), insulin growth factor (IGF-1), IL-6, tumor necrosis factor (TNF-α) (143). Epidemiological studies show that the visceral fat accumulation ”the predominant driving force behind the MetS (144)” is the most important determinant factor for VEGF circulating levels. During embryogenesis, adipose tissue development is spatially and temporally associated with microvessel growth. In developing embryos, the formation of

primitive fat organs occurs at the perivascular site (143). Endothelial cells isolated from different adipose tissues differ in their proliferative capacity, which suggests that adipocytes play both guidance and maintenance roles in vascular development. A recent study suggests that adipocytes and their accompanying endothelial cells might share a common progenitor that could differentiate into adipocytes or endothelial lineages depending upon exposure to different environments (145). Accumulating evidence shows that capillary endothelial cells communicate with adipocytes via paracrine signaling pathways, extracellular components, and direct cell-cell interactions (145).

Adipose tissue has been long known to promote wound healing and to revascularize ischemic tissues including myocardium (145) . These findings suggest that adipose tissue produces angiogenic molecules (145). Experimental angiogenesis assays show that conditioned media obtained from preadipocytes and tissue homogenates from omentum or subcutaneous fat induce angiogenesis in the chick chorioallantoic membrane (CAM) and in the mouse cornea (145). It seems that bone marrow-derived circulating endothelial precursor cells do not significantly participate to adipose neovascularization although these cells are known to contribute in neovascularization in other tissues. For instance, VEGF is a potent chemoattractant factor for inflammatory cells and for mobilization of bone marrow-derived circulating endothelial precursor cells, which involve in tumor neovascularization. Interestingly, expression levels of VEGF are only moderately up regulated in growing adipose tissue although it is a main angiogenic factor in omentum (143).

In rapidly extending adipose tissue, hypoxia is another important factor for vascular growth and remodeling. In response to hypoxia, adipose tissues produce hypoxia inducible factor 1a–induced angiogenic factors such as VEGF, leptin, TNF-α, and PAI-1, which regulate angiogenesis and vasculogenesis (146). Thus it is reasonable to speculate that expansion of

adipose tissue is associated with local hypoxia, which contributes to angiogenesis by induction of a number of growth factors.

Leptin is an adipocyte-derived hormone that regulates food intake and energy homeostasis. Functional impairment of leptin leads to severe obesity, diabetes, and infertility. Interestingly, leptin is also defined as a potent angiogenic factor. In addition to its direct angiogenic activity, leptin upregulates VEGF mRNA expression via activation of the Jak/Stat3 signaling pathway (147). Among all adipose tissues examined in the body, omentum expresses the highest level of VEGF (143). Localization studies have shown that adipocytes are the primary source of VEGF, which may act as an angiogenic and vascular survival factor for the omental vasculature. Additionally, adipose- infiltrated inflammatory cells and adipose stromal cells also significantly contribute to VEGF production (143). Anatomically, visceral adipose tissue which is the main indicator of waist circumference, is present mainly in the mesentery and omentum, and drains directly through the portal circulation to the liver (148).

Several documents indicate that serum VEGF concentrations are positively correlated with body mass index and visceral obesity (149;150). Michaela Loebig et al (151) have reported a positive correlation between plasma VEGF concentrations and BMI over a large range of BMI groups in healthy population. They have also demonstrated significantly higher concentrations of plasma VEGF in obese subjects as compared with normal and low weight individuals.

Lian-Yu Lin et al (152) in their proposed model, have demonstrated that obesity directs to an inflammatory process, which could be the precursor of MetS components including insulin resistance, dyslipidemia and hypertension.

Gaby Kressel et al (54) have investigated the relationship between vascular and systemic markers of low-grade inflammation such as hs-CRP, soluble vascular adhesion molecule

1(sVCAM-1), soluble intracellular adhesion molecule-1(sICAM-1), plasminogen activating inhibitor-1(PAI-1), fibrinogen and cardiovascular traditional risk factors and the MetS.

It seems that there is no long time that the studying of association of circulating VEGF with MetS in human samples is being started. Therefore, the documentable reports in this field are scarce (153-156). However, among these few publications recent reports have shown an association of circulating VEGF with the MetS as well as with the number of its components (153;154). There are some evidences indicate the associations of some single nucleotide polymorphisms (SNPs) with MetS via the genome wide association study (157) and candidate gene approach (158;159). Taken together, it is not clear whether the elevated VEGF level is a deleterious factor or a physiologic/negative feedback response to prevent the progress of ischemia in metabolic linked ischemic status as well as in MetS.

2.3.3.7.7. Atherosclerosis

From endothelial dysfunction to plaque rupture, VEGF directly and indirectly attracts inflammatory cells into the intima in various stages of atherogenesis. Human monocytes express the receptor VEGFR-1 (flt-1), and administration of VEGF has been shown to significantly increase the number of mature macrophages in the arterial endothelium within weeks. This accumulation of macrophages was associated with increased total plaque size. VEGF primes endothelial cells to secrete increased amounts of E-selectin, an adhesion molecule necessary for trans-epithelial migration of leukocytes. Endothelial cells ‘‘pretreated’’ with VEGF also produce significantly higher amounts of tissue factor after TNF-a exposure than cells exposed to TNF-a alone. If the plaque contents later become exposed, the accumulation of tissue factor could increase the risk of a thrombotic event. Not surprisingly then, serum VEGF level has been shown to predict adverse cardiac events in (160) patients with known atherosclerosis. For example, men with unstable angina and serum

VEGF concentrations ≥0.3 pg/L were 2.5 times more likely to die of myocardial infarction within six months than those with serum VEGF levels <0.3 pg/L (161).

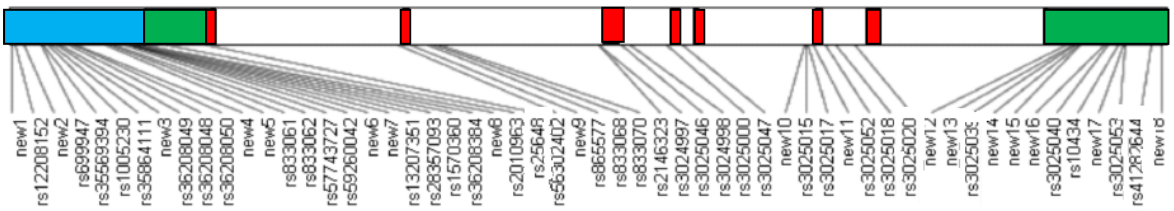
In atherosclerosis, localized hypoxia and oxidative stress in the vessel wall accelerates activation of hypoxia inducible factor 1alpha (HIF-1a) and expression of VEGF (162). Subsequent intra-plaque angiogenesis leads to neovascularization of the plaque vasavasorum, leaky capillaries, hemorrhage, and progression to a vulnerable plaque phenotype.

VEGF plays an important role in this cascade by not only stimulating the formation of new intra-plaque capillaries, but also attracting macrophages and T lymphocytes into the subintimal space (162;163). These cells further produce pro-angiogenic factors such as IL-8, TNF-a, IL-17, as well as more VEGF (163). When fragile new intra-plaque capillaries bleed, macrophages phagocytize the lipid-rich debris, become foam cells, and eventually add to the plaque's necrotic core (163-165). Taken together, the contribution of VEGF to athergenesis has been challenged.

2.3.3.8. VEGF gene single nucleotide polymorphisms and associated diseases

Several single nucleotide polymorphisms in the *VEGF* gene have been associated with VEGF protein levels and/or with a susceptibility to (or the severity of) different diseases such as CVDs, rheumatologic diseases or immunity system disorders and cancers (166;167). As an increased VEGF expression has been associated with some disorders for example; CVDs and cancers, these diseases associations may well indirectly reflect the effect of genetic variation on VEGF levels. Among the *VEGF* SNPs, those frequently reported to be associated with cancers and CVDs are: rs699947, rs833061, rs1570360, rs2010963, rs3025039 and rs25648 (167). We have also listed in the Table 7, the most prominent single nucleotide polymorphisms those have been associated with CVDs.

Figure 9: Schematic representation of SNPs of *VEGF* gene



The exons are shown in red, introns in white, regulative regions in green, and promoter region in blue. The position of the 56 SNPs identified in the gene is also indicated.

Table 7: **Studies reporting association of the VEGF polymorphisms with CVDs and related complications**

Clinical events or diseases	**SNPs**	**Ref**
Coronary artery disease	rs2010963, rs699947	(168)
Myocardial infarction	rs2010963, rs699947	(169)
Chronic heart failure	rs2010963	(170)
Heart failure after myocardial infarction	rs2010963	(171)
Death after coronary bypass graft (CABG)	rs2010963	(172)
Diabetic retinopathy	rs2010963, rs3025039	(173)
Hypertension	rs2010963	(174)
Hypertensive nephropathy	rs1570360, rs3025039	(175)
Neovascular age related macular degeneration	rs833061, rs2010963, rs1413711, rs735286, rs2146323, rs3025021, rs3025024, rs3025039	(176;177)
Severe pre-eclampsia	rs699947	(178)
Coronary artery disease in diabetic individuals	rs2010963, rs699947	(179)
Severe retinopathy in type 1 diabetes	rs3025021	(180)

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Influences of pre-analytical variables on VEGF gene expression and circulating concentrations (The second publication).

Objectives:

● Which of VEGF blood specimens (plasma or serum) is preferential for laboratory interpretation?

● Which type of anticoagulant is recommended for VEGF quantification?

● What are the effects of storage time before centrifugation on different anti-coagulated VEGF levels?

● Does the freeze-thawing cycles affect the VEGF levels in different anticoagulants tubes?

● What are the effects of storage time on VEGF gene expression in peripheral blood mononuclear cells?

● Does the storage time change the result of VEGF expression in non-tumoral tissues?

The extended role of VEGF in human pathophysiology underpins the importance of improving knowledge on the pre-analytical and analytical variation factors and thus the accuracy of laboratory analysis of VEGF. Previous works of our lab arrived to some biological variants which affect the plasma levels of VEGF such as age, platelet count and oral contraceptive pills. Also, our colleagues have defined the heritability of plasma VEGF concentrations and the reference values of VEGF levels in EDTA plasma (107;181). However, our knowledge about the probable differences between serum and plasma levels of VEGF and which of them is preferable for clinical interpretation needs to be improved. Furthermore, the type of anticoagulant recommended for VEGF quantification needs to be

assessed, as well as whether the storage time before centrifugation and freeze-thawing cycles, as the pre-analytical variants, may influence the circulating /expression of VEGF levels.

Therefore, the aim of the first part of this thesis was to determine which of serum or plasma is recommended for clinical laboratory goals and then to evaluate the effects of pre-analytical factors on VEGF circulating levels as well as its gene expression in peripheral blood mononuclear cells (PBMCs) and non-tumor tissues. Specifically, we evaluated the influence of the presence and the nature of anticoagulants [Ethylenediaminetetraacetic acid (EDTA), acid citrate dextrose (ACD), hirudin] of the blood storage delay before centrifugation and of the number of freeze-thaw cycles on circulating levels of VEGF and on $VEGF_{165}$ mRNA expression, the most abundant of VEGF isoforms, in PBMCs. We also evaluated the influence of non-tumor tissue freezing delay on VEGF protein expression. Therefore, in a collaborative study, blood samples from 27 healthy donors from the "Investigation Clinique et Accès aux Ressources Biologiques" (ICAReB) Platform at Institut Pasteur and from 9 healthy donors from Picardie Biobank were collected in order to examine the role of the mentioned pre-analytical variations of VEGF. For the assessment of VEGF gene expression on non-tumoral tissue, 16 muscle biopsies that did not show any sign of necrosis, inflammation or myopathic changes were collected from patients suffering from muscle symptoms.

Results

● *Effect of the blood collection tube type on circulating VEGF levels*

The measured VEGF levels were significantly higher in serum than in any type of plasma [264 pg/ml (158.5-383.5) ; $p<0.0001$]. Among the three different types of plasma, VEGF levels were significantly higher in EDTA [130 pg/ml (92.5-183.5)] than in hirudin [65 pg/ml (40-95); $p<0.0001$], or in ACD [76 pg/ml (42-92); $p=0.0002$].

● *Effect of blood storage at 4°C before centrifugation on serum/plasma VEGF*

When serum samples were only thawed once before analysis, there was no significant difference between 2 and 4 hours, neither was there between 2 and 48 hours of blood storage at 3-8°C. There was a significant increase between 2 and 48 hours of serum pre-centrifugation delay when the samples were analyzed after 2 freeze-thaw cycles.

In EDTA and hirudin plasma samples, no significant difference was observed between 2 and 4 hour- pre-centrifugation delay, whatever the number of freeze-thaw cycles. In contrast, there was a significant increase between 2 and 48 hours, for all numbers of freeze-thaw cycles for EDTA and hirudin. The impact of ACD on VEGF analysis was different. Although a significant increase was observed between 2 and 4 hours and between 2 and 48 hours with only one freeze-thaw cycle, this increase was no longer detectable after multiple freeze-thaw cycles.

● *Effect of the number of serum/plasma freeze-thaw cycles on measured VEGF*

The VEGF level in serum is not affected by the number of freeze-thaw cycles whatever the delay of blood storage at 3-8°C after sampling. VEGF levels in EDTA plasma significantly increased with the number of freeze-thaw cycles and this increase was similarly observed for the three blood storage delays. The VEGF increase was greater after 10 than after 2 freeze-thaw cycles. VEGF levels in hirudin plasma remained the same after 1 and 2 freeze-thaw cycles and showed a slight increase after 10 cycles. The most important effect of freeze-thawing was observed in ACD plasma.

● *Effect of blood storage at 3-8°C on VEGF expression*

A significant increase in the VEGF/β-2 microglobulin expression ratio in PBMCs was observed after 48 hours of blood storage, as compared to 2 h, with the three anticoagulants (EDTA and hirudin: $p=0.0003$; ACD: $p<0.0001$)**.**

● *Effect of freezing delay on VEGF protein expression levels in muscle tissues*

Mean standardized fluorescence from VEGF-A stained muscle biopsy sections frozen after 3 different delays showed a trend to increase with time from 15 to 60 min but with a peak of expression varying from one sample to another, either at a 30 or 60 min delay. Combining 30 and 60 min time points, we proved a significant variation in mean standardized fluorescence reflecting higher levels of VEGF expression after a 30 min delay before freezing the sample. Peak VEGF levels under hypoxic condition reached higher values.

Discussion

VEGF has been proposed as a possible prognostic biomarker in several cancers (182;183), a predictive biomarker for response to treatment in chronic inflammatory diseases (184) , and as a physiological marker of vascular remodeling and growth regulation (185). Reference values for healthy and diseased individuals must be determined to allow correct interpretation of patient's laboratory reports. Moreover, these values need to be reproducible from one laboratory to another, which means that pre-analytical and analytical parameters should be well defined.

In the present study, we have shown that VEGF measurements in serum and in plasma containing different anticoagulants are not comparable. The concentration of VEGF in serum was higher than in the different types of plasma and it was not significantly affected by the number of freeze-thaw cycles, neither was it by the storage delay at 3-8°C before

centrifugation. Using anticoagulants, VEGF values increased after 2 freeze-thaw cycles. There was an even higher increase after 10 freeze-thaw cycles in EDTA and hirudin plasma. VEGF was much higher in serum than in any anticoagulant-collected plasma and this was probably due to a massive VEGF release from platelets during the coagulation process in the first 30 minutes after blood collection. This is in accordance with the work conducted by Banks et al (186) who have shown that VEGF increased in a first step during clotting, and the maximal increase was obtained after complete coagulation of the samples. Therefore, VEGF measured in serum does not correspond only to circulating but also to intracellular VEGF. We showed that the blood storage period before centrifugation has a significant influence on VEGF levels in three types of anticoagulation tubes. Hetland et al (187) have reported that EDTA plasma VEGF levels increased by 34% and 80% respectively after 1 and 2 hours of blood storage at room temperature in healthy individuals and by 28% and 64% in rheumatoid arthritis patients. Interestingly, the same pattern of VEGF increase was observed in non-tumor tissues, after only 15 min of cold ischemia.

Peripheral blood is the most easily accessible and the most reliable specimen to be used for clinical research (188) . Therefore, a PBMC transcriptome may be used as an individual's health sensor, a concept referred to as the sentinel principle (189). We have addressed here for the first time the effects of pre-analytical factors such as different types of anticoagulants and storage delays of blood samples before centrifugation, on $VEGF_{165}$ mRNA expression by PBMCs. In solid tissue, the mechanism of VEGF expression variation is a direct response to hypoxia. In our study with the use of non-tumor tissue we had to face intrinsic variation levels between samples. In addition, we show there is a peak effect of VEGF expression ranging between 30 and 60 min time points. Therefore new strategies have to be defined to better characterize the level of «degradation» of a single non-tumor tissue sample.

Our colleagues previously have defined the heritability for plasma VEGF concentrations. Also our results in this part of study on pre-analytical variations motivated us to move to the next question of the genetic heritability which leads to the second publication of this thesis.

3.2. Identification of *cis*- and *trans*-Acting Genetic Variants Explaining Up to Half the Variation in Circulating Vascular Endothelial Growth Factor Levels (The third publication).

The heritability of circulating VEGF levels is very high, ranging between 60% and 80% (107) and pharmacogenomic studies could probably help identify potential subgroups of responders of anti-VEGF pharmacological therapies, nevertheless, there is no progress in this field partly because the genetic determinants of VEGF concentrations remain poorly understood. Thus, the second aim of this thesis was to identify genetic variants which are associated with circulating VEGF levels using an unbiased genome-wide approach in a large community-based sample.

Results

In a collaborative study between Framingham Heart Study, STANISLAS cohort study and Prospective Investigation of the Vasculature in Uppsala Seniors Study and through a genome-wide association study we identified four single nucleotide polymorphisms (SNPs) mapping to 3 chromosomal regions which were independently (following a conditional GWAS analysis) associated with circulating VEGF levels: rs6921438 and rs4416670 (6p21.1, $P=6.11\times10^{-506}$ and $P=1.47\times10^{-12}$), rs6993770 (8q23.1, $P=2.50\times10^{-16}$), and rs10738760 (9p24.2, $P=1.96\times10^{-34}$).

The most significant association was found with rs6921438 on chromosome 6p21.1, at 171 kb downstream of the *VEGF* gene, and close to the mitochondrial ribosomal protein L14 gene (*MRPL14*). Also, another SNP in 6p21.1 (rs4416670) yielded an independent genome-wide significant association

In the 8q23.1 region, the SNP yielding the most significant association with VEGF levels rs6993770 is located in the zinc finger protein, multitype 2 (*ZFPM2*) gene and 980.4 kb away from the low-density lipoprotein receptor-related protein 12 gene (*LRP12*).

The most significant association on 9p24.2 was observed with rs10738760, located close to the very low-density lipoprotein receptor (*VLDLR*) and potassium voltage-gated channel subfamily V, member 2 (*KCNV2*) genes.

We computed a genetic score including the 4 SNPs yielding genome-wide significant associations with VEGF levels in the conditional GWAS (see the publication part). This score explained 47.6% of serum VEGF variability ($P = 2.19 \times 10^{-644}$).

Six of the SNPs including rs16873365, rs16873402, rs6993770, rs16873291, rs2375980 and rs910611 that reached genome-wide significance in the genome-wide association study were significantly associated with $VEGF_{121}$ mRNA levels in peripheral blood mononuclear cells. Ingenuity pathway analyses showed plausible biological links between VEGF and 2 novel genes in these loci (*ZFPM2* and *VLDLR).*

Discussion

Candidate gene studies exploring associations between *VEGF* polymorphisms and circulating VEGF levels have yielded controversial results (see the third publication). A number of studies have found significant associations with candidate polymorphisms in the promoter, 5' and 3' untranslated regions of the *VEGF* gene. However, several other studies did not identify any association with these and other *VEGF* SNPs (see the third publication). Using a hypothesis-free genome-wide approach, we found novel associations with 140 SNPs. Of these, 68 SNPs are located on chromosome 6, approximately 150 kb downstream from the 3' end of the *VEGF* gene, far from previously tested candidate SNPs.

Although several studies have examined the association of candidate genetic variants with *VEGF* gene expression in pathological tissues, little is known about the genetic variants influencing *VEGF* expression in normal cells. Our data suggest that 6 of the SNPs associated with circulating VEGF levels in our GWAS also modulate the expression of the $VEGF_{121}$ splice variant in PBMCs of community-based persons.

Our data suggest that almost half of the inherited component of circulating VEGF levels is explained by genetic variants located downstream from the *VEGF* gene on chromosome 6p21.1. The conditional GWAS suggests that this region could harbor at least 2 distinct loci that are independently associated with circulating VEGF levels. Although located relatively far from the *VEGF* gene, results from our transcriptomic analysis indicate that this region could indeed contain functional variants modulating *VEGF* gene expression.

Genome-wide significant associations with circulating VEGF levels were also identified for SNPs located on chromosomes 8q23.1 and 9p24.2. Although these *trans* effects explain a much smaller proportion of the heritability of VEGF levels, they provide important clues about the pathways involved in the regulation of *VEGF* expression as well as suggest some possible relationships between VEGF and other molecules regulations. Indeed, we found some associations of VEGF circulating levels with SNPs located near genes linked to endothelial cell biology (ZFPM2). These associations were evidences that supported the VEGF involvement in CVDs by genetic and transcriptomic levels.

The identification of these SNPs leads us to the hypothesis whether they have an impact on other CVDs risk factors. In the following publication of this thesis, we were interested to study the associations of VEGF/VEGF identified SNPs with a number of endothelial cell dysfunction related markers such as adhesion molecules and the well known inflammatory molecules linked to CVDs including TNF-α, IL-6 and CRP. *(*For further information see the third publication).

3.3. Associations of VEGF with adhesion and inflammation molecules in a healthy population (The fourth publication).

VEGF has been linked with a number of vascular pathologies including CVDs (ischemic heart disease, heart failure and stroke). Elevated circulating VEGF levels have also been observed in several types of cancer and in various other disorders, including diabetes, cognitive decline and dementia, reproductive, immune-inflammatory disorders (190). Especially $VEGF_{145}$, has a key role during the vascularization of the human ovarian follicle and corpus luteum, in placentation and embryonic period as well as in bone and wound healings (190;191).

The existence of common molecular pathway(s) interplaying between VEGF and cellular adhesion molecules (CAM) and inflammation molecules could probably explain this broad range of VEGF functions in different pathologies.

Indeed, complex relationships have been observed between VEGF, CAM and inflammation markers in many diseases and especially in cardiovascular-related phenotypes. For instance, some studies reported the involvement of E-, P- and L-selectin, vascular cell adhesion molecule 1 (VCAM), intercellular adhesion molecule 1 (ICAM-1) and interleukin 6 (IL-6) in angiogenesis (192). Also, hypoglycemia has been shown to increase circulating levels of VEGF, IL-6, E-selectin and ICAM-1 in healthy individuals and in patients with type 1 diabetes (193). Moreover, it is noteworthy that several reports linked angiogenesis and inflammation, highlighting a key role of TNF-α (tumor necrosis factor- α) and CRP (194;195). IL-6, that mediates inflammation, is a primary determinant of the hepatic production of CRP . Recent studies indicated the simultaneous increasing of IL-6 and VEGF circulating levels in some pathological conditions such as ovarian hyperstimulation syndrome induced ascites, diabetes, visceral obesity, cancer and hypertension (196;197). Serum levels of soluble adhesion molecules have also been correlated with serum levels of some acute

phase proteins such as C-reactive protein (CRP) in individuals with breast cancer (198). In addition, in patients with hepatic cancer, the levels of soluble P-selectin have been associated with plasma levels of $VEGF_{165}$ (199).

The assessment of these relationships in different pathologies is progressing; however, due to the complexity of the pathophysiological mechanisms implicated in a disease, the origin of these biological connections is difficult to be interpreted. Therefore, their investigation in the healthy state, where there is no implication of evident pathology, could offer important information concerning the physiological connections between these molecules before the development of a specific disease. This could also be the key for the better understanding of the VEGF biological roles. However, to our knowledge, there is no published study concerning the link between VEGF and both CAM and inflammation markers in a healthy population.

Therefore, the next objective of this thesis was to examine the associations between VEGF and ICAM-1, VCAM-1, E-, L- and P-selectins, TNF-α, IL-6 and CRP in plasma of supposed healthy individuals. Furthermore, we sought to investigate whether there was a relationship in expression profile of these molecules in peripheral blood mononuclear cells (PBMCs). Ultimately, we thought to assess possible genetic links between these molecules. Thus, we aimed to determine possible associations of (rs6921438, rs4416670, rs6993770 and rs10738760) with plasma levels of CAM/inflammation markers, as well as their effect on gene expression in PBMCs.

Results

● *Associations of VEGF plasma levels with plasma levels of CAM and inflammation molecules*

VEGF plasma levels were associated with ICAM-1 levels (beta=0.043, *P*=0.023) and with E-selectin (beta=0.085, *P*=0.039).

● *Associations of VEGF isoforms mRNA and gene expression of CAM and inflammation molecules*

In assessing analysis between four VEGF isoforms and gene expression of ICAM-1, P- and L-selectin, TNF-α, and IL-6 in PBMCs we found that $VEGF_{145}$ isoform was significantly associated with ICAM-1 mRNA (beta=0.353, *P*=0.021), L-selectin mRNA (beta=0.611, *P*=0.001) and TNF-α mRNA (beta=0.616, *P*=0.001).

● *Associations of rs6921438, rs4416670, rs6993770, rs10738760 and their epistatic interactions with plasma levels and transcripts of CAM and inflammation molecules and epistatic interactions*

Significant epistatic interactions were identified for E-selectin, TNF-α, ICAM-1 and IL-6 levels (see the publication number 4). A significant association was observed between rs4416670 and mRNA levels of L-selectin (beta=0.196, *P*=0.039).

Discussion

In this part of study, we assessed the relationship between VEGF and CAM and inflammation molecules in healthy state conditions and by a multi-dimensional approach: associations in plasma, in gene expression in PBMCs, common genetic determinants and possible functional effects of these determinants on gene expression. In all these levels, significant results that

may explain the physiological relationships between these molecules were observed. VEGF plasma levels were positively associated with ICAM-1 and E-selectin. To the best of our knowledge, this is the first study to report a relationship between VEGF, the main angiogenic factor, ICAM-1, the mediator of leukocytes firmed-adhesion to endothelial cells and E-selectin, the mediator of leukocytes rolling on vascular endothelium (200)**.** We demonstrated that $VEGF_{145}$ isoform mRNA is positively associated with ICAM-1, L-selectin and TNF-α expression. These associations could indicate the existence of common transcriptional factors or other molecular mechanisms that regulate the expression of these molecules in PBMCs in non-pathological situations. The multi-dimensional approach used in this study can be considered as a strong point of this work in the assessment, validation and explanation of the relations between VEGF and adhesion/inflammation molecules in healthy individuals, thus giving new perspectives and hypotheses for further studies in order to identify the exact molecular mechanisms of VEGF implication in the development of CVDs and other chronic pathologies.

The association of VEGF/VEGF identified SNPs with MetS was the next expected study as we found associations of VEGF levels with energy expenditure linked gene (MRPL14) as well as with lipid metabolism related genes including VLDLR and LRP12.

3.4. Associations of vascular endothelial growth factor cis and trans-acting genetic variants with metabolic syndrome and its related components (The fifth and sixth publications).

The metabolic syndrome (MetS) is a cluster of interrelated risk factors that are associated with impaired angiogenesis and appear to directly promote the development of CVDs (42;156;201;202). Central obesity is considered as the core of this complex. Several documents indicate that serum VEGF concentrations are positively correlated with body mass index and visceral obesity (149;150). Epidemiological studies show that accumulation of visceral fat and not subcutaneous fat is the most important determinant factor for VEGF circulating levels (49;145).

Kressel et al (54) have investigated the relationship between vascular and systemic markers of low-grade inflammation such as hs-CRP, soluble vascular adhesion molecule 1(sVCAM-1), soluble intracellular adhesion molecule-1(sICAM-1), plasminogen activating inhibitor-1(PAI-1), fibrinogen and cardiovascular traditional risk factors and the MetS. High-density and low-density lipoproteins "HDL-C(as a component of MetS) , LDL-C respectively" are considered as independent risk factors for the development of CVD (38). Increased levels of VEGF have been found in subjects with uncomplicated hyperlipidemia in a small sample-size study (203) and similar finding has been shown in a pilot study in hypercholesterolemia patients (204). Significant associations were found between HDL-C and VEGF levels in a supposed healthy population from Japan(205), while in a supposed healthy population of SAPHIR study, VEGF was negatively correlated with LDL-C, total cholesterol (TC) and apolipoprotein B only in women (206).

Therefore, VEGF could be a key molecule that links obesity, inflammation, MetS and its related components. The reports of VEGF relationships with MetS and its related

components are limited (153-156). However, some publications have shown the associations of circulating VEGF with MetS as well as with a number of its components (153;154).

Furthermore, MetS seems to have a heritable background, since there are data that indicate associations of some SNPs with MetS via genome-wide associations studies (158;207;208) . However, the possible effect of VEGF-related SNPs on MetS and its related components has not been examined. As mentioned above, we have previously identified 4 SNPs (rs6921438, rs4416670, rs6993770, rs10738760) 47.6% of VEGF levels heritability through a genome-wide association study (209). Interestingly, in the second step of this study we have found plausible biological links between VEGF and very low density lipoprotein receptor *VLDLR* gene (one of important related components in lipid metabolism) as well. Therefore, the fourth objective of this thesis aimed to assess possible associations of MetS and its related components with circulating VEGF levels and VEGF-related SNPs in two healthy populations, STANISLAS cohort Study and another European healthy group.

Results

No statistically significant associations were observed between VEGF plasma levels and MetS as well as with its related components in this population.

Concerning the effect of the assessed SNPs, rs10738760 was significantly associated with presence of MetS. The presence of the minor allele A was associated with decreased risk for MetS. The results remained significant after adjustment for smoking. A trend for association between rs10738760 and triglycerides levels was observed (beta=-0.028, P-value=0.056).

Significant associations were observed between two variants' rs6921438 and rs6993770, and the levels of HDL-C. Specifically, the presence of the minor allele A of rs6921438 is associated with decreased in HDL-C, while the minor allele T of rs6993770 is associated with increased levels. These findings were verified in the replication population only for rs6921438. These results remained significant in both populations even after adjustment for

smoking and hypertension. The gene*environment interactions assessment revealed that rs4416670 was interacting with hypertension for apolipoprotein E. The minor allele of the polymorphism was associated with lower levels of apolipoprotein E in hypertensive participants.

Among three previous GWAS (158;207;208) performed on MetS and related components, rs4416670 and rs10738760 were not included in the significant SNPs (genotyped or in LD with another SNP). Therefore, these are considered as novel polymorphisms for MetS and one of its components, HDL-C.

Discussion

In this part of study, we examined the associations of MetS, its related components and some CVDs linked factors with cis and trans-acting VEGF genetic variants. We reported lack of association between VEGF levels, MetS and its components in our studied population. Furthermore, association of rs10738760 with MetS, independently of its components was observed.

We did not obtain a significant statistical relationship between MetS or its related components and VEGF levels. Lieb and colleagues (154) have reported a significant association of circulating VEGF with MetS. It appears that the differences between our and their samples size could explain these diversities. Tarantino and others (155) in a case control study have found a higher circulating VEGF levels in individuals with MetS compared to lean subjects. Based on another report, there is an association between VEGF and MetS, however, it seems that they have used the Adult Treatment Panel III (ATPIII) definition of (156) MetS which is less sensitive than International Diabetes federation (IDF) definition (used in the current study) for predicting diabetes and CVDs (210). Therefore, it is not clear whether elevated

VEGF level is a deleterious factor or a physiologic/negative feedback response to prevent the progress of ischemia in metabolic linked ischemic status as well as in MetS.

The polymorphism rs10738760, a novel polymorphism for MetS, is located on 9p24.2, and it is an intergenic SNP close to the very low-density lipoprotein receptor (*VLDLR*) and potassium voltage-gated channel subfamily V, member 2 (*KCNV2*) genes (209). In the second part of this study, we have discussed that rs10738760 explains≈ 5% of variation of circulating VEGF (209). Furthermore, our ingenuity pathway analyses in the mentioned study showed plausible biological links between VEGF and VLDLR gene in related loci. In this part of study, we have found a trend for association between rs10738760 and triglyceride in addition to its relationship with MetS. In insulin resistance and in hyperglycemia, the Mitogen-activated protein kinase (MAPK) pathway remains functional and even hypersensitive, whereas the pathways leading to activation of phosphatidylinositol 3-kinases (PI-3K), which is " the normal pathway of insulin functions and VEGF", is blocked (211). However, VEGF induces its anti-apoptotic and proliferative functions via the MAPK pathway (212). Interestingly, based on Miyokawa-Gorin evidence, chronic MAPK activation results to VEGF expression in adipose tissue (145;213). Furthermore, Karakelides and colleagues (214) have documented that insulin deficiency increases VEGF gene expression in muscle cells. *Kcnv2* encodes the voltage-gated potassium channel subunit Kv8.2, which also called Kv11.1 and RCD3B, and is a silent subunit when expressed as a homotetramer (215). However, when co-formed as a heterotetramer with Kv2 family members, Kv8.2 influences membrane translocation and biophysical properties of these channels (216) . A growing body of evidences indicates that there is an impairment of coronary ion channels including Kv and ca^{2+} channels in Mets which play an important role in the alterations in the control of coronary blood flow (217-221). The Kv2.1 has a negative regulatory effect on insulin secretion in animal samples while its inhibition in pancreatic beta cells enhances glucose-

dependent insulin secretion (222). Furthermore VEGF has been shown to act as a survival factor for human pancreatic islets and improve their functions of insulin secretion (223-225). Watada et al in an animal study have proposed the possible VEGF-induced compensatory beta cells activation, nevertheless they have not discussed that how their proposed model affect the insulin secretion. So far, based on our knowledge, it seems that there is no evidence to precise molecular pathways by which explain the mechanism of VEGF-induced insulin secretion. Therefore, our study for the first time might suggest that VEGF may influence the construction of heterotetramer of the voltage-gated potassium channel subunit Kv8.2 members and then alter the function of Kv2.1 in pancreatic cells that could lead to increasing insulin secretion. Thus, it seems that elevated levels of VEGF in MetS may be a compensatory procedure in which contributes; in accompany or independent of other possible factors, to maintenance the effective levels of insulin. Further studies are needed to reveal the possible mechanisms that lead to insulin secretion via the mentioned heteratetramerization as well as to determine that which other conditions may induce this molecular assembling that lead to insulin secretion. Further studies should examine the reactive stimulatory effects of VEGF on K+ channels expression in coronary microcirculation of MetS.

In the discovery population, rs6921438 and rs6993770 were associated with HDL-C plasma levels. The minor alleles of rs6921438 had a negative effect, while rs6993770 seems to be protective through increase of HDL-C levels. Furthermore, the negative association of rs6921438 with HDL-C was observed also in replication sample.

The rs6921438 is an intergenic SNP in chromosome 6, close to VEGFA gene and located between the mitochondrial ribosomal protein L14 gene (MRPL14) and the MCG45491 gene (C6orf223). The rs6993770 is an intronic polymorphism in zinc finger protein, multitype 2 (*ZFPM2*) gene and 980.4 kb away from the low-density lipoprotein receptor-related protein

12 gene (*LRP12*) in chromosome 8. These SNPs have been negatively associated with VEGF levels during the second step of our study in a GWAS. Consequently, it appears that rs6921438 could have a negative effect in cardiovascular system through decrease of HDL-C and rs6993770 could have a positive effect although they both decrease VEGF levels. Therefore, the mechanisms that modify HDL-C levels through these SNPs should be independent from the relative mechanisms that are implicated in VEGF regulation. Nevertheless, as the effect of rs6993770 was not significant in the replication set, this finding should be treated with caution.

In conclusion, although we did not arrive to a significant point in association of VEGF levels with MetS and its related components in this studied population, the regulatory effects of rs10738760, rs4416670 and rs6993770 on VEGF and their relationships with MetS and HDL-C might lead us to propose a compensatory elevated VEGF levels in MetS and consequently likely protect afflicted peoples from ischemic conditions.

In the next step, we decided to have a comparison study between STANISLAS cohort study and an Iranian population.

3.5. High Prevalence of Metabolic Syndrome in Iran in Comparison with France (The seventh publication).

The prevalence of MetS varies greatly among countries and ethnic groups and it fluctuates between 20% and 30% among Europeans and white Americans, with a similar distribution for men and women (211). However, in Asian countries, particularly in Middle Eastern societies, MetS seems to be more prevalent, with a different pattern of related components and a different distribution among genders. Population-based studies and comparative scientific reports for these east–west differences are scarce. The last objective of this thesis was to compare the prevalence of MetS and its components in a French population, the STANISLAS cohort study, with an Iranian population and to analyze the eventual differences observed. Therefore, in order : 1) to compare the prevalence of MetS and its components between the two countries, 2) to compare the (genetic) heritability of MetS between the two populations 3) to replicate our findings related to associations of VEGF cis- and trans-acting genetic variants with MetS in a different (non European) individuals in a collaborative study with Mashhad university of Medical Sciences in Iran, we examined the differences concerning the MetS and its related components pattern between the two countries.

The prevalence of MetS was significantly higher in Iranian women (55.0%), followed by Iranian men (30.1%), than in French men (13.7%) and French women (6.6%). Iranian women were characterized by high rates of abdominal obesity (65.0%), hypertension (52.1%), hypertriglyceridemia (43.1%), and low HDL-C (92.7%). Iranian men were characterized by high rates of hypertension (48.9%), hypertriglyceridemia (42.8%), and low HDL-C (81.8%). French men had high rates of hypertension (44.7%) and mild rates of hypertriglyceridemia (28.6%) and hyperglycemia (23.9%).

Discussion

This is the first study conducted in French and Iranian populations to investigate common points as well as differences in MetS between the two countries. The main findings of this study are a high prevalence of MetS and prevalence of low HDL-C and high waist circumference in Iranian women compared with French women.

These findings agree with previous studies that advised screening for the progression of MetS in Iran, including the measurement of waist circumference, TG, and HDL-C (226;227). In addition, HDL-C was associated with waist circumference in our analysis, which suggests a relationship between increased waist circumference and decreased HDL-C concentrations. For both countries, our analysis showed correlation between waist circumference and TG, and an inverse relationship with HDL-C. In fact, our findings highlighted the central obesity as the main core of MetS.

Although it seems that environmental factors, including diet and drinking patterns as well as physical activity may play a considerable role in explaining the main differences in the prevalence of MetS, particularly in women of the two countries. The contribution to these differences made by genetic factors should ultimately be considered. The following issues have been scheduled to be investigated in the future collaborations: 1) the investigation of genetic differences of MetS between the two countries 2) the comparison of (genetic) heritability of MetS between the two populations 3) the replication of our findings related to associations of VEGF cis- and trans-acting genetic variants with MetS and its related components particularly HDL-C in a different (non European) population.

CHAPTER 4

GENERAL DISCUSSION

The present investigation was designed to elucidate the likely associations of VEGF and its cis-and trans-genetic acting variants with CVDs. In particular, we examined the hypothesis that VEGF and its related regulatory genetic polymorphisms play pivotal role in MetS. The main findings of this study are:

1) The identification of the genetic variants that explain 47.6% of circulating VEGF levels, including rs6921438, rs4416670, rs6993770 and rs10738760.

2) The associations of VEGF and its identified genetic variants in a tridimensional approach (genetics, transcriptomics and protein levels) with a number of novel risk factors of CVDs such as adhesion molecules (ICAM-1 and E, L selectins) as well as a number of inflammatory molecules such as TNF-α, IL-6 and CRP.

3) The inverse association of VEGF-related polymorphism, rs10738760, with MetS.

4) The relationship between VEGF regulatory variant, rs6921438, and HDL-C. The minor allele of this variant was associated with low levels of Apolipoprotein E in hypertensive participants.

5) A high prevalence of MetS and prevalence of low HDL-C and high waist circumference in Iranian women compared with French women.

The basic and prerequisite results of this study showed that:

1) The best conditions for measuring both circulating VEGF and its gene expression are to reduce time between blood collection and centrifugation, and to avoid multiple freeze-thaw cycles.

2) Serum is the most stable circumstance rather than different anticoagulants

3) The optimal conditions for measuring non-tumor tissue VEGF levels are to freeze the tissue within 15 min following excision.

CVDs constitute the leading cause of death worldwide and accounts for as much as 30% of global mortality (228;229). T2D, obesity, hypertension and dyslipidemia are considered as the traditional risk factors for CVDs. Furthermore, the increased concentrations of pro-inflammatory factors such as TNF-α, IL-6 CRP as well as elevated levels of endothelial dysfunction markers for instance; intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin,P-selectin and L-selectin which together represent a low grade inflammation, are included in the group of novel risk factors of CVDs (230). Central obesity, the main underlying factor of MetS, is associated with increased secretion of certain adipokines, such as interleukin-6, tumor necrosis factor-α, which cause endothelial dysfunction as well as an increase in the expression of adhesion molecules and CRP (49). This interaction between adipose tissue and endothelial cells is associated with an alteration in the activation of PI3K molecular pathways and increase or decrease in the expression of several transcription factors (hypoxia inducible factor, peroxisome proliferator-activated receptors, CCAAT-enhancer-binding proteins, carbohydrate responsive element-binding proteins and sterol regulatory element-binding proteins) that play a crucial role in the regulation of distinct metabolic pathways related to the MetS.

VEGF is a multifunctional cytokine that shows distinguished functions in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis (190). VEGF has been linked with a number of vascular pathologies including CVDs (ischemic heart disease, heart failure, and stroke) (190;209). Moreover, elevated circulating VEGF levels have been also observed in various cardiovascular related disorders/ risk factors including MetS, T2D, hypertension, pre-eclampsia, chronic kidney disease and polycystic ovarian syndrome (209). VEGF induces its physiologic functions such as cell survival, proliferation, migration and vessel permeability

through its binding with the corresponding receptor via the PI3K pathway (190). Some recent reports have shown only the increased VEGF levels in individuals with MetS (154-156;202;231). However, they have not addressed any explanation to clarify the role of VEGF in this circumstance.

The contribution of VEGF to athergenesis has been challenged. Fist, systemic adenoviral gene transfer of VEGF-A, -B, -C, or –D as well as recombinant VEGF administration, did not alter plaque area or macrophage influx in LDL/Apo B48 double knockout mice. Second, periadvantitial and intra-arterial genetransfer of VEGF-A, -C, and –D has inhibited neointimal growth in many animal study. Third, there has been no evidence of increased atherogenesis in clinical traials using VEGF-A protein or gene transfer. Indeed, VEGF-A polymorphism causing higher VEGF expression recently was found to be associated with a lower risk of coronary artery disease (232).

In this study, VEGF was associated with ICAM-1 and E-selectin in plasma. In PBMCs, $VEGF_{145}$mRNA was associated with ICAM-1, L-selectin and TNF-α expression. Interactions of the VEGF genetic variants were shown to affect ICAM-1, E-selectin, IL-6 and TNF-α plasma levels, while rs4416670 was associated with L-selectin expression. Our findings propose a biological connection between VEGF and CAM/inflammation markers. Common genetic and transcriptional mechanisms may link these molecules and control their effect in healthy conditions. In fact, the interactions between VEGF and CAM/inflammatory molecules may lead to healthy circumstance maintenance against the inflammatory cascades of CVDs. Yäl-herttuala and colleagues have described that low physiological amount of VEGF is required for blood vascular homeostasis, endothelial cell survival and production of nitric oxide and prostacyclin, resulting in vasodilatation, antithrombosis and suppression of smooth muscle cells proliferation, i.e, are vasculoprotective (232). By this study, we have

documented this fact that some parts of these VEGF capacities in healthy circumstance may result from the interaction of VEGF with adhesion molecules as well as with TNF-α, IL-6.

Our results showed a significant association between rs10738760 and rs4416670 which located near the corresponding genes for very low density lipoprotein metabolism (VLDLR) and cell energy expenditure (MRPL14) and circulating VEGF levels. For this reason, the last but not the least part of this study was particularly aimed to investigate the possible relationships between VEGF and its identified polymorphisms and MetS. As we expected our results showed significant associations, however with an inverse direction, between rs10738760 and MetS, an inverse relation between rs6921438 and HDL-C and direct association between rs6993770 and HDL-C. Nevertheless, our analysis has not arrived to significant association between VEGF levels and MetS and its related components in the studied population. The VEGF regulatory variants" rs10738760, rs6921438 were associated with MetS and HDL-C respectively. Therefore, the likely influences of VEGF on the voltage-gated potassium channels 2, very low density lipoprotein receptor, mitochondrial ribosomal protein L14, MCG45491 (C6orf223) genes functions in this pre-diabetic condition will be the subjects of next studies.

Finally, the present study also revealed some differences of MetS components prevalence as well as their distribution pattern between Iran and France. The main finding of the last part of this study was the high prevalence of low HDL-C concentrations in Iranian population, especially in Iranian women, compared with French women. Our finding about elevated prevalence of MetS in Iranian women agrees with other Middle –Eastern epidemiological studies (227;233;234). However, the wide differences in HDL-C levels between two populations should lead to future studies in order to investigate the environmental and genetic explanatory factors (particularly VEGF genetic acting variants) which may cause these wide differences of the components of MetS between the two countries.

CHAPTER 5

CONCLUSION AND PERSPECTIVES

VEGF participates in some crucial physiologic processes and involves in several pathologic circumstances. This study proposes that the possible interactions between VEGF and CAM/inflammatory molecules may lead to healthy circumstance maintenance against the inflammatory cascades of CVDs as we found some associations between VEGF and CAM/inflammation markers in healthy population. Also, as we observed some associations between cis- and trans genetic acting variants of VEGF, located near the five genes that may encode a number of products linked with lipid metabolism, insulin secretion and energy expenditure, with MetS, HDL-C and apolipoprotein E. So, it could be conclude that there is a relationship between VEGF regulatory variants and MetS as well as with its related components. Our study suggests that further researches are needed in order to find possible therapeutic targets for pharmaceutical intervention to prevent T2D and CVDs.

Several future studies are needed to find the possible answers for the results and hypotheses of this work: 1) the associations of VEGF levels with VLDLR in MetS and T2D, 2) the relationships of VEGF with mitochondrial ribosomal proteins in MetS and T2D , 3) the impact of VEGF on hetra-tetramerization of potassium voltage-gated channels 2 and consequently resulted effects on the pancreas beta cells stimulation in insulin secretion, 4) the impact of VEGF on potassium voltage-gated channels 2 in cardiac microcirculation and consequently resulted effects on the myocardial ischemia. 5) the relationship between HDL-C and mitochondrial ribosomal protein L14, zinc finger protein multitype 2 and low density lipoprotein receptor 12, MCG4591(C6orf223) genes and their coded proteins. 6) the study of possible environmental factors as well as the genetic determinants (corresponding to MetS components particularly HDL-C and obesity susceptibility) which could explain the wide differences of MetS and its component distribution pattern between Iranian and French populations.

Also, but independently of the above suggested ideas, it seems that there is a tolerance threshold in VEGF functions/expressions in the body while in healthy circumstances it interacts with other effective factors to maintenance the normal conditions and protects against the inflammatory cascades. Nevertheless, after the onset of a disease, the elevated level of VEGF has deleterious effects and increases the morbidity and mortality of diseases; for example cardiovascular events and cancer metastasis. Thus, determination of this tolerance threshold needs some comprehensive and multidimensional studies.

A number of studies have described the possibility of VEGF as both a therapeutic tool and target based on its specific actions on vascular proliferation and cell survival. Recent clinical and experimental evidences have shown the feasibility of using VEGF administration to protect ischemic tissues such as the myocardium, the brain and the kidney via stimulation of microvascular proliferation and repair (235). On other side, anti-VEGF therapies are at the fore-front of treatment of many solid tumors, but blockade of VEGF carries collateral effects such as hypertension and renal damage largely due to abnormalities in the microvasculature. On the other hand, VEGF inhibitors such as bevacizumab and sorafenib have been successfully used to inhibit angiogenesis in several tumors, in macular degeneration and in rheumatoid arthritis(209). However, despite the considerable inversed effects associated with VEGF inhibitor drugs, there have been no pharmacogenomic studies to identify potential subgroups of responders, partly because the genetic determinants of VEGF concentrations were poorly understood before our study. The new findings concerning the genetic variants which affect VEGF levels could help the researchers to improve our knowledge about some therapeutic intervention outcomes and drug inverse effects in the previously mentioned pathologies above via the pharmacogenetics studies.

SYNTHESE EN FRANÇAIS

AVANT-PROPOSE

Les maladies cardiovasculaires (MCV) représentent la principale cause de décès à travers le monde, bien que la mortalité qui leur est attribuable diminue progressivement en raison des progrès réalisés dans le domaine diagnostique et thérapeutique. D'après les données de l'Organisation Mondiale de la Santé (OMS), le taux de mortalité dû aux MCV en 2008 était de 214 à 455 pour 100 000 décès ; ce taux étant de moindre ampleur dans les pays développés. Au sein de la Communauté européenne, les MCV sont également la principale cause de décès à l'âge adulte. En 2005, le taux de mortalité attribuable à des maladies de l'appareil circulatoire était de 241,2 pour 100 000 (295,4 chez les hommes et 196 chez les femmes). Ce taux a diminué pour atteindre 226,1 (273 et 183 respectivement) en 2006 grâce à la mise en place de stratégies de prévention et de traitement. L'identification de personnes apparemment en bonne santé qui présentent un risque accru de développement d'une maladie, notamment de MCV, est considérée à présent comme une priorité majeure pour les systèmes de santé.

Le syndrome métabolique (SM) est un ensemble de facteurs de risque interdépendants associés à une altération de l'angiogenèse et semblent promouvoir directement le développement du diabète de type 2 et de MCV. Chez des personnes souffrant de SM, le risque de développer une MCV ou un diabète de type 2 est multiplié par deux et par cinq respectivement. Ces personnes présentent des concentrations élevées d'insuline et du facteur de croissance de l'endothélium vasculaire (VEGF) mais, fait intéressant, un dysfonctionnement de la néovascularisation/angiogenèse. L'élucidation de ce paradoxe (taux élevés de VEGF associés à une altération de l'angiogenèse) pourrait donc permettre d'identifier de nouvelles stratégies préventives/thérapeutiques visant à empêcher le passage de l'état pré-pathologique/pré-diabétique à l'état pathologique.

Le laboratoire de "Génétique Cardiovasculaire" EA 4373 offre l'infrastructure essentielle nécessaire à la réalisation d'une étude visant à identifier les facteurs génétiques et environnementaux, ainsi que les interactions gène-environnement, qui jouent un rôle dans le

syndrome métabolique. À cet effet, l'étude de la cohorte STANISLAS (SC) ou l'Étude familiale STANISLAS (SFS), qui comprend 1006 familles, a été mise en place principalement dans le but d'identifier des interactions gène-gène et gène-environnement dans un contexte de MCV. Les familles de cette cohorte ont été recrutées entre 1993 et 1995 (visite initiale) au Centre de Médecine Préventive de Vandœuvre-lès-Nancy lors d'un bilan de santé périodique. Afin de pouvoir évaluer les effets de la génétique sur la variabilité des phénotypes intermédiaires dans des conditions physiologiques sans être influencé par un traitement médical ou une maladie autre, les familles étaient supposées saines et ne présentaient pas de maladies aiguës et/ou chroniques. La nature longitudinale de cette étude devait permettre d'observer l'évolution des phénotypes intermédiaires en fonction des facteurs génétiques lors de la période de suivi. La deuxième visite a eu lieu entre 1998 et 2000 ; le taux de participation à cette visite était de 75 %. Au cours de ces deux visites, ainsi que lors de la troisième visite entre 2004 et 2005, des échantillons de sérum, de plasma, d'ADN et des cellules mononucléaires du sang périphérique ont été recueillis. De plus, une base de données a été créée, combinant les données de l'examen de santé et les données spécifiques à l'étude STANISLAS (données cliniques, biologiques et génétiques).

Nos collègues du laboratoire EA 4373 ayant auparavant identifié des variables biologiques qui agissent sur les taux de VEGF et évalué l'héritabilité du VEGF, nous avons décidé de déterminer, en collaboration avec les études *Framingham Heart* et *Prospective Investigation of the Vasculature in Uppsala Seniors* (PIVUS), quels facteurs génétiques influencent les taux de VEGF circulant au moyen d'une étude d'association pangénomique (GWAS). À la suite de l'identification par GWAS de variants génétiques responsables de la variation des taux du VEGF, nous avons étudié l'association des polymorphismes ainsi identifiés et des taux de VEGF avec le SM et ses composants. En même temps, nous avons comparé les différences des profils des composants du SM dans des populations française (cohorte STANISLAS) et

iranienne (cohort de Mashhad) lors d'un projet commun avec l'Université des Sciences Médicales de Mashhad. En parallèle, grâce aux données issues de différents examens biochimiques et cliniques en rapport avec les MCV qui sont disponibles dans notre biobanque/Centre de Ressources Biologiques (CRB), nous avons évalué les relations entre les polymorphismes identifiés et les taux de VEGF (circulant et niveau de transcription) et les molécules inflammatoires et d'adhésion les plus impliquées dans les MCV. Le pré-requis pour toutes les études que nous avons conçues était bien évidemment d'améliorer notre connaissance des variations biologiques et pré-analytiques qui influencent la mesure du VEGF. De ce fait, nous avons élaboré et réalisé une projet en commun avec l'Institut Pasteur à Paris et l'Université de Lyon.

CHAPITRE F1

HYPOTHÈSE ET OBJECTIFS

Alors que le poids des MCV ne fait que s'accroître à travers le monde, il est de plus en plus important de mettre en œuvre une démarche de prévention dynamique et d'étudier les facteurs de risque génétiques et environnementaux éventuellement impliqués dans ces maladies multifactorielles, et ce à un niveau mondial. La prise en charge préventive et le traitement d'individus présentant des MCV, le dépistage précoce de patients en l'absence de symptômes, l'identification de personnes apparemment en bonne santé qui présentent un risque accru de développement de la maladie sont aujourd'hui considérés comme des priorités de santé publique.

Le syndrome métabolique (SM) est une constellation de facteurs de risque interdépendants d'origine métabolique — *facteurs de risque métaboliques*— qui sont associés à une altération de l'angiogenèse et semblent promouvoir directement le développement de MCV. Chez les personnes souffrant de SM, le risque de développer une MCV ou un diabète de type 2 est multiplié par deux et par cinq respectivement. Le risque de mortalité observé chez les français souffrant de SM est de 1,82 pour les hommes et de 1,80 pour les femmes. Les principaux facteurs de risque sous-jacents pour le SM semblent être l'obésité abdominale et l'insulinorésistance ; d'autres conditions associées sont le manque d'activité physique, le vieillissement, l'inflammation de faible intensité et le déséquilibre hormonal.

Le VEGF est une cytokine multifonctionnelle qui joue différents rôles dans les processus d'angiogenèse, de lymphangiogenèse, de perméabilité vasculaire et d'hématopoïèse.

Il a été montré que le VEGF est impliqué dans plusieurs pathologies vasculaires, dont les MCV (cardiopathie ischémique, insuffisance cardiaque et AVC). Par ailleurs, des taux élevés de VEGF circulant ont été également observés dans divers troubles/facteurs de risque cardiovasculaires dont le SM, le diabète de type 2, l'hypertension, la pré-éclampsie, l'insuffisance rénale chronique et le syndrome des ovaires polykystiques ainsi que d'autres

troubles tels que la démence/déclin cognitif, des troubles de la reproduction et immunoinflammatoires et plusieurs types de cancer. Le VEGF joue également un rôle essentiel lors de la vascularisation du follicule ovarien et du corps jaune chez la femme, du développement placentaire et de la période embryonnaire, ainsi que lors de la cicatrisation osseuse et tissulaire.

Pour quelle raison le VEGF présente-t-il une telle diversité de fonctions tout au long vie ? La première hypothèse élaborée afin de répondre à cette question était que le vaste éventail de fonctions attribuables au VEGF pourrait être expliqué par l'existence de voies de signalisation moléculaires communes entre le VEGF et des molécules inflammatoires ou d'adhésion cellulaires.

Il convient de préciser qu'en présence d'obésité, le principal facteur sous-jacent de SM/MCV, des concentrations élevées de VEGF (le facteur majeur de l'angiogenèse) ont été observées. D'autre part, il existe un dysfonctionnement de l'angiogenèse et de l'état de néovascularisation dans le SM. Le VEGF joue un rôle important dans le développement de la masse graisseuse de par l'angiogenèse qu'il induit. Les cellules endothéliales capillaires, les principales cellules exprimant le VEGF, communiquent avec les adipocytes par l'intermédiaire de cascades de signalisation paracrines, de composants extracellulaires et d'interactions intercellulaires directes pour induire l'angiogenèse de la graisse viscérale et enfin le développement de graisse. Quelle est la réalité derrière le paradoxe créé par la co-existence de taux élevés de VEGF et d'un dysfonctionnement de l'angiogenèse dans le syndrome métabolique ? Le VEGF pourrait représenter un des liens entre l'obésité, l'inflammation et le SM, ou jouer un rôle protecteur contre le développement de diabète de type 2/MCV chez des individus souffrant de SM ? Toutefois, il semblerait que l'implication du VEGF dans le SM et ses composants associés n'ait pas été étudiée de manière approfondie à ce jour. Le VEGF et le SM dépendent tous les deux du contexte génétique ; néanmoins, peu de variants génétiques permettant

d'expliquer une part significative de leur héritabilité ont été identifiés. L'étude d'éventuels facteurs génétiques communs entre le VEGF et le SM, ainsi que ses composants associés, serait donc d'un intérêt considérable et pourrait permettre de répondre aux interrogations précédentes.

Nos objectifs étaient donc 1) d'examiner le contexte génétique derrière les variations des taux de VEGF, 2) de définir la relation entre les taux de VEGF/les SNP associés au VEGF et les molécules inflammatoires et d'adhésion, et 3) d'étudier l'association des taux de VEGF et des SNP associés au VEGF avec le SM et ses composants. Afin de garantir une approche holistique, la détermination des facteurs de variation pré-analytiques et analytiques, et donc de la précision de l'analyse du VEGF au laboratoire, figurait également parmi les objectifs de notre travail.

Objectifs principaux :

1) Plasma ou sérum ? Quel type d'anticoagulant est à préconiser pour la quantification du VEGF ?

2) Détermination des variables pré-analytiques agissant sur le taux de VEGF circulant et son expression par les cellules mononucléaires du sang périphérique et des tissus non tumoraux.

3) Identification de variants génétiques associés au VEGF circulant afin de déterminer le score d'héritabilité dans des populations saines.

4) Étude des associations potentielles entre les taux de VEGF et les SNP associés et les molécules inflammatoires et d'adhésion.

5) Étude des relations entre les taux de VEGF et les SNP associés au VEGF et le SM et ses composants.

6) Élucidation des différences de prévalence du SM entre la France et l'Iran, et recherche des facteurs biologiques responsables dans les deux populations.

CHAPITRE F2

RÈSULTATS ET DISCUSSION

F.2.1. <u>Influence des variables pré-analytiques sur l'expression génétique et les taux de VEGF circulant (La deuxième publication).</u>

● Quel type de prélèvement sanguin (plasma ou sérum) est à préférer pour l'interprétation en laboratoire ?

● Quel type d'anticoagulant est préconisé pour la quantification du VEGF ?

● Quelle est l'influence de la durée de conservation avant centrifugation des échantillons avec différents anticoagulants sur les taux de VEGF mesurés ?

● Les cycles gel/dégel ont-ils un effet sur les taux de VEGF dans les différents tubes d'anticoagulants ?

● Quelle influence la durée de conservation a-t-elle sur l'expression génétique du VEGF dans cellules mononucléaires du sang périphérique ?

● La durée de conservation modifie-t-elle les résultats de l'expression du VEGF dans les tissus non tumoraux ?

Le rôle étendu du VEGF dans la physiopathologie humaine souligne combien il importe d'améliorer nos connaissances des facteurs de variation pré-analytiques et analytiques, et donc la précision de l'analyse du VEGF au laboratoire. Des travaux ultérieurs entrepris dans notre laboratoire ont permis d'identifier quelques variants biologiques qui agissent sur les taux plasmatiques de VEGF tels que l'âge, la numération plaquettaire et la prise de contraceptifs oraux. Nos collègues ont également défini l'héritabilité des concentrations plasmatiques de VEGF et les valeurs de référence pour les taux de VEGF dans du plasma EDTA (107, 181). Cependant, notre connaissance des différences potentielles entre les taux de VEGF dans le sérum et le plasma mérite d'être améliorée, ainsi que le fait de savoir s'il est préférable d'utiliser du sérum ou du plasma pour l'interprétation clinique. Par ailleurs, le type

d'anticoagulant à recommander pour la quantification du VEGF doit être défini, ainsi que l'impact de la durée de conservation avant centrifugation et des cycles de gel/dégel car ces variations pré-analytiques pourraient avoir une influence sur la mesure des taux circulants et de l'expression du VEGF.

De ce fait, le but de la première partie de cette thèse était de déterminer quel milieu, sérum ou plasma, permettait d'obtenir les meilleurs résultats cliniques en laboratoire, puis d'évaluer les effets des facteurs pré-analytiques sur les taux de VEGF circulant, ainsi que son expression génétique dans les cellules mononucléaires du sang périphérique (PBMC) et des tissus non tumoraux. Plus spécifiquement, nous avons évalué l'influence de la présence et de la nature d'anticoagulants [acide éthylènediaminetétraacétique (EDTA), acide citrate dextrose (ACD) et hirudine] , de la durée de conservation du sang avant centrifugation et du nombre de cycles gel/dégel sur les taux de VEGF circulant et l'expression de l'ARNm du $VEGF_{165}$, l'isoforme la plus abondante du VEGF, dans les PBMC. Nous avons également évalué l'influence du délai avant congélation de tissus non tumoraux sur l'expression protéique du VEGF. Ainsi, lors d'une étude collaborative, des prélèvements sanguins ont été recueillis chez 27 donneurs sains de la plateforme Investigation Clinique et Accès aux Ressources Biologiques (ICAReB) de l'Institut Pasteur et chez 9 donneurs sains de la Biobanque de Picardie afin d'étudier le rôle des variations pré-analytiques du VEGF précédemment citées. Pour l'évaluation de l'expression génétique du VEGF dans des tissus non tumoraux, 16 biopsies musculaires, ne présentant aucun signe de nécrose, d'inflammation ou de modification myopathique, ont été recueillies chez des patients souffrant de symptômes musculaires.

Résultats

Effet du type de tube de prélèvement sanguin sur les taux de VEGF circulant

Les concentrations de VEGF mesurées étaient significativement plus élevées dans le sérum que dans tout type de plasma [264 pg/ml (158,5 à 383,5) ; $p < 0,0001$]. Parmi les trois différents types de plasma, les concentrations de VEGF étaient significativement plus élevées sur EDTA [130 pg/ml (92,5 à 183,5)] que sur hirudine [65 pg/ml (40 à 95) ; $p < 0,0001$] ou ACD [76 pg/ml (42 à 92) ; $p = 0,0002$].

Effet de la conservation du sang à 4 °C avant centrifugation sur les taux de VEGF mesurés dans le sérum et le plasma

Aucune différence significative n'a été observée entre une durée de conservation de 2 et 4 heures si les échantillons de sérum n'étaient décongelés qu'une fois avant leur analyse, ni si le sang était conservé pendant 48 heures à une température de 3 à 8 °C. Une augmentation significative a été constatée après une durée de conservation pré-centrifugation du sérum de 48 heures si les échantillons étaient analysés à la suite de deux cycles de gel/dégel.

Dans des échantillons de plasma sur EDTA et hirudine, aucune différence significative n'a été observée entre une durée de conservation pré-centrifugation de 2 et de 4 heures, quel que soit le nombre de cycles de gel/dégel. En revanche, l'augmentation était significative après une durée de conservation de 48 heures, aussi bien pour les échantillons de plasma sur EDTA et sur hirudine et quel que soit le nombre de cycles de gel/dégel. L'impact des tubes ACD sur l'analyse du VEGF était différent. Bien qu'une augmentation significative fût observée entre une durée de conservation de 2 et de 4 heures, et entre une durée de conservation de 2 et de 48 heures avec un cycle de gel/dégel unique, cette augmentation devenait indétectable à la suite de plusieurs cycles de gel/dégel.

Effet du nombre de cycles de gel/dégel du sérum/plasma sur les taux de VEGF mesurés

Le nombre de cycles de gel/dégel n'a pas d'influence sur le taux de VEGF mesuré dans le sérum, et ce quel que soit la durée de conservation du sang entre 3 et 8 °C après prélèvement. Les taux de VEGF dans le plasma EDTA ont augmenté de manière significative avec le nombre de cycles de gel/dégel ; cette augmentation était similaire pour les trois durées de conservation du sang. L'augmentation du taux de VEGF était plus importante après 10 cycles qu'après 2 cycles de gel/dégel. Les taux de VEGF dans le plasma contenant de l'hirudine étaient identiques après 1 ou 2 cycles de gel/dégel ; une légère augmentation était observée après 10 cycles. L'effet de cycles de gel/dégel était plus important pour les échantillons de plasma ACD.

Effet de la conservation du sang entre 3 et 8 °C sur l'expression du VEGF

Une augmentation significative du rapport d'expression VEGF/β-2 microglobuline était observée dans les PBMC après une durée de conservation de 48 heures par rapport à une durée de 2 heures, et ce avec les trois anticoagulants (EDTA et hirudine : $p = 0,0003$; ACD : $p < 0,0001$)**.**

Effet du délai avant congélation sur les taux d'expression protéique du VEGF dans des tissus musculaires

Le fluorescence moyenne normalisée du VEGF-A dans des coupes de biopsie musculaire colorées après 3 délais distincts a montré que l'expression tend à augmenter en fonction du temps entre 15 et 60 minutes, mais avec un pic d'expression variable d'un échantillon à l'autre, intervenant après un délai de 30 ou de 60 minutes. En combinant les points chronologiques à 30 et à 60 minutes, nous avons mis en évidence une variation significative de la fluorescence moyenne normalisée et donc démontré que les taux d'expression du VEGF sont plus élevés si

l'échantillon est congelé plus de 30 minutes après son prélèvement. Les taux de VEGF maximaux étaient plus élevés dans des conditions hypoxiques.

Discussion

Il a été proposé que le VEGF pouvait constituer un biomarqueur prognostique pour plusieurs types de cancer (182,183), un biomarqueur prédictif pour la réponse au traitement dans des maladies inflammatoires chroniques (184), et un marqueur physiologique de la régulation du remodelage et du développement vasculaire (185). Il importe de déterminer les valeurs de référence pour des individus sains et malades pour permettre une interprétation correcte des rapports de laboratoire des patients. Par ailleurs, ces valeurs doivent être reproductibles d'un laboratoire à un autre, ce qui signifie que les paramètres pré-analytiques et analytiques doivent être correctement définis.

Dans la présente étude, nous avons démontré que la quantification du VEGF dans le sérum et le plasma contenant différents anticoagulants n'est pas comparable. La concentration du VEGF dans le sérum était plus élevée que dans les différents types de plasma et n'était pas modifiée de manière significative par le nombre de cycles de gel/dégel, ni par la durée de conservation entre 3 et 8 °C avant centrifugation. En la présence d'anticoagulants, les valeurs de VEGF mesurées ont augmenté après deux cycles de gel/dégel. Dans le plasma contenant de l'EDTA ou de l'hirudine, cette augmentation était encore plus importante après 10 cycles de gel/dégel. Le taux de VEGF était bien plus élevé dans le sérum que dans tout échantillon de plasma contenant des anticoagulants. Ceci est vraisemblablement dû à une libération du VEGF par les plaquettes pendant le processus de coagulation qui se produit au cours des premières 30 minutes après le prélèvement du sang. Ces résultats sont en accord avec le travail de Banks et coll. (186) qui ont démontré que le taux de VEGF augmentait progressivement pendant la coagulation pour atteindre un niveau maximal après la

coagulation complète des échantillons. Ainsi, le taux de VEGF mesuré dans le sérum n'est pas seulement du VEGF circulant mais également du VEGF intracellulaire. Nous avons démontré que la durée de conservation du sang avant centrifugation exerce une influence significative sur les taux de VEGF mesurés dans les trois types de tubes contenant des anticoagulants. Hetland et coll. (187) ont signalé que les taux de VEGF dans du plasma EDTA augmentaient respectivement de 34 % et de 80 % après 1 heure et 2 heures de conservation du sang à température ambiante chez des individus sains et de 28 % et de 64 % chez des patients souffrant de polyarthrite rhumatoïde. De façon intéressante, le même profil d'augmentation des taux de VEGF était observé dans des tissus non tumoraux, et ce après une période d'ischémie froide de seulement 15 minutes.

Le sang périphérique représente le type de prélèvement le plus accessible et le plus fiable pour la recherche clinique (188) . Il a donc été envisagé d'utiliser le transcriptôme des PBMC en tant qu'indicateur de la santé de chaque individu, un concept désigné sous le nom de principe de la sentinelle (189). Dans cette étude, nous avons abordé pour la première fois les effets de facteurs pré-analytiques tels que les différents types d'anticoagulants et la durée de conservation des échantillons de sang avant centrifugation sur l'expression de l'ARNm du $VEGF_{165}$ par les PBMC. Au sein d'un tissu solide, le mécanisme de variation de l'expression du VEGF répond directement au niveau d'hypoxie. Dans notre étude où nous avons utilisé des tissus non tumoraux, nous avons dû prendre en compte la variation intrinsèque des taux de VEGF entre les échantillons. De surcroît, nous avons démontré qu'il existe un pic d'expression du VEGF entre les points chronologiques à 30 et 60 minutes. Ainsi de nouvelles stratégies doivent être définies pour mieux caractériser le niveau de « dégradation » d'un échantillon de tissu non tumoral unique.

Nos collègues ayant auparavant défini l'héritabilité des concentrations plasmatiques de VEGF et compte-tenu des résultats que nous avons obtenus sur les variations pré-analytiques dans

cette partie de l'étude, nous avons décidé d'examiner la question de l'héritabilité génétique qui constitue donc le sujet de la deuxième publication de cette thèse.

F.2.2. Identification de variants génétiques agissant en *cis* et en *trans* expliquant jusqu'à la moitié de la variation des taux circulants du facteur de croissance de l'endothélium vasculaire (La troisième publication).

L'héritabilité des taux de VEGF circulant est très importante (compris entre 60 % et 80 %) (107) et des études pharmacogénomiques pourraient vraisemblablement permettre d'identifier des sous-groupes d'individus susceptibles de répondre à des thérapies pharmacologiques anti-VEGF. Cependant, peu de progrès n'ont vu le jour dans ce domaine en partie car les déterminants génétiques des taux de VEGF sont encore mal compris. Ainsi, le deuxième objectif de cette thèse était d'identifier des variants génétiques associés aux taux de VEGF circulant à l'aide d'une approche pangénomique objective et communautaire de grande envergure.

Résultats

Au moyen d'un projet commun entre l'étude *Framingham Heart*, l'étude de cohorte STANISLAS et l'étude *Prospective Investigation of the Vasculature in Uppsala Seniors* (étude prospective de la vasculature chez des personnes âgées à Uppsala) et au moyen d'une étude d'association pangénomique, nous avons identifié quatre SNP localisés dans 3 régions chromosomiques qui sont indépendamment associés (à la suite d'une analyse GWAS conditionnelle) aux taux de VEGF circulant : rs6921438 et rs4416670 (6p21.1, $p = 6{,}11\times10^{-506}$ et $p = 1{,}47\times10^{-12}$), rs6993770 (8q23.1, $p = 2{,}50\times10^{-16}$) et rs10738760 (9p24.2, $p = 1{,}96\times10^{-34}$).

L'association la plus significative était celle de rs6921438 sur le chromosome 6p21.1, à 171 kb en aval du gène du *VEGF* et proche du gène de la protéine ribosomale mitochondriale L14 (*MRPL14*). Un autre SNP localisé en 6p21.1 (rs4416670) montrait une association pangénomique significative et indépendante.

Au niveau de la région 8q23.1, le SNP rs6993770 montrait l'association la plus significative avec les taux de VEGF. Ce SNP est localisé dans le gène d'une protéine à doigt de zinc multitype 2 (*ZFPM2*), à une distance de 980,4 kb du gène de la protéine associée au récepteur de la lipoprotéine de basse densité 12 (*LRP12*).

L'association la plus significative identifiée au niveau de la région 9p24.2 était le SNP rs10738760, localisé très près du gène du récepteur de la lipoprotéine de très basse densité (*VLDLR*) et du gène du membre 2 de la sous-famille V des canaux potassiques dépendants du voltage (*KCNV2*).

Nous avons calculé un score génétique comprenant les 4 SNP qui présentaient des associations significatives avec les taux de VEGF lors de l'étude pangénomique conditionnelle (voir le texte de la publication). Ce score permettait d'expliquer 47,6 % de la variabilité des taux de VEGF dans le sérum ($p = 2{,}19\times10^{-644}$).

Six des SNP dont l'association atteignait un niveau signification pangénomique lors de l'étude d'association, dont rs16873365, rs16873402, rs6993770, rs16873291, rs2375980 et rs910611, étaient associés de manière significative aux taux d'expression de l'ARNm du $VEGF_{121}$ dans les cellules mononucléaires du sang périphérique. L'analyse des différentes voies de signalisation à l'aide du logiciel Ingenuity a mis en évidence des liens biologiques convaincants entre le VEGF et deux nouveaux gènes à ces loci : *ZFPM2* et *VLDLR*.

Discussion

Les études de gènes candidats visant à explorer les associations entre des polymorphismes *VEGF* et les taux de VEGF circulant ont donné des résultats controversés (voir la troisième publication). Plusieurs études ont signalé des associations significatives avec des polymorphismes candidats dans le promoteur et au niveau des régions 5' et 3' non traduits du gène du *VEGF*. Cependant, plusieurs autres études n'ont pu déceler d'association pour ces mêmes SNP ainsi que d'autres SNP *VEGF* (la troisième publication). À l'aide d'une approche

pangénomique sans hypothèse de départ, nous avons décrit de nouvelles associations pour 140 SNP. Parmi celles-ci, 68 des SNP associés étaient localisés sur le chromosome 6, à environ 150 kb en aval de l'extrémité 3' du gène du *VEGF*, c'est-à-dire à une distance importante des SNP candidats précédemment analysés.

Bien que plusieurs études aient étudié l'association de variants génétiques candidats avec l'expression génetique du *VEGF* dans des tissus pathologiques, relativement peu de choses sont connues sur les variants génétiques qui pourraient exercer une influence sur l'expression du *VEGF* dans des cellules normales. Nos données suggèrent que six des SNP identifiés lors notre étude pangénomique et associés aux taux de VEGF circulant modulent également l'expression de l'isoforme $VEGF_{121}$ dans les PBMC d'un échantillon communautaire.

De même, nos résultats semblent indiquer que presque la moitié de la composante héréditaire des taux de VEGF circulant peut être expliquée par les variants génétiques localisés en aval du gène du *VEGF* sur le chromosome 6p21.1. Les résultats de l'étude GWAS conditionnelle insinuent que cette région pourrait comprendre au moins deux loci distincts indépendamment associés aux taux de VEGF circulant. Bien que localisé à une distance relativement importante du gène du *VEGF* , les résultats de notre analyse transcriptomique indiquent que cette région pourrait en effet comprendre des variants fonctionnels modulant l'expression génétique du *VEGF* .

Des associations pangénomiques significatives avec les taux de VEGF circulant ont également été identifiées pour des SNP localisés sur les chromosomes 8q23.1 et 9p24.2. Bien que ces effets agissant en *trans* permettent d'expliquer une part bien plus réduite de l'héritabilité des taux de VEGF, ils fournissent des informations importantes concernant les voies impliquées dans la régulation de l'expression du *VEGF* ainsi que sur des liens éventuels entre le VEGF et la régulation d'autres molécules. En effet, nous avons trouvé une association entre les taux de VEGF circulant et des SNP localisés proches de gènes impliqués dans la

biologie des cellules endothéliales (ZFPM2). Ces associations constituent donc des preuves soutenant l'implication du VEGF dans les MCV aussi bien au niveau génétique que transcriptomique.

L'identification de ces SNP nous a conduit à nous interroger sur leur impact sur d'autres facteurs de risque des MCV. Dans la partie suivante, nous nous sommes intéressés aux associations entre le VEGF, les SNP associés au VEGF et plusieurs marqueurs du dysfonctionnement des cellules endothéliales tels que les molécules d'adhésion et des molécules inflammatoires associées aux MCV dont TNF-α, IL-6 et CRP. (La troisième publication).

F.2.3. Association du VEGF à des molécules d'adhésion et de l'inflammation dans une population saine (la quatrième publication).

Il a été démontré que le VEGF est impliqué dans plusieurs pathologies vasculaires, dont des MCV (cardiopathie ischémique, insuffisance cardiaque et AVC). Des taux élevés de VEGF circulant ont été également observés dans plusieurs types de cancer et divers troubles, dont le diabète, la démence/déclin cognitif, et des troubles de la reproduction et immunoinflammatoires (190). Le $VEGF_{145}$ joue un rôle essentiel lors de la vascularisation du follicule ovarien et du corps jaune chez la femme, lors du développement placentaire et de la période embryonnaire, ainsi que lors de la cicatrisation osseuse et tissulaire (190 ;191).

L'existence de voies de signalisation communes entre le VEGF, les molécules d'adhésion cellulaire (CAM) et des molécules de l'inflammation pourrait permettre d'expliquer la large gamme de fonctions du VEGF dans différentes situations pathologiques.

En effet, des relations complexes entre le VEGF, les CAM et des marqueurs de l'inflammation ont été décrites dans de nombreuses maladies, particulièrement dans les phénotypes d'origine cardiovasculaire. Par exemple, certaines études ont signalé l'implication des sélectines E, P et L, de la molécule d'adhésion des cellules vasculaires 1 (VCAM), de la molécule d'adhésion intercellulaire 1 (ICAM-1) et de l'interleukine 6 (IL-6) dans l'angiogenèse (192). Il a également été démontré que les taux circulant de VEGF, d'IL-6, de sélectine E et d'ICAM-1 augmentent dans une situation d'hypoglycémie chez des individus sains et chez les patients souffrant de diabète de type 1 (193). Par ailleurs, il est intéressant de remarquer que plusieurs travaux ont établi un lien entre l'angiogenèse et l'inflammation, et soulignent le rôle clé du TNF-α (tumor necrosis factor) et de la CRP (194;195). L'IL-6, un médiateur de l'inflammation, est un des facteurs principaux de la production de CRP dans le foie. Des études récentes ont signalé une augmentation simultanée des taux d'IL-6 et de VEGF circulant dans certaines pathologies telles que les ascites induites par le syndrome d'hyperstimulation

ovarienne, le diabète, l'obésité viscérale, le cancer et l'hypertension (196 ; 197). Les concentrations sériques de molécules d'adhésion solubles sont également corrélées avec celles de protéines de phase aiguë comme la protéine C réactive (CRP) chez des femmes souffrant d'un cancer du sein (198). De surcroît, chez les patients souffrant de cancer du foie, les taux de sélectine P soluble ont été associés aux concentrations de $VEGF_{165}$ dans le plasma (199).

L'évaluation de ces relations dans différentes situations pathologiques progresse, cependant, en raison de la complexité des mécanismes physiopathologiques sous-jacents à chaque maladie, l'origine de ces interactions biologiques est difficile à interpréter. Il importe donc de les étudier chez l'individu sain en l'absence de toute manifestation pathologique afin de recueillir des informations importantes concernant les interactions physiologiques entre ces molécules à l'état normal avant tout développement d'une maladie donnée. Cela pourrait également permettre de mieux comprendre les rôles biologiques du VEGF. Pourtant, à notre connaissance, aucune étude publiée n'a encore abordé les interactions entre le VEGF, les CAM et les marqueurs de l'inflammation dans une population en bonne santé.

De ce fait, l'objectif suivant de cette thèse était d'examiner les associations entre le VEGF et l'ICAM-1, la VCAM-1, les sélectines E, L et P, le TNF-α, l'IL-6 et la CRP dans le plasma d'individus apparemment en bonne santé. De plus, nos travaux visaient à déterminer s'il existait un lien entre le profil d'expression de ces molécules dans les cellules mononucléaires du sang périphérique (PBMC). Enfin, nous avons évalué les liens génétiques éventuels entre ces molécules. Ainsi, nous avons cherché à définir les associations éventuelles entre les SNP rs6921438, rs4416670, rs6993770 et rs10738760 et les taux plasmatiques de CAM et de marqueurs de l'inflammation, ainsi que leur effet sur l'expression génétique de ces molécules dans les PBMC.

Résultats

Associations entre les taux plasmatiques de VEGF, les CAM et les molécules inflammatoires

Les taux plasmatiques de VEGF étaient associés aux concentrations d'ICAM-1 (beta = 0,043, p = 0,023) et de sélectine E (bêta = 0,085, p = 0,039).

Associations entre l'ARNm des isoformes du VEGF et l'expression génétique des CAM et des molécules inflammatoires

Lors de l'analyse de l'association des quatre isoformes du VEGF et de l'expression génétique d'ICAM-1, des sélectines P et L, de TNF-α et d'IL-6 dans les PBMC, nous avons trouvé que l'isoforme $VEGF_{145}$ était associée de manière significative avec l'ARNm d'ICAM-1 (bêta = 0,353, p = 0,021), l'ARNm de la sélectine L (bêta = 0,611, p = 0,001) et l'ARNm du TNF-α (bêta = 0,616, p = 0,001).

Associations de rs6921438, rs4416670, rs6993770, rs10738760 et leurs interactions épistatiques avec les taux plasmatiques et le niveau de transcription des CAM et des molécules inflammatoires

Des interactions épistatiques significatives ont été identifiées pour les concentrations de sélectine E, de TNF-α, d'ICAM-1 et d'IL-6 (voir le texte de la publication n° 3). Une association significative était observée entre rs4416670 et les niveaux de l'ARNm de la sélectine L (beta = 0,196, p = 0,039).

Discussion

Dans cette partie de l'étude, nous avons évalué les liens entre le VEGF, les CAM et molécules inflammatoires à l'état sain au moyen d'une approche pluridimensionnelle : associations des taux plasmatiques, de l'expression génétique dans les PBMC, des déterminants génétiques communs et effets fonctionnels éventuels de ces déterminants sur l'expression génétique. Des résultats significatifs ont été constatés à tous les niveaux et pourraient permettre d'expliquer les interactions physiologiques entre ces molécules. Les taux plasmatiques de VEGF étaient

associés positivement à l'ICAM-1 et à la sélectine E. Pour autant que nous sachions, notre étude est la première à démontrer un lien entre le VEGF, le principal facteur angiogénique, l'ICAM-1, qui permet une adhésion solide des leucocytes aux cellules endothéliales, et la sélectine E, qui permet le roulement des leucocytes sur l'endothélium vasculaire (200)**.** Nous avons démontré que l'expression de l'ARNm de l'isoforme $VEGF_{145}$ est associée de manière positive à l'expression d'ICAM-1, de sélectine L et de TNF-α. Ces associations pourraient indiquer l'existence de facteurs transcriptionnels communs ou d'autres mécanismes moléculaires régulant l'expression de ces molécules dans les PBMC dans des situations non pathologiques. L'approche pluridimensionnelle employée lors de cette étude doit être considérée comme un des points forts de ce travail d'évaluation, de validation et d'élucidation des liens entre le VEGF et les molécules d'adhésion et inflammatoires chez des individus en bonne santé. Elle ouvre ainsi de nouvelles perspectives et hypothèses pour des études ultérieures qui viseront à identifier précisément les mécanismes moléculaires sous-jacents à l'implication du VEGF dans le développement de MCV et d'autres pathologies chroniques.

L'étude de l'association du VEGF et de SNP associés au VEGF avec le syndrome métabolique était donc la prochaine étape car nous avons découvert des associations entre les taux de VEGF et le gène MRPL14 lié à la dépense énergétique, ainsi que les gènes VLDLR et LRP12 impliqués dans le métabolisme des lipides.

F.2.4. Association des variants génétiques du VEGF agissant en *cis* et en *trans* avec le syndrome métabolique et ses composants (Les cinquième et sixième publications).

Le syndrome métabolique (SM) est un ensemble de facteurs de risque interdépendants qui sont associés à une altération de l'angiogenèse et semblent promouvoir directement le développement de MCV (42,156,201,202), étroitement lié à l'obésité. Plusieurs publications ont signalé la corrélation positive entre les taux sériques de VEGF, l'indice de masse corporelle et l'obésité viscérale (149,150). Des études épidémiologiques ont pu montrer qu'une accumulation de graisse viscérale, et non de graisse sous-cutanée, représente le principal facteur déterminant des taux de VEGF circulant (49 ;150). Kressel et coll. (54) ont étudié le lien entre les marqueurs vasculaires et systémiques de l'inflammation de faible intensité, tels que hs-CRP, la molécule d'adhésion vasculaire soluble 1 (sVCAM-1), la molécule d'adhésion intracellulaire soluble (sICAM-1), l'inhibiteur du plasminogène 1 (PAI-1), le fibrinogène et les facteurs de risque cardiovasculaires traditionnels ainsi que le SM. Les lipoprotéines de haute et de basse densité, la HDL-C (en tant que composant du SM) et la LDL-C respectivement, sont considérées comme des facteurs de risque indépendants pour le développement de MCV (38). Des taux accrus de VEGF ont été décelés dans des sujets souffrant d'hyperlipidémie sans complications lors d'une étude de population de faible taille (203) ; des résultats similaires ont été signalés dans une étude pilote chez des patients atteints d'hypercholesterolémie (204). Des associations significatives ont été trouvées entre les taux de HDL-C et de VEGF dans une population apparemment saine au Japon(205), alors que dans la population supposée saine de l'étude SAPHIR, une corrélation négative a été mise en évidence entre le VEGF et la LDL-C, le cholestérol total (CT) et l'apolipoprotéine B, et ce uniquement chez la femme (206). Ainsi, le VEGF pourrait constituer une molécule clé reliant l'obésité, l'inflammation, le SM et ses composants associés. Les publications traitant de la relation entre le VEGF et le SM ainsi que ses composants associés sont peu nombreuses (153-156).

Toutefois, certains travaux ont mis en évidence des associations entre le VEGF circulant et le SM ainsi que qu'avec certains de ses composants (153 ;154). De plus, un contexte héréditaire semble exister pour le SM car les données d'études d'association pangénomiques indiquent que certains SNP sont associés au SM (158,207,208) . Toutefois, l'effet éventuel de SNP associés au VEGF sur le SM et ses composants n'a pas encore été examiné. Comme décrit précédemment, nous avons identifié quatre SNP (rs6921438, rs4416670, rs6993770 et rs10738760) permettant d'expliquer environ 48 % de l'héritabilité des taux de VEGF au moyen d'une étude d'association pangénomique (209). De façon intéressante, dans la deuxième partie de cette étude, nous avons trouvé des liens biologiques convaincants entre le VEGF et le gène du récepteur de la lipoprotéine de très basse densité *VLDLR* (un des composants associés jouant un rôle important dans le métabolisme des lipides). De ce fait, le quatrième objectif de cette thèse visait à évaluer les possibles associations entre le SM et ses composants avec les taux de VEGF circulant et les SNP associés au VEGF dans une population en bonne santé (étude de cohorte STANISLAS).

Résultats

Aucune association statistiquement significative n'a pu être observée entre les taux plasmatiques de VEGF et le SM, ainsi que ses composants, dans cette population.

Lors de l'analyse des SNP, il a été démontré que rs10738760 était associé de manière significative à la présence de SM. La présence de l'allèle mineure A était associée à un risque réduit de SM. Les résultats étaient encore significatifs à la suite de l'ajustement pour le tabagisme. Une tendance à l'association entre rs10738760 et la concentration de triglycérides était observée (bêta = -0,028, p = 0,056). Des associations significatives étaient observées entre les variants du VEGF ayant un rôle régulateur, rs6921438 et rs6993770, et la HDL-C et ainsi que d'une interaction entre 4416670 et de l'hypertension pour l'apolipoprotéine E. L'allèle mineur du polymorphisme était associé à des niveaux moins élevés de HDL-C chez

les participants hypertendus. rs6993770 et rs10738760 n'étaient pas compris parmi les SNP significatifs (génotypés ou en LD avec un autre SNP) lors des trois études d'associations pangénomiques (158,207,208) précédemment réalisées sur le SM et ses composants. Il s'agit donc de nouveaux polymorphismes pour le SM et un de ses composants, la HDL-C.

Discussion

Dans cette partie de l'étude, nous avons examiné l'association du SM, de ses composants, et de certains facteurs liés aux MCV avec les variants génétiques du VEGF agissant en *cis* et en *trans*. Nous n'avons pas pu mettre en évidence d'association entre les taux de VEGF, le SM et ses composants dans la population étudiée. De plus, une association a été observée entre rs10738760 et le SM, indépendamment de ses composants.

Aucune relation statistiquement significative n'a été obtenue entre le SM ou ses composants associés et les taux de VEGF. Pourtant, Lieb et coll. (154) ont signalé une association significative entre le VEGF circulant et le SM. Il apparait donc que la différence entre la taille de leur échantillon et le nôtre pourrait expliquer ces divergences. Lors d'une étude cas-contrôle, Tarantino et coll. (155) avaient signalé des taux de VEGF circulant plus élevés chez des individus souffrant de SM que chez des sujets minces. Sur la base d'une autre étude, une association a été démontrée entre le VEGF et le SM, cependant il semblerait que les auteurs aient utilisé la définition de SM du *Adult Treatment Panel III* (ATPIII) (156) qui présente une sensibilité moindre que celle de la Fédération Internationale pour le Diabète (IDF) (utilisée dans l'étude actuelle) pour la prédiction du diabète et des MCV (270). De ce fait, il ne ressort pas clairement si un taux de VEGF élevé correspond à un facteur délétère ou s'il s'agit d'une boucle de régulation physiologique/négative pour éviter un accroissement de l'ischémie d'origine métabolique ainsi que celle due au SM. Le polymorphisme rs10738760 est un nouveau polymorphisme pour le SM et est localisé en 9p24.2. Il s'agit d'un SNP intergénique localisé très près du gène du récepteur de la lipoprotéine de très basse densité (*VLDLR*) et d'un

gène de la sous-famille V des canaux potassiques dépendants du voltage (*KCNV2*) (209). Dans la deuxième partie de cette étude, nous avons examiné les résultats montrant que rs10738760 explique environ 5 % de la variation des taux de VEGF circulant (209). De surcroît, dans cette même partie, l'analyse des voies de signalisation à l'aide du logiciel Ingenuity a mis en évidence des liens biologiques convaincants entre le VEGF et le gène VLDLR situé au locus associé. Dans cette partie de l'étude, nous avons trouvé, en plus de sa relation avec le SM, une tendance à l'association entre rs10738760 et le taux de triglycérides. Dans un contexte d'insulinorésistance et d'hyperglycémie, la voie de signalisation des protéines kinases activées par des facteurs mitogènes (MAPK) reste fonctionnelle et montre même une hypersensibilité, alors que les voies de signalisation conduisant à l'activation des phosphatidylinositol 3-kinases (PI-3K), qui représente « la voie de signalisation normale des fonctions attribuables à l'insuline et au VEGF », sont inhibées (211). Cependant, le VEGF exerce ses fonctions anti-apoptotiques et prolifératives par l'intermédiaire de la voie de signalisation des MAPK (212). De manière intéressante, d'après les données de Miyokawa-Gorin, une activation chronique des MAPK induit l'expression du VEGF dans le tissu adipeux (145 ; 213). Il a également été démontré par Karakelides et coll. (214) qu'un déficit en insuline augmente l'expression génétique du VEGF dans des cellules musculaires. *Kcnv2* code pour la sous-unité de canal potassique dépendant du voltage Kv8.2, appelé également Kv11.1 et RCD3B, qui lorsque exprimée sous la forme d'un homotétramère n'ayant aucune activité fonctionnelle (215). En revanche, lorsqu'elle s'associe à des membres de la famille Kv2 pour former une hétérotétramère, Kv8.2 agit sur la translocation membranaire et les propriétés biophysiques de ces canaux (216). Des éléments de plus en plus nombreux indiquent qu'il existe un dysfonctionnement des canaux ioniques coronariens, dont Kv et des canaux $Ca^{2+,}$ dans le SM. Ces canaux joueraient un rôle important dans la dérégulation du contrôle du flux sanguin coronarien (217-221). Kv2.1 régule négativement la sécrétion d'insuline dans des

échantillons d'origine animale et son inhibition dans des cellules bêta du pancréas conduit à une augmentation de la sécrétion d'insuline dépendante du glucose (222). De surcroît, il a été montré que le VEGF pouvait agir comme facteur de survie pour les îlots pancréatiques humains et améliorer leur sécrétion d'insuline (223-225). Une étude chez l'animal réalisée par Watada et coll. suggérait une activation compensatoire des cellules bêta induite par le VEGF. Cependant les auteurs n'ont pas discuté comment leur modèle pourrait influencer la sécrétion d'insuline. Jusqu'à présent, et pour autant que nous le sachions, il semblerait qu'aucune publication ne présente de résultats permettant d'établir par quels mécanismes moléculaires le VEGF induit la sécrétion d'insuline. Ainsi, pour la première fois, notre étude suggère que le VEGF pourrait agir sur l'assemblage du hétérotétramère comprenant les membres Kv8.2 de la famille de canaux potassiques dépendants du voltage, et modifier la fonction de Kv2.1 dans les cellules pancréatiques, ce qui aboutit à une augmentation de la sécrétion d'insuline. Il semblerait donc que les taux élevés de VEGF observés dans le SM pourraient être le résultat d'un processus de compensation, lequel contribuerait, avec ou indépendamment d'autres facteurs éventuels, à maintenir une concentration d'insuline active. Des études complémentaires seront nécessaires pour déterminer les mécanismes conduisant à la sécrétion d'insuline par l'intermédiaire de la hétérodimérisation précédemment décrite ainsi que les autres conditions induisant cet assemblage moléculaire. Ces études ultérieures devront examiner l'effet de stimulation par le VEGF sur l'expression des canaux potassiques dans l'appareil microcirculatoire coronarien de patients souffrant de SM. Les SNPsrs6921438 et rs4416670 sont des SNPs intergéniques sur le chromosome 6, à proximité du gène du VEGF et localisé entre le gène de la protéine ribosomale mitochondriale L14 (MRPL14) et le gène MCG45491 (C6orf223). rs6993770 est un polymorphisme intronique du gène de la protéine à zinc doigt gène multitype 2 (*ZFPM2*) localisé à 980,4 kb du gène de la protéine associée au récepteur de la lipoprotéine de basse densité 12 (*LRP12*) sur le chromosome 8. Nous avons pu

démontrer, dans la deuxième partie de cette étude, que ces SNP étaient associés de manière négative aux taux de VEGF. Par conséquent, il apparaît que ces SNP pourraient exercer un effet protecteur au niveau du système cardio-vasculaire par l'intermédiaire d'une augmentation des niveaux de HDL-C et d'apolipoprotéine E.

En conclusion, bien que nous n'ayons pas pu mettre en évidence d'association significative entre les taux de VEGF et le SM ou ses composants associés dans la population étudiée, les effets régulateurs des polymorphismes rs10738760, rs4416670,rs6921438 et rs6993770 sur le VEGF ainsi que les liens découverts entre le SM et la HDL-C nous amènent à suggérer que les taux élevés de VEGF observés dans le SM représentent une mesure compensatoire et pourraient protéger les personnes souffrant de SM de conditions ischémiques.

Dans une prochaine étape, nous avons décidé de réaliser une étude comparative entre la cohorte STANISLAS et une population iranienne.

F.2.5. Prévalence élevée du syndrome métabolique en Iran en comparaison avec la France (Septième publication).

La prévalence du SM varie énormément entre différents pays et groupes ethniques ; entre 20 % et 30 % de la population européenne et blanche américaine en serait victime, avec une distribution similaire chez les hommes et les femmes (211). Dans les pays asiatiques, notamment dans les sociétés du Moyen Orient, la prévalence du SM est encore plus importante, avec un profil de composants associés différent et une distribution inégale entre les sexes. Les études de population et les travaux de comparaison scientifiques documentant ces divergences Est–Ouest sont rares. Le dernier objectif de cette thèse était donc de comparer la prévalence du SM et de ses composants entre une population française, celle de la cohorte STANISLAS, et d'une population iranienne afin d'analyser les éventuelles disparités observées. De ce fait, afin de 1) comparer la prévalence du SM et de ses composants entre les deux pays, 2) comparer l'héritabilité génétique du SM entre les deux populations, 3) reproduire nos observations quant à l'association des variants génétiques du VEGF agissant en *cis* et en *trans* au SM dans une population distincte (non européenne) lors d'une étude collaborative avec l'Université des sciences médicales de Mashhad en Iran, nous avons examiné les différences du profil du SM et de ses composants associés entre les deux pays.

La prévalence du SM était significativement plus élevée chez les femmes iraniennes (55,0 %) et les hommes iraniens (30,1 %) que dans la population française, 6,6 % et 13, 7 % chez les femmes et les hommes respectivement. Chez les femmes iraniennes, le syndrome était caractérisé par des taux élevés d'obésité abdominale (65,0 %), d'hypertension (52,1 %), d'hypertriglycéridémie (43,1 %) et un niveau faible de HDL-C (92,7 %). Chez les hommes iraniens, le syndrome était caractérisé par des taux élevés d'hypertension (48,9 %), d'hypertriglycéridémie (42,8 %) et un niveau faible de HDL-C (81,8 %). Les hommes français

souffrant de SM présentaient des taux élevés d'hypertension (44,7 %) et des taux moyennement élevés d'hypertriglycéridémie (28,6 %) et d'hyperglycémie (23,9 %).

Discussion

Cette étude est la première réalisée chez des populations française et iranienne dans un but d'identifier les points communs ainsi que les différences qui caractérisent le SM dans les deux pays. Les résultats principaux de cette étude sont la prévalence élevée du SM, se traduisant par un niveau faible de HDL-C et un tour de taille élevé, chez les femmes iraniennes en comparaison avec les femmes françaises. Ces observations concordent avec celles d'études antérieures qui recommandaient de suivre de la progression du SM en Iran en incluant le tour de taille, le taux de TG et le taux de HDL-C dans les mesures effectuées (226 ;227). De plus, dans notre analyse le niveau de HDL-C était associé avec le tour de taille ce qui suggère un lien entre un tour de taille importante et une baisse de la concentration de HDL-C. Notre analyse a mis en évidence une corrélation entre le tour de taille et le taux de TG, ainsi qu'une relation inverse du premier avec la HDL-C, et ce dans les deux pays.

En effet, nos observations ont souligné que l'obésité centrale représentait le noyau dur caractérisant le SM, malgré le rôle considérable qui pourrait être attribuable à des facteurs environnementaux, dont les comportements alimentaires et l'activité physique, dans la prévalence du SM, notamment chez les femmes des deux pays. La contribution de facteurs génétiques aux différences entre les deux populations devra enfin être abordée. L'étude des problématiques suivantes a été prévue lors de collaborations à venir : 1) l'analyse des différences génétiques du SM entre les deux pays, 2) la comparaison de l'héritabilité génétique du SM entre les deux populations, 3) la reproduction de nos observations quant à l'association des variants génétiques du VEGF agissant en *cis* et en *trans* au SM et ses composants associés, notamment la HDL-C, dans une population distincte (non européenne).

CHAPITRE F3

DISCUSSION GÈNÈRALE

La présente étude a été conçue dans le but d'identifier les associations vraisemblables qui existaient entre le VEGF, ses variants génétiques agissant en *cis* et en *trans* et les MCV. Plus particulièrement, nous avons examiné l'hypothèse selon laquelle le VEGF et ses polymorphismes régulateurs associés pourraient jouer un rôle central dans le SM. Les résultats principaux de cette étude sont :

1) l'identification de variants génétiques du VEGF qui expliquent environ 48 % des taux de VEGF circulant, dont rs6921438, rs4416670, rs6993770 et rs10738760,

2) l'association du VEGF et de ses variants génétiques connus au moyen d'une approche tridimensionnelle (génétique, transcriptomique et protéique) avec plusieurs marqueurs de risque majeurs des MCV tels que des molécules d'adhésion (ICAM-1 et les sélectines E et L) ainsi que plusieurs molécules inflammatoires telles que TNF-α, IL-6 et CRP,

3) l'association inverse du polymorphisme rs10738760 associé au VEGF avec le SM,

4) la relation entre le variant de VEGF ayant un rôle régulateur, rs6921438, et la HDL-C. L'allèle mineur de cette variante a été associée à de faibles niveaux de l'apolipoprotéine E chez les participants hypertendus

5) la prévalence élevée du SM, se traduisant par un niveau faible de HDL-C et un tour de taille élevé, chez les femmes iraniennes en comparaison avec les femmes françaises.

Les résultats de base et préalables de cette étude ont montré que :

1) la mesure à la fois des taux de VEGF circulant et de son expression génetique est optimale en réduisant la durée entre le recueil du sang et sa centrifugation, et en évitant des cycles de gel/dégel répétés,

2) le sérum représente l'environnement le plus stable (plutôt que l'utilisation de divers anticoagulants),

3) la détermination des niveaux de VEGF dans des tissus non tumoraux est optimale si ceux-ci sont congelés moins de 15 minutes après leur excision.

Les MCV représentent la principale cause de décès à travers le monde et sont à l'origine de jusqu'à 30 % de la mortalité mondiale (228 ;229). Le diabète de type 2, l'obésité, l'hypertension et la dyslipidémie sont considérés comme des facteurs de risque classiques pour les MCV. À présent, un nouveau groupe de facteurs de risque pour les MCV se dégage et comprend des taux accrus de facteurs pro-inflammatoires tels que le TNF-α, l'IL-6 et la CRP ainsi que des niveaux élevés de marqueurs d'un dysfonctionnement endothélial, à savoir la molécule d'adhésion intercellulaire 1 (ICAM-1), la moléculaire d'adhésion vasculaire 1 (VCAM-1) et les sélectines E, P et L qui dénotent une inflammation de faible intensité (230). L'obésité centrale, le facteur sous-jacent principal du SM, est associée à une sécrétion accrue de certaines adipokines, telles que l'Il-6 et le TNF-α, qui sont à l'origine d'un dysfonctionnement endothélial et d'une augmentation de l'expression de molécules d'adhésion et de la CRP (49). Cette relation entre le tissu adipeux et les cellules endothéliales est associée à une altération de l'activation des voies de signalisation de la PI3K et à une augmentation ou diminution de l'expression de plusieurs facteurs de transcription (facteur inductible par l'hypoxie [HIF], récepteurs activés par les proliférateurs des péroxisomes [PPAR], protéines fixant la séquence activatrice CCAAT [C/EBP], protéines fixant l'élément de réponse aux glucides [ChREBP] et protéines fixant l'élément de régulation des stérols [SREBP]) qui jouent un rôle essentiel dans la régulation de voies métaboliques distinctes impliquées dans le SM.

Le VEGF est une cytokine multifonctionnelle qui joue différents rôles dans les processus d'angiogenèse, de lymphangiogenèse, de perméabilité vasculaire et d'hématopoïèse (190). Il a été montré que le VEGF est impliqué dans plusieurs pathologies vasculaires, dont des MCV

(cardiopathie ischémique, insuffisance cardiaque et AVC) (190 ;209). Par ailleurs, des taux élevés de VEGF circulant ont été également observés dans divers troubles/facteurs de risque cardiovasculaires dont le SM, le diabète de type 2, l'hypertension, la pré-éclampsie, l'insuffisance rénale chronique et le syndrome des ovaires polykystiques (209). Le VEGF exerce ses fonctions physiologiques, telles que la survie, la prolifération et la migration cellulaires et la perméabilité vasculaire, en se fixant à son récepteur et en activant la voie de signalisation de la PI3K (11). Plusieurs publications récentes ont fait état de taux accrus de VEGF chez des individus souffrant de SM (154-156 ; 202 ;203). Cependant, ces études n'ont pas fourni d'explication permettant de préciser le rôle du VEGF dans ce contexte.

Dans la présente étude, nous avons démontré que le VEGF est associé à l'ICAM-1 et à la sélectine E dans le plasma. Dans les PBMC, l'ARNm du $VEGF_{145}$ est associé à l'expression d'ICAM-1, de la sélectine L et de TNF-α. Il a été démontré que les interactions des variants génétiques du VEGF avaient un impact sur les taux plasmatiques d'ICAM-1, de sélectine E, d'IL-6 et de TNF-α, et que rs44116670 était associé à l'expression de la sélectine L. Nos résultats suggèrent un lien biologique entre le VEGF et les marqueurs de l'inflammation et les CAM. Des mécanismes génétiques et transcriptionnels communs pourraient donc impliquer ces molécules et contrôler leurs effets chez des individus en bonne santé. En effet, les interactions entre le VEGF et les marqueurs de l'inflammation et les CAM pourraient servir à faire prévaloir l'état sain et empêcher la mise en place des voies de signalisation inflammatoires caractéristiques des MCV. Yäl-Herttuala et coll. ont signalé qu'un niveau physiologique faible de VEGF est nécessaire pour maintenir l'homéostasie vasculaire, la survie des cellules endothéliales et la production de monoxyde d'azote et de prostacycline qui conduisent à la vasodilatation, à un effet antithrombotique et à l'inhibition de la prolifération des cellules musculaires lisses, c'est-à-dire des effets protecteurs (232). Au moyen de cette étude, nous avons pu documenter le fait que, chez des personnes en bonne santé, certaines de

ces fonctions du VEGF pourraient être dues à l'interaction du VEGF avec des molécules d'adhésion ainsi qu'avec le TNF-α et l'IL-6.

Nos résultats ont mis en évidence une association significative entre rs10738760 et rs4416670, qui sont localisés à proximité des gènes impliqués dans le métabolisme de la lipoprotéine de très basse densité (VLDLR) et de la dépense énergétique cellulaire (MRPL14), et les taux de VEGF circulant. De ce fait, la dernière partie, mais non la moindre, de cette étude visait à examiner plus particulièrement les relations éventuelles entre le VEGF, ses polymorphisme identifiés et le SM. Comme attendu, nos résultats ont permis d'identifier une association significative, quoique inversée, entre rs10738760 et le SM, ainsi qu'une relation directe également inversée entre rs6921438 et la HDL-C. Pour autant, notre analyse n'a pas permis de déceler d'association significative entre les taux de VEGF et le SM ou ses composants associés dans la population étudiée. Il existe des relations entre les variants de VEGF ayant un rôle régulateur, rs10738760, rs6921438, et le SM et la HDL-C. L'impact du VEGF sur les fonctions des canaux potassiques dépendants du voltage 2, du récepteur de la lipoprotéine de très basse densité, de la protéine ribosomale mitochondriale L14, MCG45491(C6orf223) dans un contexte pré-diabétique seront donc le sujet de travaux à venir. Enfin, cette étude a également permis de faire état de différences de prévalence pour certains composants du SM ainsi qu'un profil de distribution distinct entre la France et l'Iran. Le résultat principal de cette dernière partie de l'étude était la prévalence élevée des faibles taux de HDL-C dans la population iranienne, particulièrement chez les femmes en comparaison avec les femmes françaises. Notre observation concernant la prévalence du SM chez les femmes iraniennes est en accord avec d'autres études épidémiologiques sur le Moyen-Orient (227 ;233 ;234). La recherche de l'origine de la différence importante du taux de HDL-C entre les deux populations, pourraient constituer la base de travaux à venir.

CHAPITRE F4

CONCLUSION ET PERSPECTIVES

Le VEGF est impliqué dans de nombreux processus physiologiques essentiels ainsi plusieurs situations pathologiques. Sur la base des associations que nous avons détectées entre le VEGF, les marqueurs de l'inflammation et les CAM dans une population en bonne santé, les résultats de cette étude suggèrent que d'éventuelles interactions entre le VEGF, les marqueurs de l'inflammation et les CAM pourraient servir à faire prévaloir l'état sain et empêcher la mise en place des voies de signalisation inflammatoires caractéristiques des MCV. Nous avons également observé des associations entre les variants génétiques du VEGF agissant en *cis* et en *trans*, localisés à proximité de cinq gènes codant pour des protéines impliquées dans le métabolisme des lipides, la sécrétion d'insuline et la dépense énergétique, et le SM, la HDL-C et l'apolipoprotéine A1. Par conséquent, nous pouvons conclure qu'il existe une relation entre les variants du VEGF ayant un rôle régulateur et le SM et ses composants associés. Notre étude montre que des travaux supplémentaires seront nécessaires afin d'identifier d'éventuelles cibles thérapeutiques pour des stratégies pharmaceutiques destinées à prévenir la survenue du diabète de type 2 et des MCV. Plusieurs aspects devront être abordés pour répondre aux questions suscitées et aux hypothèses issues de ce travail, à savoir : 1) les associations entre les taux de VEGF et le VLDLR dans le SM et le diabète de type 2, 2) les interactions entre le VEGF et les protéines ribosomales mitochondriales dans le SM et le diabète de type 2, 3) l'impact du VEGF sur l'héterotétramérisation des canaux potassiques dépendants du voltage 2 et par conséquent son effet sur la stimulation de la sécrétion d'insuline par les cellules bêta du pancréas, 4) l'effet du VEGF sur les canaux potassiques dépendants du voltage 2 au niveau de la microcirculation cardiaque et par conséquent son effet sur l'ischémie myocardique, 5) la relation entre la HDL-C et les gènes de la protéine ribosomale mitochondriale L14, de la protéine à zinc de doigt multitype 2 et du récepteur de lipoprotéine de basse densité 12 et les protéines pour lesquelles ils codent, 6) l'étude d'éventuels facteurs environnementaux ainsi que des déterminants génétiques (correspondant aux composants du SM, notamment la HDL-

C et la susceptibilité à l'obésité) qui permettraient d'expliquer les différences importantes du profil de distribution des composants du SM entre les populations iranienne et française.

D'autre part, mais indépendamment des idées suggérées ci-dessus, il semblerait qu'il existe un seuil de tolérance pour les fonctions/l'expression du VEGF chez les personnes en bonne santé qui permet de maintenir un état normal par une interaction avec d'autres facteurs actifs et évite l'activation de voies de signalisation inflammatoires. Toutefois, après la survenue d'une maladie, le taux élevé de VEGF aurait un effet délétère et accroîtrait leur morbidité et leur mortalité, par exemple lors d'événements cardiovasculaires et de métastases cancéreuses. La détermination de ce seuil de tolérance nécessitera la mise en place d'études compréhensives et pluridimensionnelles.

Plusieurs études ont décrit la possibilité d'utiliser le VEGF à la fois en tant qu'outil et cible thérapeutique en raison de la spécificité de son action sur la prolifération vasculaire et la survie cellulaire. Des données cliniques et expérimentales récentes ont démontré la faisabilité de l'administration de VEGF pour protéger des tissus ischémiques tels que le myocarde, le cerveau et le rein par la stimulation de la prolifération et de la réparation microvasculaire (235). À l'inverse, des thérapies anti-VEGF sont au premier plan pour le traitement de nombreuses tumeurs solides, mais l'inhibition du VEGF comporte des effets collatéraux tels que l'hypertension et des lésions rénales dues en grande partie à des anormalités de la vasculature. Des inhibiteurs du VEGF tels que le bévacizumab et le sorafénib ont été utilisés avec succès pour inhiber l'angiogenèse dans plusieurs types de tumeurs, la dégénérescence maculaire et la polyarthrite rhumatoïde (209). Cependant, en dépit des effets contraires importants associés aux médicaments à base d'inhibiteurs du VEGF, aucune étude pharmacologique n'a été entreprise dans le but d'identifier des sous-groupes d'individus susceptibles de répondre à ce type de thérapie, et ce en partie parce que les déterminants génétiques des taux de VEGF sont encore mal compris. Grâce à des études pharmacologiques,

les résultats de nos travaux sur les variants génétiques agissant sur les taux de VEGF pourraient permettre aux chercheurs de mieux prévoir l'issue des différentes stratégies thérapeutiques et comprendre comment certains médicaments produisent des effets inverses dans différentes situations pathologiques.

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Invited critical review

Cardiovascular diseases and genome-wide association studies

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ABSTRACT

Genome-Wide Association Studies (GWAS) on cardiovascular diseases and related quantitative traits revealed numerous genetic variants, which however have been partially replicated, probably due to the heterogeneity of the clinical phenotypes and the populations studied. Even if novel biological pathways have been identified through these studies, there is still a long way until the validation of causal variants and their use in clinical practice as factors for prevention, risk assessment and as targets for the development of new medications. GWAS methodologies should, in the following years, integrate gene–gene and gene–environment interaction analyses in a global research strategy and also involve subsequent transcriptomic and proteomic investigations. The GWAS era is very promising but it is just at the beginning.

Contents

1. Introduction: cardiovascular diseases: common disorders with important genetic component

Cardiovascular diseases (CVDs) are the leading cause of death in the world. Based on World Health Organization, 17.1 million people died from CVDs in 2004 and it is estimated that by 2030, approximately 23.6 million deaths will be recorded due to CVDs, mainly from heart disease and stroke [1]. The aetiology of CVDs is multifactorial, where a complex combination of environmental, genetic and clinical risk factors seem to play determinant role [2]. In fact, the pathogenesis of coronary heart disease (CHD) is known to be influenced by smoking, diabetes, hypertension, obesity, physical inactivity, alcohol intake and psycho social conditions [3–5]. Genetic elements contribute to the development of CAD [6,7]. This complex architecture and its genetic background [8,9] are still poorly understood and substantial discrepancies remain in estimating the heritability of numerous CVDs-related quantitative traits (QTs) [10]. Risk algorithms such as the Framingham Risk Score [11] have traditionally incorporated classical clinical and environmental risk factors such as age, gender, blood lipid concentrations, blood pressure (BP), body mass index, family history and

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smoking habit for primary prevention strategies purposes. The incorporation of genetic risk factors in the risk assessment algorithms can be beneficial for the improvement of preventive public health strategies and could open new landscapes for more effective treatment (personalised medicine) and reduced complications [12]. The identification and validation of genetic variants that predispose to CVDs and related QTs may lead to better understanding of the fundamental pathogenetic pathways and to the establishment of new biological pathways and novel risk factors.

Over the last 2 decades, genetics of chronic diseases and related QTs were assessed by candidate gene approaches [13]. Twins and families studies were used to estimate heritability, while population-based studies were used to identify variants that are associated with a phenotype and to explore interactions between genetic and environmental factors. Candidate genes are those considered to influence complex phenotypes due to their participation in biological pathways or due to their location close to a region of interest [13]. This "hypothesis-driven" research has provided the foundation for the development of genetic CVDs risk profiles [12]. However, since 2007, genome-wide association studies (GWAS) have brought a revolution in the field of genetic background of chronic diseases like CVDs and their associated QTs [14–16]. The recent technological advances are enabling the rapid and accurate assessment of millions of single-nucleotide polymorphisms (SNPs) in large populations, leading to an increased number of GWAS for different CVDs phenotypes and related traits. These studies resulted in the identification of hundreds of genetic factors, some robustly replicated, however with small individual effects [17,18]. Moreover, GWAS revealed previously unsuspected pathological pathways [8,19]. Nevertheless, the identification of causal variants explaining the high heritability of the different CVDs phenotypes, which could be used for prevention, diagnosis, treatment and prognosis of CVD patients, has not yet been achieved with satisfactory results through the GWAS [20].

In this work we review GWAS findings concerning CVDs and their related QTs, the reasons for missing heritability and future challenges.

2. Methods

2.1. Literature search

The GWAS investigator of HuGENavigator engine [21] and the NHGRI Catalog of published GWAS (http://www.genome.gov/gwasstudies, assessed May 2011) [22] were used in order to assess GWAS published between 2007 and 2011, using the keywords of "cardiovascular diseases", "coronary heart disease", "coronary artery disease", "diabetes", "obesity", "hypertension", "blood pressure", "anthropometric measurements", "response to medication" and "lipids". In order to confirm the retrieved findings, we have checked the related articles and their reference lists in the MEDLINE engine using the same previous keywords along with 'GWAS' or 'single nucleotide polymorphism (SNPs)'. In addition, we restricted the search results to articles published in English language and conducted on humans.

2.2. Data extraction

Two authors independently extracted the following information from each study: CVDs traits, variants and their near gene(s), genomic region, corresponding trait(s), reference(s), P-values, number of subjects in each study (discovery and replication), the variant risk allele and the effect size. Genome-wide significance was considered when P-value$<5\times10^{-8}$.

3. Brief overview of GWAS on cardiovascular diseases and their related quantitative traits

To date, a vast number of GWAS focusing on about 150 distinct diseases and QTs have reported several hundreds significant SNP-trait associations. GWAS have been conducted on cardiovascular events (CHD) and specifically to myocardial infraction (MI) and they have reported the association of at least 16 genetic variants found in 15 genes (Supplementary Table 1). They have identified SNPs on chromosomal regions 1p13, 1q41, 2q36, 3q22, 6p24, 6q25, 7q22, 9p21, 10q11, 10q23.2, 10p11.23, 11q22.3, 12q24, 15q25 and 15q22 that are associated with risk of CHD or its main complication, MI [23–28]. The associations between SNPs on 9p21 and CHD or MI were the most strongly replicated findings in the majority of the assessed ethnic groups [29–31].

However, caution is needed when interpreting data found in GWAS for CVDs, given the weak sample size of the majority of these studies. The genetic variants are rarely robustly linked to the cardiovascular events. Similarly, the lack of suggestive associations stressed out the value of using accurate clinical subtype classification for the phenotypes and standardised methodology for multi-centre studies.

GWAS of CVDs-related QTs could provide an alternative approach for better comprehending the phenotypic diversity of a given CVD as stated by the Wellcome Trust Case Control Consortium [32].

A significant proportion of GWAS has been focused on obesity. The identified genetic variants, including some paediatric studies, have been replicated in less than 2/3 of them. Interestingly, the *FTO* gene was identified as the first locus harbouring common variants with an unequivocal impact on obesity predisposition, diabetes and fat mass in population studies. The most important reports are summarised in Supplementary Table 1.

More than 1/4 of all GWAS for CVDs related QTs have studied fasting plasma glucose and diabetes (Supplementary Table 1). Up to 90% of them have been replicated in European, American and Asian populations. In two distinct studies, Zeggini et al. [33,34] reported the association of type 2 diabetes with 22 SNPs close to the following genes: *CDKN2A, CDKN2B, FTO, HHEX, IGF2BP2, KCNJ11, CDKAL1, TCF7L2, THADA, TSPAN8, LGR5, VEGF, NOTCH2, ADAM30, PPARG, SLC30A8, JAZF1, SYN2, CDC, ADAMTS9, CDC123, CAMK1D*. Of these, 2 SNPS in the vicinity of *TCF7L2* had the greatest association with type 2 diabetes (Supplementary Table 1).

Also, lipid profile and BP studies represent a considerable part of CVD GWAS. About 2/3 of SNPs associated with lipid levels have been successfully replicated, notably 3 in the vicinity of *CETP* (Supplementary Table 1). It is important to mention that, although rs3764261 in *CETP* was strongly associated with total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) (P-value $=7\times10^{-380}$) [35], it was not associated with CHD. Interestingly, a number of GWAS have achieved to find some loci which were associated with both lipid levels and CHD risk. For example, there are some replicated SNPs in *CETP, APOC1, APOB, APOA1* genes that are associated with more than one related trait in CVD risk (Supplementary Table 1).

It should be noted also that the meta-analyses on hyperlipidaemic and diabetic individuals [25,35,36], have included some common subsamples. Therefore, similar association results will not be surprising [37].

In addition to lipid profiles, replication studies have been relatively successful in confirming BP and hypertension (HTN) key loci [38–40]. In the largest joint meta-analysis published in May 2009, by CHARGE [41] and GlobakBPgen [8] consortia, researchers discovered 14 loci associated with systolic BP, diastolic BP and/or HTN. Recently, Takeuchi et al. [38] succeeded in replicating 7 loci in Japanese subjects, whereas Liu et al. [39] and Hong et al. [40] have confirmed some of them in Chinese Hans and Koreans respectively. These main

findings constitute a milestone in bringing genome knowledge into BP regulation in diverse populations, as it will be discussed below [42].

In 2009, a GWAS assessed the possible associations between several hundred thousand SNPs and warfarin dose in about 1000 Swedish patients. The results showed 3 significant SNPs around *CYP2C9* and *VKORC1*. After removing the effects of those SNPs through multiple regression adjustment, an additional signal was observed, implicating another SNP around the cytochrome P450 gene (*CYP4F2*) [43]. Similar results were presented in Japanese population [44]. These findings raise the probability that testing patients for variations in the two mentioned genes might provide information that could improve clinical algorithms currently used to guide the administration of warfarin.

Also, the role of genetic factors in differences of lipid-lowering response to statin treatment seems to be influenced by several loci with small individual contributions that are compounded when simultaneously inherited. Although several loci have been investigated in relationship with efficacy of statin using the candidate gene approach, the combined contribution of these genotypes explains a relatively small proportion of the variation in statin lipid-lowering efficacy [45– 47]. GWA analysis of lipid-lowering response to statin treatment has previously failed to identify novel loci. In order to find eventual genetic variants, Barber MJ et al. [48] have done a combined analysis on 3 statin GWA studies. They have eventually found that the most provocation is the association of rs8014194 in *CLMN* gene with changes in total cholesterol in response to statin treatment. They have observed strongest association of this SNP in one of three populations. However, this genetic variation was less strongly associated with LDL-C reduction.

4. GWAS strengths and limitations

4.1. Current GWAS designs

GWAS involve various tiered designs including case–control and cohort studies for the identification of trait/disease-SNP associations. These strategies allow affordable procedures to be carried out by conducting a genome-wide scan in a discovery set in order to select a core of significantly associated SNPs that will be subsequently genotyped in larger replication sets for confirmation [49]. These intricate designs involve more and more tiers according to the complexity and originality of the trait of interest [50] and allow the elimination of previous spurious associations (false positives) [18]. Traditionally, genetic associations having causal functionality and appearing in multiple populations and studies are considered reliable [18,49].

Testing the association of millions of SNPs with a specific trait or disease implies a large number of statistical tests (at least one per SNP) making nominal significance (0.05) inappropriate for originally selecting associated variants. Such designs also require large populations [51] and this need led to the recruitment of large international consortia, in which substantial pooled GWAS have been conducted. Indeed, significant progress in the management of the methodological weaknesses of GWAS has been made under the auspices of large consortia providing numerous independent populations for pooling analyses and replications in specific fields, such as the Glucose and Insulin Related Traits Consortium (MAGIC) [52]. These pooling strategies required controls for differences in allele frequencies [13] to minimise false positive findings and reinforce the validity of genetic variants found to be associated with specific traits or pathologies.

Only 12% of SNPs discovered by all the GWAS performed to date and associated with traits are located in the vicinity of protein-coding regions of genes, although SNPs in protein-coding regions are heavily over-represented on genotyping arrays [22]. Forty percent falls in intergenic regions, and another 40% is located in introns. The repeated replication of signals falling consecutively in the so-called “gene deserts”, although it initially raised concern in the scientific community, has sharpened the focus on the potential roles of intronic and particularly intergenic regions in regulating gene expression [14].

4.2. Future challenges

Over the past few years, GWAS have reported a large number of novel genetic variants associated with CVDs [53,54]; nevertheless, many challenges remain.

There is a need to perform GWAS on populations with diverse geographic ancestries, which have undergone more mutations and greater recombination events. This type of studies could give greater degrees of genetic variation and shorter stretches of linkage disequilibrium allowing better localisation of genome-wide association signals [20,32]. Several different ethnicities such as Japanese [38], African Americans [55] and mainly Europeans [8,41] have been examined. European populations are closely related [42], and their genome has undergone less recombination events than for example those of Africans [56], an advantage that has increased the number of studies concerning this specific descendent. Will the initial findings be replicated in non-European populations? Recent GWAS do not support this probability. The researchers believe that different populations have each one a unique population structure (different modifier genes and different gene-gene and gene-environmental interactions), different lifestyles and different environmental factors, which may expose or mask the risk of a SNP, for example rs6903956, in CAD [28]. Moreover, based on GWAS report in 2009 [57] there is a significant association between rs1252453 in *PHACTR1* on chromosome 6p24 and early onset MI in some European and American populations. However, according to HapMap data, not only the minor allele frequency of rs1252453 in Chinese population is 0%, but also none of the 113 SNPs in or near *PHACTR1* was associated with CAD in Chinese population [28]. Therefore, the public health policies and strategies, if they will include genetic factors in preventive medicine, should depend on ethnic group and may not be applicable worldwide [58].

Another interesting question is why numerous worldwide replication studies confirmed loci but not SNPs. Genetic variants having high occurrence in Europeans may have different frequencies across different populations. Also, many SNPs are rare in non-Europeans [59]. Moreover, allelic frequencies may be different even if the same SNP is found in diverse populations [60].

In this context, GWAS involving African populations are potentially of interest. Low linkage disequilibrium between genetic variants is one of their main characteristics, due in part to a long history of recombination and mutations. When SNPs extend over short genomic regions, further sequencing may be very effective in defining the true risk variant [42].

What will be the application of these findings in clinical practice and for predicting CVDs risk?

Based on the success of GWAS, some commercial companies are already offering to the public *in vitro* diagnostic genotyping assay to assess for example the CHD risk, although the risk attributable to any individual variant has been modest to date [61]. We believe that commercial assays do not give complete genomic coverage, as certain regions are not covered. Therefore, the influence of variation at these missing regions in determining CHD risks is very likely to have been underestimated, and some novel loci may have been missed completely. Nevertheless, even if valid genetic variants with modest effects explain a substantial population-attributable risk fraction they do not necessarily provide clinically useful prediction for individuals or specific population, groups [62]. However, combining multiple SNPs having modest effect into a global genetic risk score could improve the identification of high-risk populations and improve individual's risk assessment. Anderson et al. [61], have demonstrated the feasibility and the potential utility of simultaneously considering the joint effects of 5 different SNPs (rs599839 in *CELSR2*, rs2383206 in

9p21.3, rs289715 in *CETP*, rs78739461 in *ApoF* and rs1799963 in *F2)* retrieved from candidate gene studies and GWAS in not only improving the net risk classification of intermediate-risk individuals but in also predicting the risk of premature CHD [61].

Similarly to Anderson et al. [61], Kathiresan et al. [63] integrated several SNPs highly associated in GWAS with LDL-cholesterol (rs7575840 in ApoB, rs4420638 in APOE cluster, rs12654264 in HMGCR, and rs1529729 in LDLR) and HDL-cholesterol levels (rs3890182 in *ABCA1*) into a cardiovascular risk score [63,64]. However, in the study of Kathiresan et al. [63] the clinical risk prediction was not improved by genotype scores, nevertheless, there was a significant improvement in risk classification.

From a pharmacogenomics point of view, more studies taking into account the GWAS results are needed to prouve the clinical usefulness of genotyping to medication.

4.3. Missing heritability in cardiovascular diseases, an Achilles' heel

Although recent GWAS have an enormous sample size reaching up to 200,000 participants and revealing hundreds of associations with extremely impressive P-values, much of the genetic risk remains unexplained, and this represents the so-called 'dark matter' of genetic risk.

A large "hidden heritability" of unknown nature that may be explained by both low minor frequency alleles (MAF) and rare variants exists. However, the role of rare variants in population screening for CVDs has not been illustrated until now. GWAS have predominantly focused on common variants (allelic frequency >5% in the general population) [56] and ignored variants with low MAF (0.5%<MAF<5% in the general population) and rare frequency (MAF<0.5%), which are believed to have a greater risk effect [56]. In contrast to common variants, which are thought to be old in the history of humanity, rare variants are more recent and therefore not geographically extended [42]. Recently, deep sequencing revealed a myriad of rare, deleterious variants [65] and assessing their associations with increased risk will be the first step to better understand their role in CVDs. When focusing on rare genetic variants, GWAS require very large populations and different statistical methods that jointly analyse variants in a locus instead of testing each variant individually. The next generation of high resolution arrays with more genome-wide coverage is making this goal feasible.

Structural large variants (>500 Kb) such as deletions and duplications (Copy number variants, CNV) represent rare variants that may affect gene expression and molecular pathways in humans [9]. These genomic imbalances occur at an allele frequency of <0.05% and are present in about 8% of the total population [9], making them rare but collectively common. Results available suggest that CNVs strongly affect gene expression, thereby affecting mRNA splicing and transcriptional activity [66].

Gene–environment interactions (GxE) are also issues that might blind many GWAS. Integrating GxE could help in highlighting different loci effects according to modifiable risk factors, particularly in CVDs. This strategy needs large consortia, methodological efforts and stratified designs in order to improve the existing tools of GWAS that do not yet incorporate complex statistics. Although, environmental pathways acting through epigenetic mechanisms to modify gene expression [67] and DNA methylation [66] have given some fruitful results in other chronic diseases [68] and exposures [69], they are yet to be studied in CVDs. In addition to the usual challenges reported for genetic association studies, a successful GxE study should take into account sample size, exposure assessment and heterogeneity, described in full elsewhere [67]. GxE in GWAS requires enormous populations [67]. Smith and Day [70] explained that detecting an interaction needed a sample size at least 4 times greater than that required for detecting a main effect of comparable magnitude. Although difficult, studying GxE interactions could help in understanding discrepancies due to heterogeneous exposure.

Gene–gene (epistatic, GxG) interactions may also play an important role in discovering genes that have not yet been found by the consensual single-locus approach. This statement has been extensively reviewed [15,71,72] and both parametric and non-parametric multi-locus methods have been developed to detect such interactions [73] in the last years. Epistatic interactions have been documented for susceptibility to cancer [74], morphology [75] and autoimmune conditions [76]. However, current epistatic designs do not have genome-wide coverage, so that their application to high-dimensional genome-wide data including all imaginable SNPs remains a crucial challenge [77].

Furthermore, we advise that all loci associated with CVDs and related QTs should be replicated in paediatric populations, as a common variant could have a continuous impact throughout life. Epidemiological and pathophysiological evidence suggests that the precursors of CVDs originate in childhood [78,79]. The atherosclerotic process starts in childhood [80] and numerous studies have shown that CVDs risk factors during childhood can affect the risk in adulthood. For example, increased BP levels during childhood strongly predict HTN in adults [81]. Isolating genetic variations that may influence a given risk factor for CVDs at childhood, where many environmental factors such as alcohol intake, stress and smoking are absent, might have major implications for public health and could be a challenge in designing primary preventive strategies.

5. Concluding remarks and perspectives

GWAS have revealed many novel genetic risk variants in CVDs, making it an auspicious era for the better understanding of genetics. For the time being, we need to grasp the unexpected involvement of certain functional and mechanistic pathways identified in CVDs processes involving many QTs. These have to be extensively investigated in parallel with the pathology itself. Future GWAS must also detect low MAF and rare variants, and include GxG, GxE and well-designed candidate gene studies (in term of statistical power, homogeneity and exposure assessment) and involve subsequent transcriptomic and proteomic investigations. It is time to pause and think, instead of digging further down for more genetic loci with even smaller phenotypic effect.

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Disclosures

No conflicts of interest.

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Publication 2

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Influence of pre-analytical variables on VEGF gene expression and circulating protein concentrations

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Influence of pre-analytical variables on VEGF gene expression and circulating protein concentrations

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ABSTRACT

Background

The extended role of vascular endothelial growth factor in human pathophysiology led us to evaluate pre-analytical parameters possibly influencing its levels in peripheral blood and tissues. The effects of storage delay (for blood and tissue) of different types of anticoagulant (for blood) and of freeze-thaw cycles (for blood) were measured on VEGF protein and mRNA expression.

Methods

Blood from healthy donors was sampled simultaneously in Ethylene Diamine Tetraacetic Acid (EDTA), Acid Citrate Dextrose (ACD-A), hirudin and serum separation tubes. For each anticoagulant, VEGF was measured by Enzyme-Linked Immunosorbent Assay (ELISA) on samples with different conditions of delay at 4°C before centrifugation (2 h, 4 h or 48 h) and of freeze-thaw cycles (1,2 and 10). The transcripts coding for the $VEGF_{165}$ isoform were quantified on peripheral blood mononuclear cells by RT-PCR. Muscle biopsy samples were frozen with 15, 30 or 60 min delay after surgery. VEGF expression was quantified on immunofluorescence stained slides.

Results

The period of storage and the number of freeze-thaw cycles increased the levels of circulating VEGF [for each anticoagulant but not for serum] and its expression in PBMCs. VEGF expression measured from muscle biopsy sections was higher with a peak at a 30 and 60 min freezing delay as compared to a 15min one.

Conclusions

The most reliable conditions for measuring both circulating VEGF and its gene expression are to reduce time between blood collection and centrifugation, and to avoid multiple freeze-thaw cycles. Serum collecting tubes with no additive and no separator were less sensitive to the pre-analytical variations analyzed in this study. Freezing delay influenced significantly VEGF protein expression in tissue samples.

Keywords

non-tumor tissues, pre-analytical variation, plasma, PBMCs, serum, VEGF

1. Introduction

Vascular endothelial growth factor (VEGF or VEGF-A) is a basic, heparin-binding, homodimeric glycoprotein of 45 kDa. It is a potent mitogen for micro- and macrovascular endothelial cells (1). Six VEGF isoforms are generated as a result of alternative splicing from a single VEGF gene, consisting of 121, 145, 165, 183, 189, or 206 amino acids. $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ are secreted forms whereas $VEGF_{183,}$ $VEGF_{189}$ and $VEGF_{206}$ are the forms bound to the cell membrane (2). $VEGF_{165}$ is the most abundant and biologically active isoform. This isoform is secreted by cells but a significant fraction remains bound to the cell membrane via the extracellular matrix (1,2).

Recent studies on circulating levels of VEGF demonstrate the prognostic importance of serum VEGF concentration in a variety of diseases including atherosclerosis, ischemic heart disease, diabetic retinopathy, inflammatory diseases, and many types of tumors, pathologic conditions often leading to hypoxia (3,4). Other papers have proposed the circulating VEGF as a possible biomarker for diagnosis, monitoring of disease activity, treatment response and erosive progression in patients with chronic diseases, such as rheumatoid arthritis (5-9). It has been shown that VEGF is constitutively produced by megacaryocytes and platelets (10). It is released from platelets after blood clotting (11), and VEGF levels are higher in serum than in plasma; the pre-centrifugation delay induces an increase of VEGF in Ethylene diamine tetraacetic acid (EDTA) plasma samples. The delay before freezing and the number of freeze-thaw cycles influence VEGF measures in urine (12). Recently, it was shown that serum VEGF is decreased after long term storage and freeze thawing (13). Some studies have shown the need for assay validation of sample handling before the start of biomarker analysis in clinical trials while their report indicates that removal of platelets reduced the plasma concentration of VEGF, if the platelets were removed before freezing plasma samples (14).

The extended role of VEGF in human pathophysiology underpins the importance of improving knowledge on the pre-analytical and analytical variation factors and thus the accuracy of laboratory analysis of VEGF. We previously reported inter-individual biological variations and defined reference values of VEGF levels in EDTA plasma (5). However, little is known on pre-analytical variations of VEGF isoforms and corresponding gene expression .The aims of the present study are to evaluate the effects of pre-analytical factor on VEGF expression in blood and non-tumor tissues. More precisely, we evaluated the influence of the presence and the nature of anticoagulants, of the blood storage delay before centrifugation and of the number of freeze-thaw cycles on circulating levels of VEGF and on $VEGF_{165}$ mRNA expression in peripheral blood mononuclear cells (PBMCs). We also evaluated the influence of non-tumor tissue freezing delay on VEGF protein expression.

2. Materials and Methods

2.1. Biospecimens collection and pre-analytical conditioning

Blood specimens from 27 healthy donors from the “Investigation Clinique et Accès aux Ressources Biologiques" (ICAReB) Platform at Institut Pasteur and from 9 healthy donors from Picardie Biobank were collected. All donors signed an informed consent ; the study was approved by the ethics committee CPP Ile-de-France I and the resulting sample collection declared to the Research Ministry [approval No: DC-2008-68]. From each donor, blood was collected within one sampling time into 3

EDTA tubes [BD Vacutainer ref. 368589], 3 acid citrate dextrose (ACD-A) tubes [BD Vacutainer ref. 366645], 3 hirudin tubes [Sarstedt ref. 04.1912.001] and 3 serum tubes [BD Vacutainer ref. 366430]. Tubes (one tube from each lot) were stored after blood collection at 4°C for 2h, 4h and 48h before centrifugation. Centrifugation was performed at 1500g for 10 min at room temperature. All frozen samples were shipped on dry ice to "Cardiovascular Genetics" research center in Nancy for laboratory measurements. From each type of tubes, different aliquots were prepared for subsequent Enzyme-linked Immunosorbant Assay (ELISA) analysis after one, two or ten freeze thaw cycles. The supernatant (serum or plasma) was aliquoted (100µl) in polypropylene cryotubes and placed in a -80°C freezer within two hours from aliquoting step- as soon as each tube was completely aliquoted. Serum or plasma aliquots were defrosted at room temperature then frozen again at -80°C. For this analysis the tubes were placed on open holders as this method ensured the most homogeneous and rapid freeze-thawing of tubes. Indeed, freeze-thawing of samples stored in 9×9 sample boxes led to a 20 min delay between the central compared to peripheral part of the sample.

PBMCs from EDTA, ACD-A and hirudin samples were isolated by centrifugation on a density gradient of Ficoll (15) (Ficoll-Paque^{TM} PLUS; Amersham BioSciences) and stored at -80°C in Instapure lysis buffer [Eurogentec (E)] until analysis of $VEGF_{165}$ mRNA expression as described below.

Sixteen muscle biopsies were collected from patients suffering from muscle symptoms after informed consent. Lidocaine was locally applied on the skin before biopsies were taken. Samples were immediately sent from the Neurologic Hospital Hospices Civils de Lyon surgery rooms to the Hospices Civils de Lyon Biological Resource Center Cardiobiotec at room temperature through a direct pneumatic tube in less than 10 minute. The time of removal was noted for each individual sample. In our experience, a freezing delay of this type of biopsies varying between 15 and 60 min does not significantly influences the quality of histological diagnosis. The minimal sample size varied between 2 to 3cm long and 1 cm thick. Each biopsy was then split into three biopsy sections and snap frozen in a mixture of liquid nitrogen and cold Isopenthane to -80°C at 15, 30 and 60 minutes respectively after removal. Pathological examination of selected samples did not show any sign of necrosis, inflammation or myopathic changes. Six µm thick serial sections were cut from each biopsy section and fixed in Acetone for 10 min before immunofluorescence analysis.

2.2. VEGF ELISA

VEGF was quantified in all serum and plasma samples using a quantitative sandwich enzyme immunoassay technique (Quantikine_VEGF ref. SVE00, R&D Systems, Abingdon, U.K.) following the manufacturer's instructions.

2.3.VEGF gene expression analysis

Total RNA was extracted from PBMCs with a MagNaPure automate, using the MagNA Pure LC RNA HP isolation kit and RNA HP Blood External lysis protocol [Roche Diagnostics, France]. Reverse transcription of total RNA was performed using 200 units of M-MuLV Reverse Transcriptase with 0.25 µg of oligos(dT) (Promega, France) according to a previously described protocol (15). Quantification of the transcripts coding for the $VEGF_{165}$ isoform, and for the β-2 microglobulin control gene (16), was performed using TaqMan® and LightCycler technologies (LC TaqMan Master Kit, Roche Diagnostics, France). Duplicate aliquots of each cDNA were analyzed. RT-PCR optimization and specificity of Real Time-PCR products were conducted using SYBR® Green technology (LC FastStart DNA Master^{PLUS} SYBR Green I kit, Roche Diagnostic, France), melting curves analysis and

agarose gel electrophoresis of the PCR amplicons, as previously described (15). Stability of the normalization gene β-2 microglobulin was assessed according to its related protocol (16), for the delay times of 2, 4 and 48 hours before centrifugation.

2.4. VEGF expression in muscle biopsies

Thawed muscle biopsy sections from each time point were stained together. VEGF staining was obtained after overnight incubation with rabbit anti-VEGF-A antibody (1/200, Clone SC-152, Santa Cruz) and 30 min incubation with Goat anti-Rabbit FITC secondary antibody (MSDS A10526). Fluorescence was measured from jpeg pictures shot with a Zeiss Axio Observer A1 microscope combined to a 1,3 Mega pixel Zeiss AxioCam at x200 power field using identical acquisition settings for each channel (3s for GFP channel, 800ms for DAPI). Fluorescence density was measured in 6 regions of interest per slide drawn manually to encompass only the cytoplasm of muscle fibers with AxioVision 4.8 software. For each time point and each biopsy a negative control was also assessed. Mean fluorescence observed for each time point was standardized by subtracting the mean fluorescence of negative controls to the mean fluorescence of samples as described previously (17).

2.5. Statistical analyses

Descriptive statistics for VEGF levels are presented as the median and 25^{th} and 75^{th} percentiles.

VEGF levels, measured from different anticoagulant types and after different freeze-thaw cycle numbers, were compared using Wilcoxon's rank sum test. We considered $p \leq 0.05$ significant for differences between variables. Results are expressed as the ratio (percentage) between a given value at a specific pre-centrifugation delay and the corresponding value obtained at the 2-hour delay, or as the ratio between a value obtained at a specific number of freeze-thaw cycles and the corresponding value obtained with only one thawing. For mRNA analysis, only the pre-centrifugation delay was studied, with just one thawing of the PBMCs in all cases.

For muscle biopsies analysis, means comparison using ANOVA test were calculated with JMP 8.0 software.

3. Results

We initially performed comparison of the values of VEGF concentrations at the baseline (2 hours pre-centrifugation delay and 1 freeze-thaw cycle) between the collection centers and no difference was observed for any of the collection tubes used. The analysis reports the aggregated results of all donors at particular time points.

3.1. Effect of blood storage at 4°C before centrifugation on serum/plasma VEGF

VEGF recovery percentages in serum and plasma sampled with different anticoagulant types and after different pre-centrifugation delays at 3-8°C are presented in Figure 1 and in Table 1. When serum samples were only thawed once before analysis, there was no significant difference between 2 and 4 hours, neither was there between 2 and 48 hours of blood storage at 3-8°C. There was a significant increase (124%; p = 0.0009) between 2 and 48 hours of serum pre-centrifugation delay when the samples were analyzed after 2 freeze-thaw cycles.

In EDTA plasma samples, no significant difference was observed between 2 and 4 hour- pre-centrifugation delay, whatever the number of freeze-thaw cycles. In contrast, there was a significant increase between 2 and 48 hours, for all numbers of freeze-thaw cycles.

Similarly, the effect of blood storage time, when using hirudin as the anticoagulant, was not significant between 2 and 4 hours, however when the delay reached 48 hours, a highly significant VEGF increase was observed, for all numbers of freeze-thaw cycles.

The impact of ACD on VEGF analysis was different. Although a significant increase was observed between 2 and 4 hours ($p=0.0046$) and between 2 and 48 hours ($p=0.006$) with only one freeze-thaw cycle, this increase was no longer detectable after multiple freeze-thaw cycles.

3.2.Effect of the number of serum/plasma freeze-thaw cycles on measured VEGF

VEGF recovery percentages in serum and plasma obtained in different anticoagulant types and after different numbers of freeze-thaw cycles are shown in Figure 2 and Table 2.

The VEGF level in serum is not affected by the number of freeze-thaw cycles whatever the delay of blood storage at 3-8°C after sampling.

VEGF levels in EDTA plasma significantly increased with the number of freeze-thaw cycles and this increase was similarly observed for the three blood storage delays. The VEGF increase was greater after 10 ($p<0.0001$) than after 2 ($p=0.0046$) freeze-thaw cycles.

VEGF levels in hirudin plasma remained the same after 1 and 2 freeze-thaw cycles and showed a slight increase after 10 cycles ($p=0.005$ and 0.008 for storage delays of 2 h and 48 h respectively).

The most important effect of freeze-thawing was observed in ACD plasma. Between 1 and 2 cycles, a very significant increase was observed ($p<0.0001$ at 2 h and 4 h time points). Interestingly, this increase was less significant when considering the 48 h delay ($p=0.0047$ and 0.0029 when comparing 1 to 2 cycles, and 1 to 10 cycles, respectively).

3.3. Effect of blood storage at 3-8°C on VEGF expression

There was no significant variation in the VEGF/β-2 microglobulin expression ratio in PBMCs isolated and stored 2 *vs* 4 hours at 3-8°C after blood sampling (Figure 3). In contrast, a very significant increase was observed after 48 hours of blood storage, as compared to 2 h, with the three anticoagulants (EDTA and hirudin: $p=0.0003$; ACD: $p<0.0001$)

3.4. Effect of the blood collection tube type on circulating VEGF levels

Plasma circulating VEGF levels were significantly different depending on the collection tube type used . The measured levels were significantly higher in serum than in any type of plasma ($p<0.0001$). Among the three different types of plasma, VEGF levels were significantly higher in EDTA than in hirudin ($p<0.0001$), or in ACD ($p=0.0002$) (table 3).

3.5. Effect of freezing delay on VEGF protein expression levels in muscle tissues

Mean standardized fluorescence from VEGF-A stained muscle biopsy sections frozen after the 3 different delays showed a trend to increase with time from 15 to 60 min but with a peak of expression varying from one sample to another, either at a 30 or 60 min delay (Figures: 4A1, 4A2 and 4A3). Despite a trend to increase, mean standardized fluorescence intensities reflecting VEGF expression were not significantly different among the 3 time points (p=0.0961). Combining 30 and 60 min time points, we proved a significant variation in mean standardized fluorescence reflecting higher levels of VEGF expression after a 30 min delay before freezing the sample (p=0.0187) (Figure 4A4). Although baseline levels of VEGF expression evaluated by optical density ranged between 0 and 320, peak levels under hypoxic condition reached higher values, from 50 to 1600.

Discussion

VEGF has been proposed as a possible prognostic biomarker in several cancers (18-22), a predictive biomarker for response to treatment in chronic inflammatory diseases (6, 23), and as a physiological marker of vascular remodeling and growth regulation (24-26). Reference values for healthy and diseased individuals must be determined to allow correct interpretation of patient's laboratory reports. Moreover, these values need to be reproducible from one laboratory to another, which means that pre-analytical and analytical parameters should be well defined.

In the present study, we have shown that VEGF measurements in serum and in plasma containing different anticoagulants are not comparable. The concentration of VEGF in serum was higher than in the different types of plasma and it was not significantly affected by the number of freeze-thaw cycles, neither was it by the storage delay at 3-8°C before centrifugation. When serum is collected for clinical immunological analyses, blood usually coagulates at room temperature for at least 30 minutes (27). However, for the purposes of this study, all pre-centrifugations delays were performed at 4°C and the coagulation after 2H at 4°C was completed.

Using anticoagulants, VEGF values increased after 2 freeze-thaw cycles. There was an even higher increase after 10 freeze-thaw cycles in EDTA and hirudin plasma. However, although significant increases represented only 20% in EDTA and hirudin plasma, whereas more than 2 times higher VEGF was recovered in ACD plasma, after only 2 freeze-thaw cycles. This could be due to a better preservation of platelets in ACD, and thus, to a greater cumulated release of VEGF from platelets as they got lysed at each freeze-thaw event. Better preservation of platelets in ACD may also explain the fact that pre-centrifugation delays induced a lower VEGF increase in ACD than in EDTA or hirudin plasma. When ACD plasma is collected for analysis of coagulation factors, blood is usually centrifuged at a higher g-force and a second centrifugation reduces the number of platelets (28). However, for the purposes of this study, platelet-rich plasma was used with all types of anticoagulants tested, including ACD. VEGF was much higher in serum than in any anticoagulant-collected plasma and this was probably due to a massive VEGF release from platelets during the coagulation process in the first 30 minutes after blood collection. This is in accordance with the work conducted by Banks et al (11) who have shown that VEGF increased in a first step during clotting, and the maximal increase was obtained after complete coagulation of the samples. Therefore, VEGF measured in serum does not correspond only to circulating but also to intracellular VEGF.

We showed that the blood storage period before centrifugation has a significant influence on VEGF levels in three types of anticoagulation tubes. Hetland et al (6) have reported that EDTA plasma VEGF levels increased by 34% and 80% respectively after 1 and 2 hours of blood storage at room temperature in healthy individuals and by 28% and 64% in rheumatoid arthritis patients. They found no differences after 2-9 freeze-thaw cycles, but an unexpected decrease of 8% after the 10^{th} cycle. This could be due to uncompleted freeze-thaw cycles. Indeed in our experiments, we first established the protocol for complete freeze-thawing of the samples at each cycle, as we observed that both the type of holding (completely open or partly closed like a box), the position of the tubes (whether central or peripheral) and the delay time were crucial to obtain a homogeneous, complete freeze-thaw cycle of a panel of tubes. Alternatively, the VEGF protein could have been damaged during the experiment, depending on the protocol used. Contrary to us, Svendsen et al (29) have reported no difference between EDTA and citrated plasma. Very recently, it was shown that VEGF in serum decreased after freeze-thawing (13). However, thawing in this study was performed at 37°C and this may have induced VEGF degradation by proteolysis. Thus, different thawing parameters such as thawing temperature, duration and conditions of each freeze-thaw cycle may significantly modify the interpretation of results. Interestingly, the same pattern of VEGF increase was observed in non-tumor tissues, after only 15 min of cold ischemia.

For clinical research, gene expression signatures can be used to better define biological processes in relation with disease or treatment outcome. Peripheral blood is the most easily accessible and the most reliable specimen to be used for this purpose (30). For the gene expression profiling of peripheral blood to become a routine clinical diagnostic tool, it is critical to better understand the factors affecting gene expression signatures in PBMCs. Use of PBMCs as representative material for high-throughput analysis of gene expression is currently being explored (15). These cells are involved in a wide variety of diseases, including infection and cancer (15). Moreover, in some evidences, characteristic sets of transcriptional changes in PBMCs were associated with physiologic or pathologic states. Therefore, a PBMC transcriptome may be used as an individual's health sensor, a concept referred to as the sentinel principle (15). In this study, we have addressed for the first time the effects of pre-analytical factors such as different types of anticoagulants and storage delays of blood samples before centrifugation, on $VEGF_{165}$ mRNA expression by PBMCs. The expression of the VEGF165 isoform was chosen as Kruizinga et al (7) recently showed that a single VEGF isoform, indeed $VEGF_{165,}$ is representative for all VEGF isoforms [121, 165, 183, and 189]. Also $VEGF_{165}$ is the most abundant isoform of VEGF (1). It was shown that in peripheral blood cells, β-2 microglobulin transcript is more stable than other housekeeping genes transcripts (31). The expression ratio VEGF/ β-2 microglobulin was not modified between 2 and 4 hours of blood storage, whatever the anticoagulant. An important increase occurred between 4 and 48 hours of blood storage, possibly due to activation of hypoxia-related expression pathways in PBMCs. Dynamic correlation between PBMC gene expression and circulating VEGF was influenced by specimen collection method; indeed, VEGF gene expression increased in plasma with all types of anticoagulants. In order to valid these results, we verified that β-2 microglobulin remained stable under all experimental conditions of this study, which was the case.

Baechler et al (32) have shown that gene expression profiles from fresh peripheral blood samples are significantly different from the profiles of samples shipped overnight. They observed that VEGF shows higher expression levels after overnight incubation of blood at room temperature. Debey et al (30) have proposed that signaling events associated with hypoxia and stress responses might be induced in peripheral blood samples analysed after a delay. The same mechanism would explain the increase in tissue VEGF, observed over increasing cold ischemia delays. Consistent with this concept, some

evidences indicate that VEGF also participates in some physical responses to hypoxia and shows higher expression levels after overnight incubation of blood (32-34). In solid tissue, the mechanism of VEGF expression variation is a direct response to hypoxia. The increase of VEGF expression is under the control of a direct effector of hypoxia, Hypoxia Inducible factor 1 (HIF1α) whose pathway targets the expression of many other genes with local pro-angiogenic effect, such as stromal cell-derived factor alpha (SDF1α), vascular endothelial growth factor receptor 1(VEGFR1), platelet deriving growth factor-beta (PDGF-ß), fibroblast growth factor-2 (FGF-2). In addition, stabilization/degradation balance of these proteins at normoxia is changed under hypoxic conditions (35). In our study based on immunofluorescence analysis of muscle biopsies, despite the standardisation of VEGF expression detection, the use of non-tumor tissue, we had to face intrinsic variation levels from 1 sample compared to the others. In addition, we show there is a peak effect of VEGF expression ranging between 30 and 60 min time points. This result means that it is not possible to predict the biological quality of a sample, i.e. biological changes related to freezing delay and hypoxia condition of one sample, only by evaluating VEGF expression. Therefore new strategies have to be defined to better characterize the level of «degradation» of a single non-tumor tissue sample. An option would be to rely on the analysis of multiple factors from these pathways.

Evidence also indicates that the handling of blood samples and the method of cell processing induces changes in the inflammatory pathway gene expression patterns (36). For example, Duvigneau et al (37) have observed in their animal (pork) model that isolated PBMCs from EDTA-anticoagulated blood have higher cytokine gene expression than PBMCs from heparinized blood samples. Similarly, the EDTA-anticoagulated blood has been successfully used to measure the production of cytokines at protein and RNA level upon stimulation with tetanus (38,39). The study of Isaksson et al (40) demonstrated that different laboratory tube types did not have significant influence on VEGF mRNA stability. This finding may be explained by different processing delays. Anticoagulant may not differentially influence VEGF gene expression shortly after blood collection but the differential effect may become visible after longer blood storage delays.

Finally, our results suggest that the best conditions for measuring both circulating VEGF and its gene expression are to reduce time between blood collection and centrifugation, and to avoid multiple freeze-thaw cycles. In the present work, we could not decipher if a specific sample collection tube type was better than the others tested. Serum is the most stable, but we have to take into account that values are also due to intracellular release. The optimal conditions for measuring non-tumor tissue VEGF levels are to freeze the tissue within 15 min following excision.

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Conflict of interest

All authors declare no conflict of interests.

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Table 1: Effect of the duration of storage at 3-8°C before centrifugation on VEGF quantification in different blood collection tubes and after different numbers of freeze-thaw cycles.

Blood collection tubes	Number of freeze-thaw cycles	4h/2h before centrifugation		48h/2h before centrifugation		48h/4h before centrifugation	
		Median(1^{st}-3^{rd} quartiles)	p	Median(1^{st}-3^{rd}quartiles)	p	Median(1^{st}-3^{rd} quartiles)	p
Serum	1	105 (96-121)	ns	104 (96-119)	ns	104 (96-117)	ns
	2	103 (96.5-109.5)	ns	110.5 (98.7-136.7)	0.0009	107 (101-144)	0.0008
	10	100.5 (89.5-111.2)	ns	106.5 (94-127.5)	ns	113.5 (103.7-154.5)	0.0015
EDTA	1	93.5 (79.7-106.2)	ns	131 (108.5-173)	0.0001	138 (102-166.5)	0.0001
	2	96 (82.5-110)	ns	127 (98-162.5)	0.002	131 (97-160.7)	0.0001
	10	98 (88-107.5)	ns	118.5 (94-176.2)	0.0006	130 (94.5-179)	0.0001
Hirudin	1	92 (69-110.7)	ns	240.5 (168-304.2)	<0.0001	243.5 (178.5-303.5)	<0.0001
	2	79 (67-98)	0.0023	232 (165.5-294.5)	<0.0001	276 (164-383)	<0.0001
	10	92 (74-107)	ns	228.5 (193.5-359)	<0.0001	240.5 (194.7-320.5)	<0.0001
ACD	1	105.5 (86.7-142.7)	0.046	126 (96-218)	0.006	130 (82.5-188.2)	0.025
	2	97.5 (86.7-115.2)	ns	91(70.5-139.7)	ns	92.5 (80.5-127.7)	ns
	10	96 (69.2-112.5)	ns	92.5 (61.7-133)	ns	95 (69.5-152.2)	ns

Values are expressed as the ratio (percentages) between 4 or 48 hours, and 2 hours of storage. p values are calculated between each ratio and the corresponding T2 value (Wilcoxon's rank test). ns: not significant.

Table 2: Effect of the number of freeze-thaw cycles on the VEGF quantification in different blood collection types and after different delays of blood storage at 3-8°C

Blood collection tubes	Delay before centrifugation (hours)	2X/1X cycles		10X/1X cycles		10X/2X cycles	
		Median(1^{st}-3^{rd} quartiles)	p	Median(1^{st}-3^{rd} quartiles)	p	Median(1^{st}-3^{rd}quartiles)	p
Serum	2	97 (87.2-109.2)	ns	97(88-104)	ns	98 (91.7-109)	ns
	4	96 (87-106)	ns	92.5 (81-99.5)	0.011	96.5 (84.2-103.5)	ns
	48	107 (96.5-117)	ns	98.5 (92-112.5)	ns	102 (85.7-112.5)	ns
EDTA	2	113 (99.5-120)	0.0046	123.5 (101.7-147.2)	0.0001	112 (102.5-122.5)	0.0002
	4	110 (95.5-119.2)	0.03	123 (108.7-146)	0.001	110 (103.5-119.5)	0.0003
	48	103 (96-109.2)	0.0453	114 (101.7-127)	<0.0001	112.5 (99-124)	<0.0001
Hirudin	2	109 (90.2-129)	ns	112 (98.2-131.5)	0.005	109 (87.2-121.5)	ns
	4	94 (83-130)	ns	110 (91-154)	ns	120 (103-157)	0.0012
	48	98 (89-107)	ns	107 (97.5-125)	0.008	111 (100.5-129)	0.0004
ACD	2	180 (125-222)	<0.0001	200 (126-254)	<0.0001	107 (94-126)	0.007
	4	143 (125-215.5)	<0.0001	149 (124-210)	<0.0001	108.5 (97-116.5)	ns
	48	124.5 (110-158.2)	0.0047	140 (114-163)	0.0029	112 (92-124)	ns

Values are expressed as a ratio between 2 or 10 cycles and 1 cycle. The last column represents the ratio between 10 and2 cycles. p values are calculated between each ratio and the corresponding T2 value (Wilcoxon's rank test). ns: not significant.

Table 3: VEGF levels in different anticoagulant tubes

	serum	EDTA	Hirudin	ACD
VEGF	264 (158.5-383.5)*	130 (92.5-183.5)*	65 (40-95)*	76 (42-92)¥

Values are expressed as the median and (the first and third) inter quartiles.

*: p value <0.0001, ¥: p value=0.0002

Figures legends

Fig 1 Box plot representing the effect of the period of storage at 3- 8°C before centrifugation on the quantification of VEGF [median, 1st and 3rd quartiles]

The number of freeze-thawing is of 1. Values at time T2 correspond to 100%.

P values are calculated between each ratio and the corresponding T2 value [Wilcoxon's rank test]. Vertical lines correspond to the 10th and 90th centiles.

Fig 2 Box plot representing the effect of the number of freeze-thawing [F/T] on the expression of VEGF. Period of storage at 3- 8°C before centrifugation is of 2 hours [median, 1st and 3rd quartiles].

Values for 1 F/T correspond to 100%. p values are calculated between each ratio and the corresponding « F/T1X » value [Wilcoxon's rank test] [median, 1st and 3rd quartiles]. Vertical lines correspond to the 10th and 90th centiles.

Fig 3 Box plot representing the effect of the period of storage at 3- 8°C before centrifugation on the expression of $VEGF_{165}$ /β-2 microglobulin.

The number of freeze-thawing is of 1. Values at time T2 correspond to 100%.

p values are calculated between each ratio and the corresponding T2 value [Wilcoxon's rank test] [median, 1st and 3rd quartiles]. Vertical lines correspond to the 10th and 90th centiles.

Fig 4A1 Expression in immunofluorescence technique of VEGF-A by muscle fibers from samples frozen after 15, 30 and 60 minutes

VEGF expression in green channel [488nm, x200] from section of 2 cases showing a peak of expression respectively at 30 [Case 1] and 60 min [Case 2] of freezing delay. Nuclei were stained in blue (DAPI). Comparison with negative control.

Fig 4A2 Individual variations of green channel optical density

Each sample was plotted with a single color for the 3 time points to show individual variation of green channel optical density.

Fig 4A3 Variations of VEGF expression calculated from mean standardized optical density calculated at 15, 30 and 60 min.

Fig 4A4 VEGF expression calculated from mean standardized fluorescence at 15min time point compared to 30 and 60 min delay samples grouped together

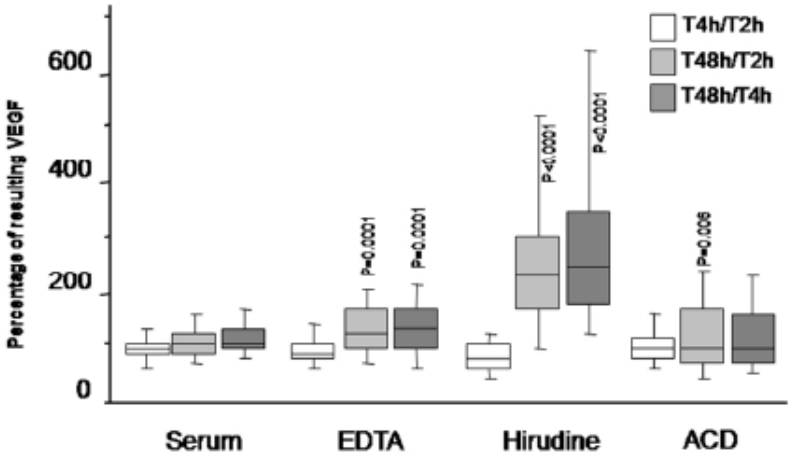

T4h/T2h
T48h/T2h
T48h/T4h
Percentage of resulting VEGF
600
400
200
0
P=0.0001
P=0.0001
P<0.0001
P<0.0001
P=0.008
Serum
EDTA
Hirudine
ACD

Figure 1

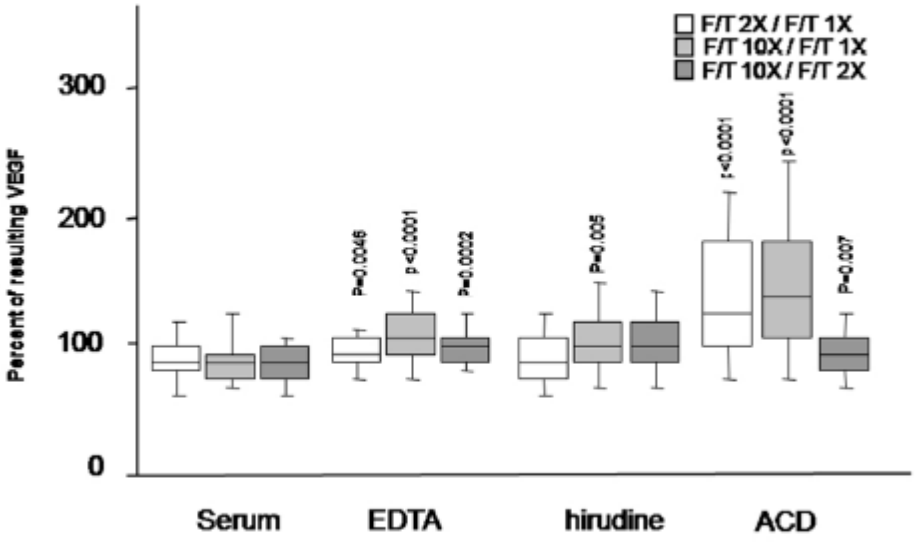

F/T 2X / F/T 1X
F/T 10X / F/T 1X
F/T 10X / F/T 2X
Percent of resulting VEGF
300
200
100
0
P=0.0046
p <0.0001
P=0.0022
P=0.005
p<0.0001
p<0.0001
P=0.007
Serum
EDTA
hirudine
ACD

Figure 2

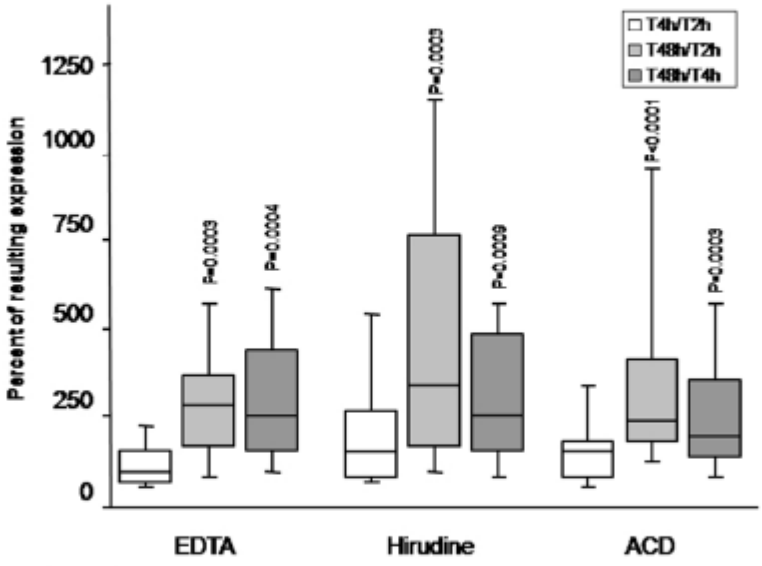
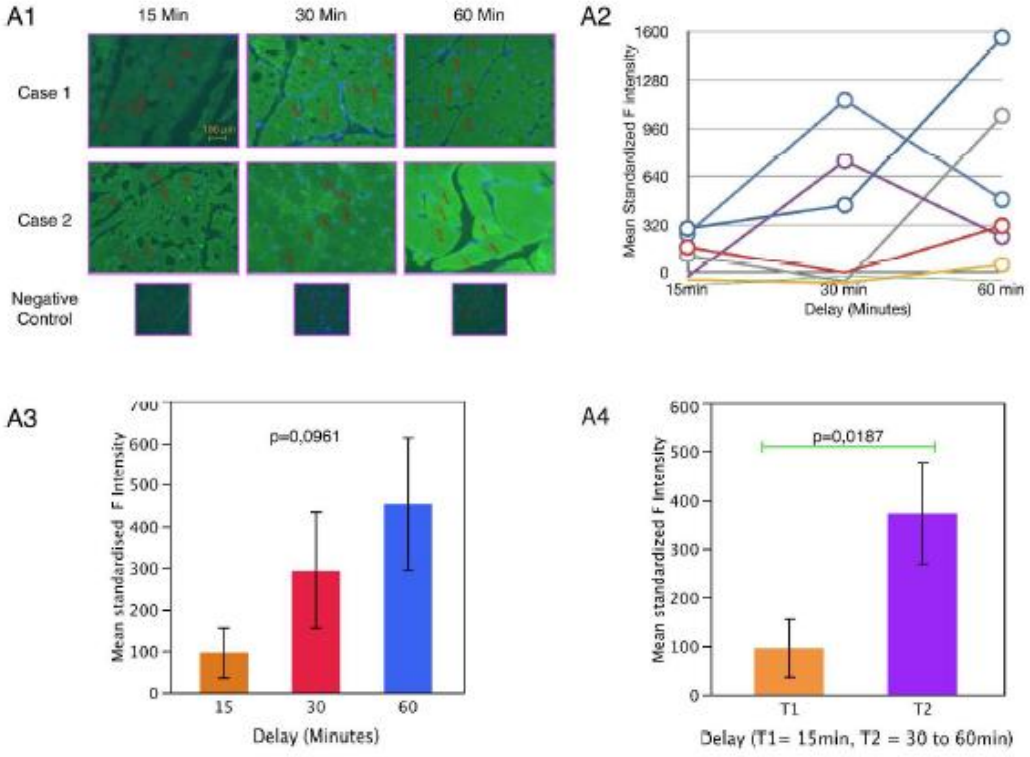

T4h/T2h
T48h/T2h
T48h/T4h
Percent of resulting expression
1250
1000
750
500
250
0
P=0.0003
P=0.0001
P=0.0009
P=0.0004
P=0.0009
P=0.0003
EDTA
Hirudine
ACD

Figure 3

Figure 4: VEGF expresion in Biopsy samples frozen after 15, 30 an 60 min delay



Publication 3

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Identification of *cis*- and *trans*-Acting Genetic Variants Explaining Up to Half the Variation in Circulating Vascular Endothelial Growth Factor Levels

Stephanie Debette, Sophie Visvikis-Siest, Ming-Huen Chen, Ndeye-Coumba Ndiaye, Ci Song, Anita Destefano, Radwan Safa, Mohsen Azimi Nezhad, Douglas Sawyer, Jean-Brice Marteau, Vanessa Xanthakis, Gerard Siest, Lisa Sullivan, Michele Pfister, Holly Smith, Seung-Hoan Choi, John Lamont, Lars Lind, Qiong Yang, Peter Fitzgerald, Erik Ingelsson, Ramachandran S. Vasan and Sudha Seshadri

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Identification of *cis*- and *trans*-Acting Genetic Variants Explaining Up to Half the Variation in Circulating Vascular Endothelial Growth Factor Levels

Stephanie Debette,* Sophie Visvikis-Siest,* Ming-Huen Chen, Ndeye-Coumba Ndiaye, Ci Song, Anita Destefano, Radwan Safa, Mohsen Azimi Nezhad, Douglas Sawyer, Jean-Brice Marteau, Vanessa Xanthakis, Gerard Siest, Lisa Sullivan, Michele Pfister, Holly Smith, Seung-Hoan Choi, John Lamont, Lars Lind, Qiong Yang, Peter Fitzgerald, Erik Ingelsson, Ramachandran S. Vasan,† Sudha Seshadri†

Rationale: **Vascular endothelial growth factor (VEGF) affects angiogenesis, atherosclerosis, and cancer. Although the heritability of circulating VEGF levels is high, little is known about its genetic underpinnings.**

Objective: **Our aim was to identify genetic variants associated with circulating VEGF levels, using an unbiased genome-wide approach, and to explore their functional significance with gene expression and pathway analysis.**

Methods and Results: **We undertook a genome-wide association study of serum VEGF levels in 3527 participants of the Framingham Heart Study, with preplanned replication in 1727 participants from 2 independent samples, the STANISLAS Family Study and the Prospective Investigation of the Vasculature in Uppsala Seniors study. One hundred forty single nucleotide polymorphism (SNPs) reached genome-wide significance ($P<5 \times 10^{-8}$). We found evidence of replication for the most significant associations in both replication datasets. In a conditional genome-wide association study, 4 SNPs mapping to 3 chromosomal regions were independently associated with circulating VEGF levels: rs6921438 and rs4416670 (6p21.1, $P = 6.11 \times 10^{-506}$ and $P = 1.47 \times 10^{-12}$), rs6993770 (8q23.1, $P = 2.50 \times 10^{-16}$), and rs10738760 (9p24.2, $P = 1.96 \times 10^{-34}$). A genetic score including these 4 SNPs explained 48% of the heritability of serum VEGF levels. Six of the SNPs that reached genome-wide significance in the genome-wide association study were significantly associated with VEGF messenger RNA levels in peripheral blood mononuclear cells. Ingenuity pathway analyses showed found plausible biological links between VEGF and 2 novel genes in these loci (*ZFPM2* and *VLDLR*).**

Conclusions: **Genetic variants explaining up to half the heritability of serum VEGF levels were identified. These new insights provide important clues to the pathways regulating circulating VEGF levels. (*Circ Res.* 2011;109:554-563.)**

Key Words: growth factors genome-wide association study gene expression pathway analysis

Vascular endothelial growth factor (VEGF, also referred to as VEGFA in contrast to other members of the VEGF family) is pivotal in many physiological and pathological processes.[1] It is primarily known for its key role in the stimulation of angiogenesis, with a potent mitogenic effect on vascular endothelial cells from arteries, veins, and lymphatics.[2] VEGF also promotes vasodilatation by inducing the production of nitric oxide and prostacyclin by endothelial cells.[3] In addition, VEGF is involved in hematopoietic development and chemotaxis of monocytes, regulation of osteoclast differentiation, and stimulation of surfactant production[1] and has neurotrophic and neuroprotective effects on neuronal and glial cells.[4] Elevated circulating VEGF levels have been observed in vascular disease (ischemic heart disease,[5,6] heart failure,[7] stroke[8]) and in various other disorders, including diabetes,[9] cognitive decline and dementia,[10,11]

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reproductive,[12–14] immune-inflammatory disorders,[15,16] and neoplastic diseases.[17,18] Administration of VEGF promotes angiogenesis in patients with critical leg ischemia as well as in animal models of coronary and limb ischemia.[19] VEGF inhibitors such as bevacizumab and sorafenib have been successfully used to inhibit angiogenesis in several tumors,[20,21] in macular degeneration,[22] and in rheumatoid arthritis.[23] However, despite the considerable toxicity associated with VEGF inhibitor drugs,[24] there have been no pharmacogenomic studies to identify potential subgroups of responders, partly because the genetic determinants of VEGF concentrations remain poorly understood.

Indeed, although the heritability of circulating VEGF levels is very high, ranging between 60% and 80%,[25–27] few studies have assessed the relation between circulating VEGF levels and genetic variants, yielding inconsistent results. The aim of the present study was to identify genetic variants associated with circulating VEGF levels using an unbiased genome-wide approach in a large community-based sample.

Non-standard Abbreviations and Acronyms

FHS	Framingham Heart Study
GWAS	genome-wide association study
IPA	Ingenuity Pathway Analysis
KCNV2	potassium voltage-gated channel subfamily V, member 2
LD	linkage disequilibrium
LRP12	low-density lipoprotein receptor-related protein 12
mRNA	messenger RNA
MRPL14	mitochondrial ribosomal protein L14
PBMC	peripheral blood mononuclear cells
PIVUS	Prospective Investigation of the Vasculature in Uppsala Seniors study
SFS	STANISLAS Family Study
SHARe	SNP-Health Association Resource
SNP	single nucleotide polymorphism
VEGF	vascular endothelial growth factor
VLDLR	very low-density lipoprotein receptor
ZFPM2	zinc finger protein, multitype 2

Methods

Study Populations

The Framingham Heart Study

The Framingham Heart Study (FHS), initiated in 1948, is a 3-generation, community-based, prospective cohort study conducted in Framingham, MA.[33–35] Serum VEGF levels were measured in third-generation cohort participants (2002 to 2005), and genome-wide genotyping was performed on these individuals at Affymetrix (Santa Clara, CA) through a National Heart, Lung, and Blood Institute–funded SNP-Health Association Resource (SHARe) project. We chose not to include participants with cardiovascular disease because it may influence VEGF levels. After excluding participants who had prevalent cardiovascular disease, which may influence their VEGF levels, or who failed to meet quality control standards, 3527 participants were enrolled.

The STANISLAS Family Study

The STANISLAS Family Study (SFS) is a 10-year, longitudinal survey involving 1006 volunteer families from Vandoeuvre-lès-Nancy, France, whose members were free of chronic disease (cardiovascular or cancer) between 1993 and 1995.[28] Plasma VEGF levels were measured at the second examination cycle (1998 to 2000) in a randomly selected subsample; of these, 859 persons from 217 families, who also had DNA and met genotyping quality control criteria, were included.

Prospective Investigation of the Vasculature in Uppsala Seniors Study

The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study is a population-based study that enrolled 1016 70-year-old individuals living in the community of Uppsala, Sweden (2001 to 2004).[29] Of these, 999 persons provided DNA for genetic studies, and, after exclusions for prevalent cardiovascular disease and inadequate genotyping quality, 868 participants were eligible.

Further details of the study samples are available in the Online Supplemental Methods, section I at http://circres.ahajournals.org.

Laboratory Measurements of VEGF Levels

VEGF levels were measured in serum for the FHS and PIVUS and plasma for the SFS (Online Supplemental Methods, section II).

Genotyping

Framingham Heart Study

Genome-wide genotyping in the FHS was performed on the Affymetrix GeneChip Human Mapping 500K Array Set and 50K Human Gene Focused Panel. Genotyping, quality control, and imputation methods are detailed in the Online Supplemental Methods, section III.

STANISLAS Family Study

The single nucleotide polymorphisms (SNPs) were genotyped by Genoscreen (http://genoscreen.fr), using a Sequenom iPLEX Gold assay–Medium Throughput Genotyping Technology.[30]

PIVUS Study

The SNPs were genotyped as part of a 96-plex assay at the SNP technology platform in Uppsala University (http://www.genotyping.se/), using the Illumina BeadXpress system from Illumina Inc.[31] Genotyping calls were done with Illumina BeadStudio software.

Statistics

VEGF levels were natural log-transformed to normalize their distribution.

Genome-Wide Association Analysis in the FHS

A linear mixed effects model accounting for familial relatedness was used to evaluate the association of each SNP with VEGF levels.[32] An additive genetic model with 1 degree of freedom was used. In a first step (model A), analyses were adjusted for age, sex, and the ninth principal component (Online Supplemental Methods, section IV). In a second step designed to explore potential mechanisms, we additionally adjusted our most significant associations for covariates previously found to be associated with serum VEGF levels[25]: Compared with model A, model B was additionally adjusted for hypertension; model C for smoking; model D for central obesity; and model E for the presence of a metabolic syndrome (Online Supplemental Methods, section V).

Genetic Association Study in the SFS and the PIVUS Studies

To confirm our findings in the FHS, we genotyped 25 SNPs in 2 independent samples. To select a parsimonious number of SNPs for replication, we used criteria of strength of association (probability value), whether the SNP was genotyped or imputed, linkage disequilibrium (LD) between SNPs and functionality (Online Supplemental Methods, section VI). A linear regression model using the same covariates and analytic strategy as in the FHS was implemented.

Table 1. Characteristics of Study Participants

Characteristics	FHS	SFS†	PIVUS Study
No. of participants	3527	859	868
Mean circulating VEGF level, ng/L, median (IQR)*	280 (294.7)	27.4 (28.2)	187.5 (210.6)
Mean age (SD) at VEGF measurement, mean (SD)	40.0 (8.7)	29.83 (14.5)	70.2 (0.2)
Women, n (%)	1890 (53.2)	428 (49.8)	454 (52.3)
Cardiovascular risk factor at VEGF measurement			
Systolic blood pressure, mean (SD)	116.7 (14.0)	120.3 (12.8)	149.6 (22.7)
Hypertension, n (%)	561 (15.9)	23 (2.7)	606 (69.8)
Diabetes mellitus, n (%)	89 (2.5)	0	68 (7.8)
Current smoker, n (%)	544 (15.3)	188 (21.9)	93 (10.7)
Central obesity, n (%)	1315 (37.2)	37 (44.3)	266 (30.6)
Metabolic syndrome, n (%)	693 (19.6)	19 (2.2)	198 (22.8)

IQR indicates interquartile range; SD, standard deviation.

*Serum levels for the FHS and the PIVUS study and plasma levels for the SFS.

†By design, SFS participants were free of chronic disorders (cardiovascular or cancer) and had no history of cardiovascular disease at the time of inclusion (VEGF levels and covariates for the present analysis were measured during the second examination cycle in 1998 to 2000); all individuals with cardiovascular disease, defined in the FHS as presence of stroke, congestive heart failure, coronary heart disease, or intermittent claudication, were excluded before analyses in FHS and PIVUS.

Joint Analysis of the FHS, SFS, and PIVUS Studies

For SNPs that were successfully genotyped in the SFS and the PIVUS study, we performed a meta-analysis of the SNP-phenotype associations, using a fixed-effects inverse-variance meta-analysis technique for the combination of results from the FHS and the PIVUS study (which had both measured VEGF levels in the serum) and an effective sample size–weighted meta-analysis for the combination of results from all 3 studies to account for the different scales of VEGF levels in serum and plasma (Online Supplemental Methods, section VII).

Genetic Score

The methods used for computing a genetic score are detailed in the Online Supplemental Methods, section VIII, and Online Table I. The phenotypic variance explained by this genetic score was separately calculated in the FHS, the SFS, and the PIVUS study, using regression models that included age and sex as covariates.

VEGF Gene Expression Analysis in Peripheral Blood Mononuclear Cells

Sample preparation and quantification of the peripheral blood mononuclear cells (PBMCs) messenger RNA (mRNA) of *VEGF* spliced forms and statistical analyses of these data are described in the Online Supplemental Methods (section X).

Biological Pathway Analysis

Methods for the biological pathway analysis are provided in the Online Supplemental Methods, section IX.

Results

Characteristics of the 5273 study participants are presented in Table 1.

Genome-Wide Association Study of VEGF Levels in the FHS

The quantile-quantile plot showed an excess of extreme probability values but no evidence of systematic inflation of the genomic control inflation factor (1.02) (Online Figure I). The genome-wide plot of probability values for the individual SNPs against their genomic position is shown in Online Figure II. A total of 140 SNPs cleared the threshold for genome-wide significance at 5 10^{-8} (Online Table II). These were located in 3 chromosomal regions: 6p21.1, 8q23.1, and 9p24.2 (Table 2). The most significant association was found with rs6921438 on chromosome 6p21.1 (P 6.11 10^{-506}), at 171 kb downstream of the *VEGF* gene, and close to the mitochondrial ribosomal protein L14 gene (*MRPL14*) and the *MCG45491* gene (*C6orf223*), encoding an uncharacterized protein. Sixty-seven other SNPs on chromosome 6p21.1 were also associated with VEGF levels at P 5 10^{-8} (Figure 1A). When running a conditional genome-wide association study (GWAS) adjusting for rs6921438, 1 other SNP in 6p21.1 (rs4416670) still yielded a genome-wide significant association, suggesting that 2 variants in this region independently modulate VEGF levels. In the 8q23.1 region, the SNP yielding the most significant association with VEGF levels (rs6993770, P 2.50 10^{-16}) is located in the zinc finger protein, multitype 2 (*ZFPM2*) gene and 980.4 kb away from the low-density lipoprotein receptor-related protein 12 gene (*LRP12*). Forty-three SNPs in LD with rs6993770 were also associated with VEGF levels at P 5 10^{-8} (Figure 1B). A conditional GWAS adjusting for rs6993770, rs6921438, and rs4416670 did not yield any other genome-wide significant association in chromosome 8q23.1. The most significant association on 9p24.2 was observed with rs10738760 (P 1.96 10^{-34}), located close to the very low-density lipoprotein receptor (*VLDLR*) and potassium voltage-gated channel subfamily V, member 2 (*KCNV2*) genes. Twenty-nine SNPs in LD with rs10738760 were also associated with VEGF levels at P 5 10^{-8} (Figure 1C). None reached genome-wide significance in a conditional GWAS adjusted for rs6921438, rs4416670, rs6993770, and rs10738760.

We computed a genetic score including the 4 SNPs yielding genome-wide significant associations with VEGF levels in the conditional GWAS (Online Table I). This score explained 47.6% of serum VEGF variability (P 2.19 10^{-644}).

Replication Studies

We sought to replicate our most significant results in 2 independent cohorts. Of the 25 SNPs selected for replication, 24 were successfully genotyped in the SFS and 20 in the PIVUS study (Table 2). Of these, 17 and 20, respectively, reached nominal significance in association with VEGF levels, with the same direction of effect (Table 2). When meta-analyzing the results of the FHS and the PIVUS study, which both used serum VEGF levels, for the 19 SNPs genotyped in both studies, all 19 SNPs were associated with VEGF levels at P 0.05 (Table 2). The joint meta-analysis of results from all 3 studies, using an effective sample size–weighted meta-analysis, is displayed in Table 2. There was statistically significant heterogeneity between studies for a

Table 2. Genome-Wide Significant SNP Phenotype Associations in Genome-Wide Association Analysis of Circulating VEGF Levels ($P<5 \times 10^{-8}$)

SNP	Chr	Position*	Function	CAF	Coded Allele	Strand	† (FHS)	SE (FHS)	*P* (FHS)	*P* (PIVUS)
rs6921438	6	44033585	Intergenic	0.51	G		0.72	0.01	6.11×10^{-506}	NA
rs4513773	6	44033504	Intergenic	0.47	G		0.70	0.01	2.08×10^{-482}	6.23×10^{-139}
rs9472159	6	44027673	Intergenic	0.50	C		0.76	0.02	4.30×10^{-452}	3.27×10^{-109}
rs9369434	6	44026385	Intergenic	0.53	C		0.84	0.02	2.15×10^{-442}	1.43×10^{-63}
rs1776717	6	44059314	Intergenic	0.21	A		0.23	0.02	8.10×10^{-20}	3.74×10^{-4}
rs1776721	6	43998961	Intronic	0.31	T		0.18	0.02	1.52×10^{-19}	3.43×10^{-8}
rs1886979	6	44012879	3 UTR	0.41	G		0.17	0.02	3.71×10^{-19}	3.23×10^{-6}
rs9472155	6	44005705	Intronic	0.22	T		0.20	0.02	4.45×10^{-19}	3.93×10^{-9}
rs844294	6	44008685	Intronic	0.52	C		0.15	0.02	1.19×10^{-14}	2.25×10^{-5}
rs4416670	6	44058431	Intergenic	0.55	T		0.13	0.02	1.47×10^{-12}	0.10
rs910611	6	44058829	Intergenic	0.08	C		0.26	0.04	2.61×10^{-10}	6.36×10^{-6}
rs6993770	8	106650704	Intronic	0.32	T		0.17	0.02	2.50×10^{-16}	3.99×10^{-8}
rs16873402	8	106658423	Intronic	0.33	T		0.15	0.02	1.97×10^{-14}	9.49×10^{-9}
rs16873365	8	106627411	Intronic	0.22	T		0.16	0.02	5.65×10^{-12}	2.09×10^{-6}
rs7013321	8	106662734	Intronic	0.49	A		0.14	0.02	6.75×10^{-12}	NA
rs6993696	8	106650460	Intronic	0.46	A		0.13	0.02	8.54×10^{-12}	1.49×10^{-4}
rs16873291	8	106597206	Intronic	0.31	T		0.13	0.02	5.30×10^{-11}	7.65×10^{-7}
rs1349319	8	106625810	Intronic	0.39	A		0.11	0.02	3.59×10^{-8}	1.32×10^{-3}
rs10738760	9	2681186	Intergenic	0.49	A		0.28	0.02	1.96×10^{-34}	1.12×10^{-8}
rs6475920	9	2663933	Intergenic	0.36	A		0.24	0.02	3.76×10^{-32}	2.40×10^{-8}
rs4741756	9	2658187	Intergenic	0.28	C		0.25	0.02	2.95×10^{-31}	8.64×10^{-5}
rs2375980	9	2682622	Intergenic	0.42	G		0.25	0.02	1.30×10^{-27}	2.25×10^{-8}
rs10122587	9	2681951	Intergenic	0.28	T		0.22	0.02	3.02×10^{-24}	NA
rs10967492	9	2671175	Intergenic	0.21	A		0.22	0.02	1.02×10^{-21}	NA
rs10967470	9	2665698	Intergenic	0.24	G		0.22	0.02	1.17×10^{-21}	NA

CAF indicates coded allele frequency; Chr, chromosome; Dir, direction of association in FHS, PIVUS, and SFS; Gene1, closest referenced gene; Gene2, second closest referenced gene; Meta-*P*, meta-analysis *P* value; and CSE, standard error.

*Genome build 36.3.

†Effect estimate for the minor allele.

‡Inverse variance meta-analysis.

§Effective sample size weighted meta-analysis.

Model A: adjusted for age and sex, as well as for the ninth principal component in FHS.

few but not all SNPs in each locus, due to differences in effect size, but not in direction of effects (Online Table III). The genetic score explained 16.6% ($P = 1.75 \times 10^{-36}$) of observed plasma VEGF variability in the SFS and 48.4% ($P = 3.31 \times 10^{-180}$) of observed serum VEGF variability in the PIVUS study.

The observed associations remained unchanged in each of the 3 cohorts after adjusting for hypertension, current smoking, central obesity, and metabolic syndrome (Online Table IV).

VEGF Gene Expression Analysis

To better characterize the functional role of the SNPs identified in the GWAS, we quantified mRNA expression of the 2 splice variants corresponding to the diffusible isoforms of VEGF, $VEGF_{121}$, and $VEGF_{165}$ in PBMCs of 220 SFS participants. The association of VEGF mRNA levels with the 24 SNPs successfully genotyped in the SFS was assessed.

At the nominal significance level, 1 SNP on chromosome 6p21.1, 4 SNPs on chromosome 8q23.1, and 1 SNP on chromosome 9p24.2 were associated with $VEGF_{121}$ mRNA levels (Table 3).

Biological Pathway Analysis

With the use of the Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, www.ingenuity.com), we explored functional relationships between *VEGF* and the genes closest to the SNPs on chromosomes 8q23.1 and 9p24.2 that were significantly associated with circulating VEGF levels. In each case, we selected the genes closest to the identified SNPs, as in Table 2, to identify plausible biological pathways. We selected 5 focus genes: *VEGF, ZFPM2, LRP12, VLDLR,* and *KCNV2*. The IPA network analysis identified relationships among 3 of these 5 focus genes (*VEGF*, *ZFPM2,* and *VLDLR*) as part of a larger network of 35 genes. The probability of finding 3 or more focus genes in a set of 35 genes randomly selected from the Global Molecular Network was $P < 10^{-8}$, suggesting that the presence of 3 of our 5 focus genes in this network was unlikely to occur by chance. In Figure 2 we

Table 2. Continued

P (SFS)	Dir	Meta-*P* (FHS PIVUS)‡	Meta-*P* (All)§	Gene1	Distance (kb)	Gene2	Distance, kb
2.93 10 39	?	6.11 10 506	4.44 10 524	MGC45491	42.7	MRPL14	155.8
NA	?	4.45 10 619	1.10 10 584	MGC45491	42.8	MRPL14	155.8
4.11 10 35	. . .	1.61 10 557	8.16 10 553	MGC45491	48.6	MRPL14	161.7
5.31 10 28	. . .	1.43 10 490	1.21 10 496	MGC45491	49.9	MRPL14	163.0
9.75 10 6	. . .	3.28 10 22	1.07 10 26	MGC45491	17.0	MRPL14	130.0
0.02	. . .	5.38 10 26	4.23 10 26	MGC45491	77.3	VEGF	136.8
0.01	. . .	6.55 10 24	1.70 10 24	MGC45491	63.4	VEGF	150.7
0.01	. . .	2.50 10 26	1.51 10 26	MGC45491	70.6	VEGF	143.5
0.09		1.41 10 18	2.46 10 18	MGC45491	67.6	VEGF	146.5
2.87 10 4		1.44 10 12	2.08 10 15	MGC45491	17.9	MRPL14	130.9
0.11		9.92 10 15	1.94 10 14	MGC45491	17.5	MRPL14	130.5
0.02		2.60 10 22	4.71 10 23	ZFPM2	0	LRP12	980.4
0.16	. . .	1.10 10 20	5.32 10 20	ZFPM2	0	LRP12	988.1
0.37	. . .	4.10 10 16	2.27 10 15	ZFPM2	0	LRP12	957.1
0.01	?	6.75 10 12	4.49 10 13	ZFPM2	0	LRP12	992.4
0.05	. . .	6.18 10 15	2.12 10 15	ZFPM2	0	LRP12	980.1
0.07	. . .	1.12 10 15	4.75 10 16	ZFPM2	0	LRP12	926.9
0.05		1.99 10 10	3.53 10 11	ZFPM2	0	LRP12	955.5
0.03		4.46 10 41	9.93 10 40	KCNV2	26.3	VLDLR	36.7
0.02	. . .	6.11 10 39	7.93 10 38	VLDLR	19.4	KCNV2	43.6
0.09		4.45 10 34	3.41 10 32	VLDLR	13.7	KCNV2	49.3
0.02		2.55 10 34	1.01 10 33	KCNV2	24.9	VLDLR	38.1
0.02	?	3.02 10 24	4.67 10 24	KCNV2	25.6	VLDLR	37.5
0.10	?	1.02 10 21	1.25 10 20	VLDLR	26.7	KCNV2	36.3
0.04	?	1.17 10 21	2.79 10 21	VLDLR	21.2	KCNV2	41.8

present a subset of this network, including only interactions between *VEGF* and the 2 other focus genes in the network, with 2 or fewer intermediate nodes.

Discussion

Principal Findings

In this first GWAS of circulating VEGF levels undertaken in 3527 community individuals of European descent, we identified novel genetic associations: 140 SNPs reached genome-wide significance. Of these, 4 SNPs were independently associated with VEGF levels (rs6921438 and rs4416670 on chromosome 6p21.1, rs6993770 on chromosome 8q23.1, and rs10738760 on chromosome 9p24.2). We found evidence of replication for selected SNPs in 1727 individuals of European descent from 2 independent community-based samples. The SNPs are located close to the *VEGF* and *MRPL14* genes (chromosome 6p21.1), within the *ZFPM2* gene (chromosome 8q23.1), and between the *VLDLR* and *KCNV2* genes (chromosome 9p24.2). In a subset of participants, we found that 6 of 25 selected SNPs yielding genome-wide significant associations with circulating VEGF levels were also associated with *VEGF* mRNA levels ($VEGF_{121}$ splice variant) in PBMCs.

In the Context of the Current Literature

Genetic Association Study

Candidate gene studies exploring associations between *VEGF* polymorphisms and circulating VEGF levels have yielded controversial results (Online Supplemental Methods, section XI; Online Table V). Eight studies have found significant associations with candidate polymorphisms (rs699947, rs1570360, rs833061, rs2010963, rs3025039, and 2549 18bp I/D) in the promoter, 5 , and 3 untranslated regions of the *VEGF* gene.[33–40] However, several other studies did not identify any association with these and other *VEGF* SNPs (Online Table V). Using a hypothesis-free genome-wide

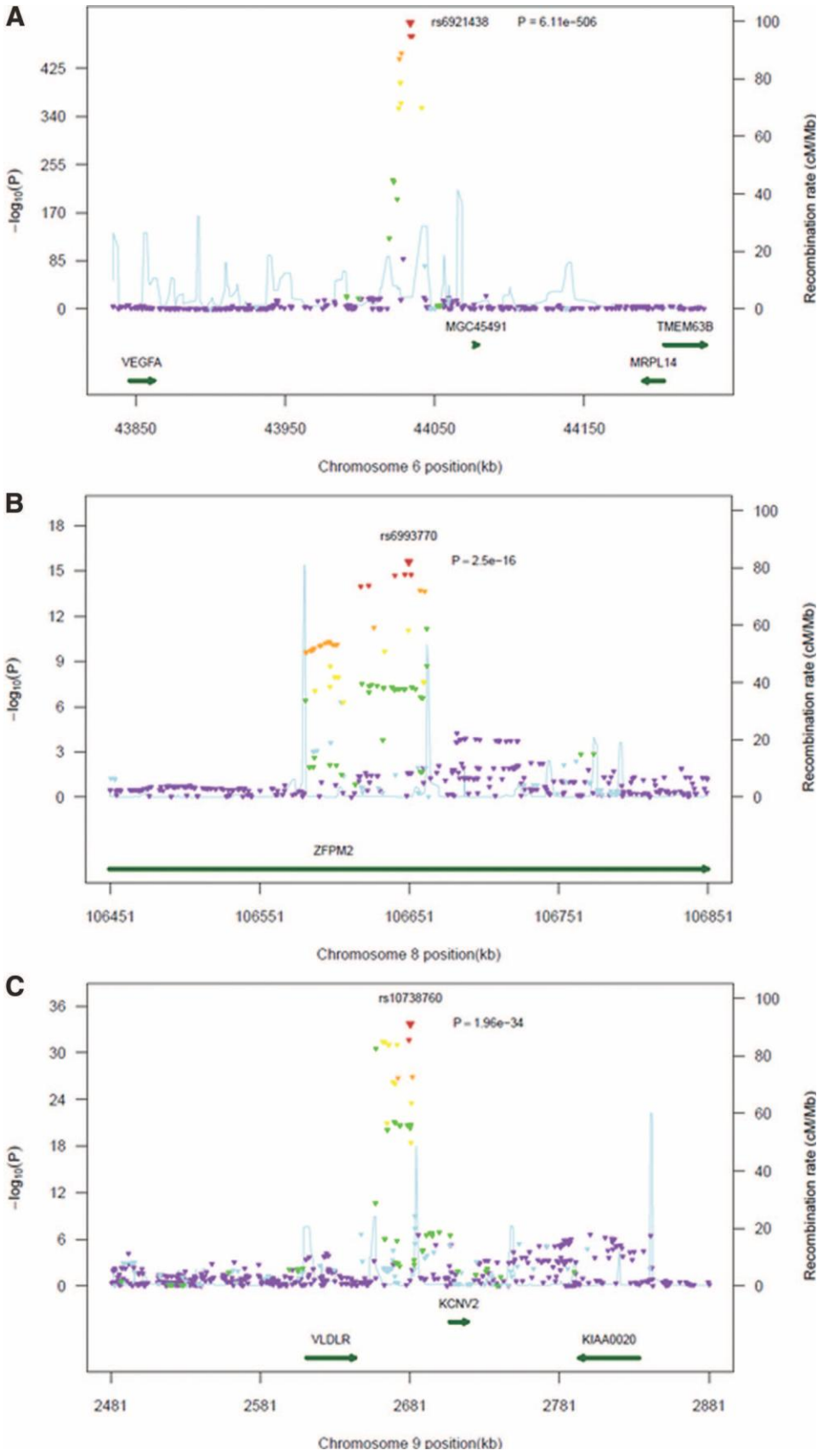

Figure 1. Regional plot for associations in region centered on rs6921438 (A), rs6993770 (B), and rs10738760 (C). All SNPs (triangles) are plotted with their meta-analysis probability values against their genomic position. The color of the triangles represents the linkage disequilibrium between each of the SNPs in the region with rs6921438: purple, r^2 0.05; light blue, 0.05 r2 0.10; green, 0.10 r^2 0.30; yellow, 0.30 r^2 0.60; orange, 0.60 r^2 0.80; and red, r^2 0.80. Light blue line represents estimated recombination rates. Genes are shown as dark green **arrows**. LD and recombination rates were drawn from Hapmap (release No. 22).

Table 3. Significant Associations Between SNPs and VEGF Transcripts

Phenotype	SNP	Chr	Position	CA	CAF	†	SE	*P*	h^2_q, %
mRNA_121	rs16873365	8	106627411	T	0.22	22.71	7.22	0.002	4.73
mRNA_121	rs16873402	8	106658423	T	0.33	12.15	5.10	0.017	2.84
mRNA_121	rs6993770	8	106650704	T	0.32	12.06	5.23	0.021	2.82
mRNA_121	rs16873291	8	106597206	T	0.31	11.95	5.37	0.026	2.47
mRNA_121	rs2375980	9	2682622	G	0.42	10.15	4.75	0.032	2.03
mRNA_121	rs910611	6	44058829	C	0.08	19.47	9.49	0.040	2.13

CAF indicates coded allele frequency; Chr, chromosome; h^2_q, variance explained; and SE, standard error.
*Log-transformed.
†Effect estimate for the minor allele.

approach, the present study revealed novel associations with 140 SNPs. Of these, 68 SNPs are located on chromosome 6, approximately 150 kb downstream from the 3 end of the *VEGF* gene, far from previously tested candidate SNPs. Although we do replicate previously described associations of 2 *VEGF* promoter polymorphisms (rs699947 and rs833061) with serum VEGF levels at *P* 5 10 ^{7} (Online Supplemental Methods, section XI; Online Table V; Online Figure III), none of the SNPs that reached genome-wide significance in our analysis, on chromosome 6p21.1, 8q23.1, and 9p24.2, had been examined previously in relation with circulating VEGF levels.

Transcriptomic Analysis

Although several studies have examined the association of candidate genetic variants with *VEGF* gene expression in pathological tissues,[41,42] little is known about the genetic variants influencing *VEGF* expression in normal cells. Our data suggest that 6 of the SNPs associated with circulating VEGF levels in our GWAS also modulate the expression of the $VEGF_{121}$ splice variant in PBMCs of community-based persons. The diffusible VEGF isoforms, $VEGF_{165}$ and $VEGF_{121}$, are released by a variety of tumor and normal cells, including PBMCs.[43] $VEGF_{121}$ lacks a heparin-binding domain and has a higher migration but lower mitogenic potency than $VEGF_{165}$.[44]

Potential Mechanisms Mediating Observed Genetic Associations

Our data suggest that almost half the inherited component of circulating VEGF levels is explained by genetic variants located downstream from the *VEGF* gene on chromosome 6p21.1. The conditional GWAS suggests that this region

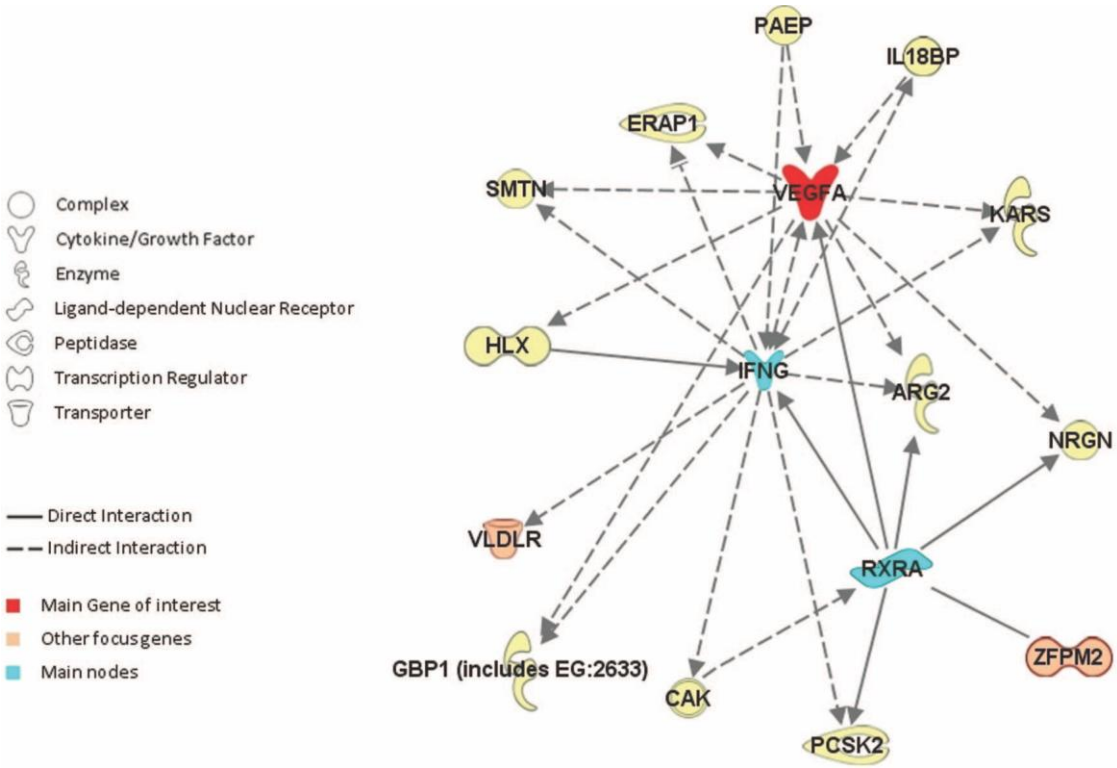

Figure 2. Putative gene network based on Ingenuity Path Analysis. Edges are displayed with labels describing the nature of the relationship between the nodes. Lines between genes represent known interactions and the nodes are displayed using various shapes that represent the functional class of the gene product (legend).

could harbor at least 2 distinct loci that are independently associated with circulating VEGF levels. Although located relatively far from the *VEGF* gene, results from our transcriptomic analysis indicate that this region could indeed contain functional variants modulating *VEGF* gene expression.

Genome-wide significant associations with circulating VEGF levels were also identified for SNPs located on chromosomes 8q23.1 and 9p24.2. Although these *trans* effects explain a much smaller proportion of the heritability of VEGF levels, they provide important clues about the pathways involved in the regulation of *VEGF* expression. The SNPs on chromosome 8q23.1 are located in introns 4 and 5 of the *ZFPM2* gene. This gene encodes a widely expressed member of the Friend of GATA family of transcription factors that modulate the activity of the GATA family proteins, which are important regulators of embryogenesis and also appear to play a significant role in endothelial cell biology.[45–47] The second closest gene to the SNPs identified on chromosome 8q23.1 is *LRP12*, encoding a low-density lipoprotein receptor-related protein that interacts with proteins related to signal transduction pathways and is differentially expressed in many cancer cells. The SNPs on chromosome 9p24.2 are located between the *VLDLR* and *KCNV2* genes. *VLDLR* encodes a lipoprotein receptor involved in the metabolism of apolipoprotein-E–containing triacylglycerol-rich lipoproteins. Like *VEGF, VLDLR* appears to modify the risk of developing age-related macular degeneration,[48] and recent data suggest that *VLDLR* could play a central role in a network of interacting angiogenic genes activated in response to hypoxia.[49] *KCNV2* encodes a member of the potassium voltage-gated channel subfamily V involved in regulation of neurotransmitter release, neuronal excitability, and heart rate. Although the present data do not permit us to formally determine which of these genes underlie the observed SNP associations with circulating VEGF levels, our in silico biological pathway analysis suggests that *ZFPM2* and *VLDLR* are the most likely candidates. Further research is needed to explore the mechanisms underlying the associations of *cis*- and *trans*-acting genetic variants with circulating VEGF levels, such as modulation of gene expression, differential splicing, or mRNA degradation.

Strengths and Limitations

The findings from this first GWAS of circulating VEGF levels emphasize the importance of screening for genetic variation modulating biomarker levels not only within and in close proximity to the gene encoding the protein under investigation, but also in more distant potentially regulatory regions, including on other chromosomes. The strength of the observed associations and the fact that we were able to replicate our findings in 2 independent cohorts suggest that these associations are real. This is further supported by the association of several of these genetic variants with *VEGF* gene expression in PBMCs. Our study also had several limitations. Whereas focusing on white populations of European descent has the advantage of minimizing potential population stratification issues, our findings cannot be generalized to other ethnic groups. We may not have identified the true causal variants but merely SNPs in LD with the latter. Plasma levels of VEGF were measured in one of the replication cohorts (SFS), whereas serum VEGF levels had been measured in the discovery cohort. Serum VEGF concentrations are higher than plasma concentrations because of the release of VEGF from platelets during the clotting process.[50] Although the vast majority of associations found in the FHS did replicate in the SFS, suggesting an important overlap between genetic susceptibility factors of serum and plasma VEGF levels, the lower proportion of VEGF variability explained by the genetic score in the SFS compared with the FHS and PIVUS study may be related to differences in plasma and serum VEGF concentrations. Finally, although our transcriptomic analysis does provide some support for a functional role of SNPs associated with mRNA levels of $VEGF_{121}$, these results are exploratory and were not corrected for multiple testing. We may have been underpowered for this analysis because of limited sample size and also because VEGF expression was measured on PBMCs only, which are not the sole contributors to circulating VEGF levels. Further studies looking at the association of genetic markers with VEGF expression in other cell types, including endothelial cells, would be of great value.

Clinical Implications

VEGF plays a key role in various diseases including atherosclerosis, inflammatory and neurodegenerative disorders, and cancer.[1] Anti-VEGF and proangiogenic VEGF-based treatments have recently been developed for several therapeutic indications.[19–23] The identification of polymorphisms linked to VEGF levels could help in identifying patients who are more likely to respond favorably to such treatments. These therapies can have major side effects,[24] and optimizing the risk-benefit ratio of their administration could lead to substantial improvements in patient care. The discovery of *trans*-acting genetic variants influencing VEGF levels could also spur the discovery of new molecular targets for proangiogenic or antiangiogenic therapies.

Conclusions

In a large population-based sample of European ancestry, we identified novel genetic variants associated with circulating VEGF levels on chromosomes 6p21.1, 8q23.1, and 9p24.2, which explain almost half of the observed phenotypic variation.

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STANISLAS Family Study

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Circulating level of vascular endothelial growth factor (VEGF) is a heritable trait (60% to 80% heritability). The underlying genetic variants have not been identified.
- VEGF has important physiological and pathophysiological roles in promoting and supporting angiogenesis, as a vasodilator and neurotrophic factor. It also promotes atherosclerosis.
- VEGF antagonists are used to treat macular degeneration and certain cancers.
- VEGF agonists may help treat limb ischemia.

What New Information Does This Article Contribute?

- Four independent novel loci are identified, using an unbiased genome-wide association study approach combined with messenger RNA expression and Ingenuity pathway analysis.
- The novel loci explain nearly half the observed variation in serum VEGF levels, in population-based samples of European descent, who were free of cardiovascular disease.
- Two novel loci are downstream of the *VEGFA* gene on chromosome 6 and may be transcription regulators.
- Two other novel *"trans"* loci are located on chromosomes 8q23.1 and 9p24.2. Potential candidate genes at these loci are zinc finger protein multitype 2 (*ZFPM2*) and very low-density lipoprotein receptor (*VLDLR*).

VEGF has a pathophysiological role in atherosclerosis, inflammatory and neurodegenerative disorders, and cancer. Circulating VEGF level is a strongly heritable trait, but the underlying genetic variants are unknown. Understanding the genetic determinants of VEGF levels is of potential pharmacogenomic importance as VEGF antagonists (with interindividual differences in toxicity and efficacy) are used to treat macular degeneration, colon cancer, and other conditions. We undertook genome-wide association study of serum VEGF levels in 3527 community-based, Framingham Heart Study participants and replicated our findings in 2 other healthy samples, the Stanislas Family Study and the Prospective Investigation of Vasculature in Uppsala Seniors. We identified 4 novel loci across 3 chromosomes: 6p21.1, 8q23.1, and 9p24.2, which together explain 48% of the observed variability in serum VEGF. In exploratory analyses, 6 of 24 SNPs studied were associated with peripheral blood mononuclear cell messenger RNA expression of VEGF. Further, Ingenuity pathway analyses revealed that genes adjacent to the 2 *trans* loci, *ZFPM2* on chromosome 8 and *VLDLR* on chromosome 9, are linked to *VEGF* along plausible biological pathways. These findings highlight the potential importance of distant regulatory regions in determining biomarker levels. These results might help target VEGF-based treatments and spur the discovery of new molecular targets for proangiogenic or antiangiogenic therapies.

Supplemental Material

Identification of Cis- and Trans-acting Genetic Variants Explaining up to Half the Heritability of Circulating VEGF levels

A. Supplemental Methods

1. Cohorts

Framingham Heart Study (FHS)
The FHS is a three-generation, single-site, community-based, prospective cohort study that was initiated in 1948 to investigate risk factors for cardiovascular disease including stroke in Framingham, MA, USA. It now comprises 3 generations of participants: the original cohort followed since 1948 (Original, Gen 1)[1]; their offspring and spouses of the offspring, followed since 1971 (Offspring, Gen 2);[2] and grandchildren of the Original cohort in 2002 (Gen 3).[3] Vascular endothelial growth factor (VEGF) levels have at this time only been measured in Gen 3 participants. Starting in 2002, 4,095 participants with at least one parent in the Offspring cohort were enrolled in the Gen 3 cohort.[3] At their first examination (2002-2005) Gen 3 participants underwent a targeted medical history, physical examination, anthropometry, and laboratory assessment of traditional cardiovascular risk factors. VEGF levels were available in 3,946 participants, among whom we excluded persons with prevalent cardiovascular disease (n=66), serum creatinine >2 mg/dl (n=1) and those in whom data for more covariates (n=125) were not available. Of the remaining 3,754 participants, 3,553 also had DNA extracted and genome-wide genotyping data available. Genome-wide genotyping was performed at Affymetrix (Santa Clara, CA) through an NHLBI funded SNP-Health Association Resource (SHARe) project and was successful in 3,527 persons. These 3,527 persons constitute the FHS sample for this study.
All study participants provided informed consent and the study was approved by the Institutional Review Board at the Boston University Medical Center.

STANISLAS Family Study (SFS)
Initiated in 1993, the STANISLAS Family Study is a 10-year longitudinal survey set up to seek out gene-gene and gene-environment interactions in the field of cardiovascular diseases.[4] 1,006 volunteer families (2 parents and at least two siblings) were initially recruited between 1993 and 1995 during a free health check-up at the Centre for Preventive Medicine in Vandoeuvre-lès-Nancy, France. Individuals with chronic disorders (cardiovascular or cancer) or having a personal history of cardiovascular disease were not included, in order to assess the effects of genetic susceptibility factors on the variability of intermediate phenotypes in physiological conditions without the influence of any long term medication and disease. Biological and clinical measurements, health and lifestyle information were thus collected from 4,488 individuals belonging to 2 generations using appropriate, validated questionnaires and procedures as described previously.[4, 5] DNA was extracted from all participants and serum/plasma/DNA biobanks were constructed. In 1998–2000, 756 of the original families attended a second check-up and the same examination and biobanking protocols undertaken at the first examination were repeated. Using the plasma collected at this examination, specific measurements like VEGF plasma concentrations were measured on a random subsample of 1,000 subjects. A third check-up took place between 2003 and 2005 and at this visit mRNA and lymphocytes extract biobanks were constructed in addition to serum/plasma/DNA biobanks. Individuals with both VEGF plasma concentrations and DNA available were selected for the present study (919 individuals from 263 families). Some parents or children were secondarily excluded because DNA amplification was not successful (n=58), or results of genotyping were ambiguous (n=2). Finally, 859 individuals from 217 families met our selection criteria. Each subject gave written informed consent for participating in this study, which was approved by the Local Ethics Committee of Nancy.

Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study
All 70-year old individuals living in the community of Uppsala, Sweden, between April 2001 and June 2004 were eligible for the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study, which

has been described in detail previously.[6] The individuals were selected randomly, and were examined within one month of their 70th birthday in order to standardize for age. Of 2,025 individuals invited, 1,016 were investigated giving a participation rate of 50%, and 999 of these individuals provided DNA for genetic studies. For the purpose of the present investigation, we excluded participants with sample call rate <90% (n=11), prevalent myocardial infarction, stroke or congestive heart failure (n=116), serum creatinine >2 mg/dl (n=1), or missing data on VEGF (n=3). Thus, 868 participants (52% women) were eligible for the present study. The PIVUS study has been approved by the Ethics Committee of Uppsala University and the participants gave written informed consent.

2. Laboratory measurements of vascular endothelial growth factor

VEGF levels were measured in serum for the FHS and PIVUS and plasma for the SFS, at the first examination cycle (2002-2005) in the FHS, the second examination in the SFS (1998-2000) and the baseline examination (2001-2004) in the PIVUS study.
In all 3 studies venous blood samples were drawn after an overnight fast, immediately centrifuged and stored appropriately (at -80°C in FHS and PIVUS and at -196°C in liquid nitrogen in SFS) until VEGF measurements were undertaken. At FHS, serum VEGF was measured using a commercial ELISA assay (R&D Inc.). In SFS and PIVUS plasma VEGF and serum VEGF quantification respectively was performed by Randox Ltd (Crumlin, UK), using a biochip array analyzer (Evidence®).[7, 8] In all studies both diffusible VEGF isoforms ($VEGF_{121}$ and $VEGF_{165}$) were detected.
The average inter-assay coefficients of variation were 2.1% for serum VEGF in the FHS, less than 9% in the SFS and less than 15% in the PIVUS study.
Since serum VEGF had been measured in the FHS and PIVUS and plasma for VEGF in the SFS, we checked the correlation between the 2 types of specimens. VEGF was measured in a subset (n=18) of matched plasma and serum samples from the SFS. Plasma VEGF was lower (42±28 ng/L, mean±SD) than serum VEGF (361±223 ng/L); There was a strong correlation between plasma and serum VEGF (r=0.76, p=0.0002) which strengthens our study.

3. Genotyping, quality controls, and imputation

Genome-wide genotyping in the FHS study was performed on the Affymetrix GeneChip Human Mapping 500K Array Set® and 50K Human Gene Focused Panel®.
The set of genotyped input SNPs used for imputation was selected based on their highest quality GWA data. From a total of 534,982 genotyped autosomal SNPs in FHS, we used 378,163 SNPs in the imputation after filtering out 15,586 SNPs for Hardy-Weinberg disequilibrium ($p<1x10^{-6}$), 64,511 SNPs for missingness >0.03, 45,361 SNPs for a test of differential missingness yielding a $p<1x10^{-9}$ (mishap test in PLINK, http://pngu.mgh.harvard.edu/purcell/plink/), 4,857 SNPs for >100 Mendel errors, 67,269 SNPs for a minor allele frequency < 0.01, 2 SNPs due to strandedness issues upon merging data with HapMap, and a further 13,394 SNPs because they were not present on HapMap. We used the Markov Chain Haplotyping (MaCH) package (http://www.sph.umich.edu/csg/abecasis/MACH, version 1.0.15 software) and imputed to the plus strand of NCBI build 36, HapMap release #22. For each imputed SNP, imputation quality was estimated as the ratio of the empirically observed dosage variance to the expected binomial dosage variance. After quality control and filtering, FHS had either genotyped or imputed data for 2,540,223 autosomal SNPs. From a total of 10,886 genotyped SNPs on the X chromosome, we used 7,795 SNPs in the imputation after filtering out 3,091 SNPs for Hardy-Weinberg $p<1x10^{-6}$ (n=159), missingness >0.03 (n=450), minor allele frequency < 0.01 (n=1851), male heterozygote count > 45 (n=12), and a further 619 SNPs because they were not present on HapMap. We used the IMPUTE package (https://mathgen.stats.ox.ac.uk/impute/impute.html, version 0.5.0) and imputed to the plus strand of NCBI Build 35, Hapmap release #21.

4. Screening for latent population substructure

FHS was screened for latent population substructure, including cryptic relatedness, using EIGENSTRAT.[9, 10] We used 882 unrelated individuals to infer eigenvectors, then projected the rest of the subjects onto these eigenvectors. Principal components associated with the outcome were handled like any associated covariate that needs to be adjusted for. We have systematically tested the association of the ten first principal

components with VEGF serum levels and found that only the ninth principal component was significantly associated with VEGF serum levels. Therefore only this principal component was included as a covariate. in the GWAS.

5. Covariate definition

In all 3 studies, hypertension was defined as systolic blood pressure ≥140, diastolic blood pressure ≥90 or use of anti-hypertensive treatment and smokers were identified based on current smoking status. Standing waist circumference (WC) was obtained at the level of the umbilicus and central obesity was defined as a WC ≥102 cm in men and ≥89 cm in women. Metabolic syndrome was defined as having any 3 of the following traits: (i) central obesity, (ii) hypertension, (iii) hyperglycemia defined as fasting glucose ≥100 mg/dL or use of anti-diabetic medications or insulin, (iv) hypertriglyceridemia defined as serum or plasma triglycerides ≥150 mg/dL or being on any lipid-lowering medication, (v) low HDL cholesterol (<40 mg/d in men and <50 mg/d in women).[11] These covariates were measured at the same time as VEGF levels, at the first Gen 3 examination cycle (2002-2005) in the FHS, at the second examination in the SFS (1998-2000) and at the baseline examination (2001-2004) in the PIVUS study.

6. Selection of SNPs for replication

From all SNPs associated with VEGF levels at a p-value < $5x10^{-8}$ in the discovery cohort, we excluded SNPs with minor allele frequencies (MAF) <0.05 as well as those imputed SNPs with low imputation quality (ratio of the empirically observed dosage variance to the expected binomial dosage variance <0.6). The remaining SNPs were grouped by "bins", each bin comprising SNPs that are in very strong linkage disequilibrium (LD) with each other, i.e. with an r^2>0.8 with the most significant SNP in the bin. Within each bin we selected one SNP for replication (except for the bin with the most significant associations where 2 SNPs were selected). Typically, we selected the SNP with the lowest p-value within each bin. In 6 bins a SNP with a slightly higher p-value was chosen either because this SNP had been directly genotyped in the discovery cohort, whereas the SNP with the lowest p-value in the bin had been imputed (rs1776721 and rs1886979) or because there were stronger arguments for functionality for the SNP with the slightly higher p-value (rs16873291, rs1349319, rs6475920 and rs10967492).

7. Meta-analysis

For the meta-analysis of results from the FHS and the PIVUS study, which both measured VEGF levels in the serum, we used a fixed effects inverse-variance weighted meta-analysis technique. Beta estimates were weighted by their inverse variance and a combined estimate was obtained by summing the weighted betas and dividing by the summed weights.
For the meta-analysis of results from all three studies (FHS, SFS and PIVUS study), an effective sample size weighted meta-analysis technique was used, to account for the fact that VEGF levels were measured in the serum in the FHS and PIVUS study and in the plasma in the SFS. This method consists of combining each study's Z-statistic, weighted by their sample size. Imputed SNPs are weighted for information content. Importantly this method does not require studies to use the same measurement scale. We do acknowledge that differences between plasma and serum levels due to differences in the various blood components that contribute to these levels cannot be addressed using the sample size weighted meta-analysis method. Hence the genetic association results for SFS alone are also presented.
Prior to meta-analysis, strand alignment was verified across all studies. After meta-analysis, the genomic control parameter was calculated and used to remove any residual population-stratification. We undertook the meta-analyses at FHS using the METAL software (http://www.sph.umich.edu/csg/abecasis/Metal/index.html).

8. Genetic score calculation

Step 1:
We selected SNPs with independent effects by running a conditional GWAS. This was done in a forward stepwise fashion. First we ran a GWAS adjusting for age, gender, the ninth principal component and the most significantly associated SNP (rs6921438). We then ran a GWAS additionally adjusting for the most

significantly associated SNP in the aforementioned conditional GWAS. This process was repeated, by adding the most significantly associated SNP as a new covariate to the regression model, until all SNPs independently associated with VEGF level at a p-value < $5x10^{-8}$ were selected. All selected independent SNPs with a minor allele frequency > 5% were used to compute the genetic score (**Supplementary Table I**). In the PIVUS study, as rs6921438 genotypes were not available, rs4513773 was used instead to compute the genetic score (r^2 with rs6921438 = 0.90)

Step 2:
Genotypes for the SNPs selected in step 1 were coded as 0, 1 or 2 for genotyped SNPs (according to the number of minor alleles) and the imputed allele dosage was used for imputed SNPs.
To compute the genetic score the genotype value was weighted by the effect size estimate from the GWAS (**Supplementary Table I**):
Risk Score = SNP1_estimate * SNP1_genotype + SNP2_estimate * SNP2_genotype + SNP3_estimate * SNP3_genotype + SNP4_estimate * SNP4_genotype

Step 3:
We estimated the proportion of phenotype variance explained by each SNP incorporated in the risk score (h^2 , see Supplementary Table I); h^2 was computed as follows, using the GWAF R-package:[12]

$$h_q^2 = \max\left(0, \frac{\sigma^2_{G.null} + \sigma^2_{e.null} - \sigma^2_{G.full} - \sigma^2_{e.full}}{Var(y)}\right)$$

Where:
Var(y)= total phenotypic variance,
$\sigma^2_{G.null}$ and $\sigma^2_{e.null}$ = the polygenic variance and error variance when modeling without the tested SNP, $\sigma^2_{G.full}$ and $\sigma^2_{e.full}$ = the polygenic variance and error variance when modeling with the tested SNP.

9. Biological pathway analysis

Genes located close to associated SNPs were investigated for relevant networks by the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com). To build networks, IPA queries the Ingenuity Pathways Knowledge Base for biological interactions between identified "focus genes", in this case genes close to SNPs significantly associated with circulating VEGF levels in the GWAS, and all other gene objects stored in the knowledge base. It then generates a set of networks with a maximum network size of 35 genes. An underlying assumption is that highly-interconnected networks are likely to represent significant biological function,[13] thus IPA optimizes for triangular relationships between genes, favoring denser networks over more sparsely connected ones. Networks are displayed graphically as "nodes" (corresponding to genes or gene products) and the biological relationships between the nodes, referred to as "edges". IPA also computes a score, representing the $-\log_{10}$(p-value), where the p-value is the probability of finding f or more focus genes in a set of n genes randomly selected. If there are n genes in the network and f of them are focus genes, the p-value is the probability of finding f or more focus genes in a set of n genes randomly selected from the Global Molecular Network. It is calculated using Fisher's exact test.

10. VEGF gene expression analysis in peripheral blood mononuclear cells (PBMCs)

Sample preparation and quantification of the messenger RNA (mRNA) VEGF spliced forms
Two-hundred and twenty samples from the SFS were randomly selected for inclusion in the PBMCs transcriptomic study. Fresh whole blood (10mL) was collected by standardized venipuncture in EDTA tubes (Vacutainer™; Becton Dickinson, NJ, USA). PBMCs were isolated by centrifugation on a density gradient of Ficoll (Ficoll-Paque™ PLUS; Amersham BioSciences) and stored at -80°c until RNA extraction according to a well-validated protocol,[14] with high recovery of lymphocyte(97.5%). Total RNA was subsequently extracted with the MagNaPure automate, using the MagNA Pure LC RNA HP isolation kit and RNA HP Blood External lysis protocol (Roche Diagnostics, France). Reverse transcription of total RNA was performed using 200 units of M-MuLV Reverse Transcriptase with 0.25 µg of oligos(dt) (Promega, France) according to a previous described protocol.[14] Quantification of the transcripts coding for

the $VEGF_{121}$ and $VEGF_{165}$ isoforms, and the beta 2 microglobulin (B2M) control gene, was performed

using TaqMan® and LightCycler technologies (LC TaqMan Master kitRoche Diagnostics, France). All experiments were performed in duplicate. The detection level for each transcript was between 1 and 10 copies for both transcripts and for B2M. RT-PCR optimization and specificity of RT-PCR products were examined using SYBR® Green technology (LC FastStart DNA Master^{PLUS} SYBR Green I kit, Roche Diagnostic, France), melting curves analysis and agarose gel electrophoresis of the PCR amplicons. Primers and probes were designed to specifically amplify the spliced forms of VEGF based on their splicing sites[15] with specific reverse primers or hydrolyzation probes spanning the variant specific exon boundaries, which also avoids amplification of contaminating genomic DNA. Hydrolyzation probes were labeled with the reporter dye FAM (6-carboxy-fluorescein phosphoramidite) at the 5' end and the quencher dye TAMRA (5 -carboxyl- tetramethyl-rhodamine) at the 3' end. For all assays, intra- and inter-run variability were 11% and 5% respectively. PCR products for each VEGF mRNA spliced variant amplification were purified with a PCR purification kit (QiaQuick, Qiagen, France). The product concentrations were measured in a spectrophotometer, the molecule concentrations were calculated, and a standard curve was generated for each transcript using serial dilutions of products ranging from 1 or 10 to 10^7 molecules/μL. The copy number of unknown samples was calculated by setting their PCR cycle number (Crossing Point: CP) to the standard curve and normalized to the housekeeping B2M gene. Results are presented as copies of the target gene product per 10^6 copies of B2M. Primer efficiencies were calculated according to the equation $E = 10[-1/slope]$. All investigated transcripts had real-time PCR efficiency rates above 1.9.

PBMCs VEGF protein measurements

PBMC VEGF (121 and 165) protein quantification was performed by Randox Ltd (Crumlin, UK), with a biochip array analyzer (Evidence®) using a high sensitivity kit as previously described.[7] PBMC VEGF concentrations were log10-transformed in all analyses in order to improve normality, were adjusted for the effect of between-run variation and regressed on mean values of all samples measured in each run. Grubbs' test was applied for detection of the extreme values in the data (log transformed VEGF) and there were no outliers at the 5% level. The average inter-assay coefficient of variation was 5.7%.

Statistical analysis

A linear mixed effects model that accounts for within family correlation was used to evaluate the association of each of the SNPs successfully genotyped in the SFS with each of the two transcript levels ($VEGF_{121}$ and $VEGF_{165}$) and with natural log-transformed PBMC VEGF concentration, assuming an additive genetic model. These analyses were adjusted for age and sex.

11. Candidate gene association studies

We searched Pubmed for original articles published before May 14 2010, which reported statistically significant associations between specific genotypes and circulating VEGF levels. We used the search terms VEGF, gene and polymorphism. We identified 14 candidate gene association studies,[16-29] who examined 11 different polymorphisms in the VEGF gene in association with plasma or serum VEGF levels (**Supplementary Table IV**). Of these, 8 studies reported a significant association of at least one polymorphism (rs699947, rs1570360, rs833061, rs2010963, rs3025039, -2549 18bp I/D) with circulating VEGF levels.[22-29] In the FHS sample we replicated the association of rs699947 and rs833061 with serum VEGF levels at $p < 5x10^{-7}$ (**Supplementary Table IV**). The other 4 polymorphisms (rs1570360, rs2010963, rs3025039, -2549 18bp I/D) had neither been genotyped nor imputed in the FHS GWAS. As these SNPs are not in the Hapmap database, we could not identify any proxy either to test their association with serum VEGF levels in the FHS dataset.

B. Supplemental Tables

Online Table I: Results of the main and conditional GWAS within the Framingham Heart Study sample for the SNPs retained in the genetic score calculation

SNP	chr	position	CA	CAF	Estimate in main GWAS*	SE in main GWAS*	p in main GWAS*	p in conditional GWAS†	h^2_q (%)
rs6921438	6	44033585	G	0.51	0.7199	0.0149	$<5x10^{-324}$	$<5x10^{-324}$	41.19
rs10738760	9	2681186	A	0.49	0.2812	0.0230	$1.96x10^{-34}$	$3.78x10^{-47}$	4.97
rs6993770	8	106650704	T	0.32	-0.1667	0.0203	$2.50x10^{-16}$	$5.45x10^{-30}$	2.03
rs4416670	6	44058431	T	0.55	0.1342	0.0190	$1.47x10^{-12}$	$4.79x10^{-9}$	1.46

CA: coded allele; CAF: coded allele frequency; Chr: chromosome; GWAS: genome-wide association study; h^2_q: percentage of phenotypic variance explained; SE: standard error; * adjusted for age, sex, and the ninth principal component; † with all four SNPs in the same model, additionally adjusted for age, sex, and the ninth principal component

Online Table II: SNPs associated with serum VEGF levels at a p-value < $5x10^{-8}$ in the discovery GWAS

SNP	Chr	Position	CA	CAF	beta	SE	P	Gene 1	Distance	Gene 2	Distance	Function	O/E ratio	Imputed
rs6921438	6	44033585	G	0,52	0,72	0,01	6.11E-506	MGC45491	42731	MRPL14	155764	INTERGENIC	0,99	0
rs7767396	6	44035028	G	0,47	-0,71	0,02	1.71E-482	MGC45491	41288	MRPL14	154321	INTERGENIC	0,99	1
rs4513773	6	44033504	G	0,46	-0,71	0,02	2.08E-482	MGC45491	42812	MRPL14	155845	INTERGENIC	1,00	0
rs9472159	6	44027673	C	0,51	0,76	0,02	4.30E-452	MGC45491	48643	MRPL14	161676	INTERGENIC	0,82	1
rs9369434	6	44026385	C	0,53	0,85	0,02	2.15E-442	MGC45491	49931	MRPL14	162964	INTERGENIC	0,66	1
rs6916314	6	44027140	G	0,38	0,92	0,02	3.13E-400	MGC45491	49176	MRPL14	162209	INTERGENIC	0,54	1
rs9472158	6	44026875	G	0,38	0,92	0,02	6.22E-400	MGC45491	49441	MRPL14	162474	INTERGENIC	0,54	1
rs6916540	6	44027394	C	0,40	0,87	0,02	3.60E-364	MGC45491	48922	MRPL14	161955	INTERGENIC	0,56	1
rs9472173	6	44041234	C	0,47	0,93	0,02	1.16E-355	MGC45491	35082	MRPL14	148115	INTERGENIC	0,47	1
rs729391	6	44025870	C	0,39	1,05	0,03	7.17E-355	MGC45491	50446	MRPL14	163479	INTERGENIC	0,38	1
rs7764227	6	44021966	G	0,26	1,32	0,04	1,41E-228	MGC45491	54350	VEGFA	159767	INTERGENIC	0,22	1
rs9369433	6	44022528	C	0,23	1,28	0,04	6,87E-225	MGC45491	53788	VEGFA	160329	INTERGENIC	0,25	1
rs1359617	6	44024870	T	0,11	1,87	0,06	1,56E-194	MGC45491	51446	VEGFA	162671	INTERGENIC	0,18	1
rs865585	6	44019529	C	0,29	0,83	0,04	3,16E-125	MGC45491	56787	VEGFA	157330	INTERGENIC	0,30	1
rs6458360	6	44028870	T	0,11	1,10	0,05	3,14E-89	MGC45491	47446	MRPL14	160479	INTERGENIC	0,28	1
rs17209449	6	44043245	C	0,09	-1,20	0,06	1,30E-76	MGC45491	33071	MRPL14	146104	INTERGENIC	0,24	1
rs10738760	9	2681186	A	0,49	0,28	0,02	1,96E-34	KCNV2	26339	VLDLR	36701	INTERGENIC	0,68	1
rs10757631	9	2680295	G	0,47	-0,26	0,02	2,30E-32	KCNV2	27230	VLDLR	35810	INTERGENIC	0,73	1
rs6475919	9	2662809	T	0,37	-0,24	0,02	3,63E-32	VLDLR	18324	KCNV2	44716	INTERGENIC	0,95	1
rs6475920	9	2663933	A	0,37	-0,24	0,02	3,76E-32	VLDLR	19448	KCNV2	43592	INTERGENIC	0,96	1
rs1020651	9	2666360	G	0,37	-0,23	0,02	8,60E-32	VLDLR	21875	KCNV2	41165	INTERGENIC	1,01	1
rs1020652	9	2667012	T	0,37	-0,23	0,02	8,69E-32	VLDLR	22527	KCNV2	40513	INTERGENIC	1,01	0
rs10738758	9	2672355	G	0,37	-0,23	0,02	9,11E-32	VLDLR	27870	KCNV2	35170	INTERGENIC	0,98	1
rs4741756	9	2658187	C	0,28	-0,25	0,02	2,95E-31	VLDLR	13702	KCNV2	49338	INTERGENIC	0,97	0
rs2375980	9	2682622	G	0,42	-0,25	0,02	1,30E-27	KCNV2	24903	VLDLR	38137	INTERGENIC	0,70	1
rs7856084	9	2672943	C	0,39	-0,22	0,02	1,75E-27	VLDLR	28458	KCNV2	34582	INTERGENIC	0,97	1
rs10125071	9	2669579	C	0,40	-0,21	0,02	5,88E-27	VLDLR	25094	KCNV2	37946	INTERGENIC	1,00	1
rs4317630	9	2671025	C	0,39	-0,21	0,02	9,65E-27	VLDLR	26540	KCNV2	36500	INTERGENIC	0,98	1

rs10122587	9	2681951	T	0,28	-0,22	0,02	3,02E-24	KCNV2	25574	VLDLR	37466	INTERGENIC	0,98	1
rs9381273	6	44084246	G	0,01	5,92	0,59	5,37E-24	MGC45491	3381	MRPL14	105103	DOWNSTREAM	0,03	1
rs7747448	6	43990902	A	0,34	-0,25	0,03	6,88E-23	MGC45491	85414	VEGFA	128703	INTRONIC	0,58	1
rs4276495	6	43991237	T	0,32	-0,23	0,02	6,46E-22	MGC45491	85079	VEGFA	129038	INTRONIC	0,69	1
rs10117473	9	2670425	G	0,21	-0,22	0,02	7,67E-22	VLDLR	25940	KCNV2	37100	INTERGENIC	1,01	1
rs10967492	9	2671175	A	0,21	-0,22	0,02	1,02E-21	VLDLR	26690	KCNV2	36350	INTERGENIC	1,01	1
rs10967470	9	2665698	G	0,24	-0,22	0,02	1,17E-21	VLDLR	21213	KCNV2	41827	INTERGENIC	0,97	1
rs10812475	9	2681546	C	0,21	-0,22	0,02	1,84E-21	KCNV2	25979	VLDLR	37061	INTERGENIC	0,98	1
rs10812474	9	2681446	G	0,21	-0,22	0,02	1,86E-21	KCNV2	26079	VLDLR	36961	INTERGENIC	0,98	1
rs10812473	9	2681329	A	0,21	-0,22	0,02	1,86E-21	KCNV2	26196	VLDLR	36844	INTERGENIC	0,98	1
rs10812471	9	2680980	T	0,21	-0,22	0,02	1,89E-21	KCNV2	26545	VLDLR	36495	INTERGENIC	0,99	1
rs10125245	9	2679244	T	0,21	-0,22	0,02	2,09E-21	KCNV2	28281	VLDLR	34759	INTERGENIC	1,00	1
rs10967512	9	2673939	C	0,21	-0,22	0,02	2,18E-21	VLDLR	29454	KCNV2	33586	INTERGENIC	1,02	0
rs10757628	9	2674656	T	0,21	-0,22	0,02	2,22E-21	VLDLR	30171	KCNV2	32869	INTERGENIC	1,01	0
rs10812472	9	2681095	G	0,20	-0,23	0,02	4,89E-21	KCNV2	26430	VLDLR	36610	INTERGENIC	0,94	1
rs7867894	9	2665867	C	0,25	-0,21	0,02	8,62E-21	VLDLR	21382	KCNV2	41658	INTERGENIC	0,98	1
rs9472175	6	44042760	T	0,15	-0,56	0,06	9,73E-21	MGC45491	33556	MRPL14	146589	INTERGENIC	0,20	1
rs1776717	6	44059314	A	0,21	-0,23	0,02	8,10E-20	MGC45491	17002	MRPL14	130035	INTERGENIC	0,86	1
rs910609	6	44059634	A	0,21	-0,23	0,02	9,14E-20	MGC45491	16682	MRPL14	129715	INTERGENIC	0,86	1
rs833623	6	44006755	C	0,41	0,18	0,02	1,07E-19	MGC45491	69561	VEGFA	144556	INTRONIC	0,92	1
rs833622	6	44008382	T	0,40	0,17	0,02	1,21E-19	MGC45491	67934	VEGFA	146183	INTRONIC	0,98	0
rs1776721	6	43998961	T	0,30	-0,18	0,02	1,52E-19	MGC45491	77355	VEGFA	136762	INTRONIC	0,99	0
rs9381267	6	44013484	G	0,41	0,17	0,02	3,15E-19	MGC45491	62832	VEGFA	151285	3PRIME_UTR	0,97	1
rs1886979	6	44012879	G	0,41	0,17	0,02	3,72E-19	MGC45491	63437	VEGFA	150680	3PRIME_UTR	0,97	0
rs10122524	9	2681667	T	0,30	-0,19	0,02	3,73E-19	KCNV2	25858	VLDLR	37182	INTERGENIC	0,99	1
rs9472155	6	44005705	T	0,22	-0,20	0,02	4,45E-19	MGC45491	70611	VEGFA	143506	INTRONIC	0,96	1
rs7767550	6	44007230	A	0,22	-0,20	0,02	6,36E-19	MGC45491	69086	VEGFA	145031	INTRONIC	0,99	0
rs3888006	6	44000916	A	0,22	-0,20	0,02	7,60E-19	MGC45491	75400	VEGFA	138717	INTRONIC	0,95	1
rs1750570	6	44000213	T	0,22	-0,20	0,02	8,79E-19	MGC45491	76103	VEGFA	138014	INTRONIC	0,95	1
rs1326141	6	44011255	A	0,22	-0,20	0,02	2,43E-18	MGC45491	65061	VEGFA	149056	INTRONIC	0,98	0
rs910608	6	44059804	C	0,22	-0,21	0,02	3,39E-18	MGC45491	16512	MRPL14	129545	INTERGENIC	0,87	1
rs7356919	6	43999343	A	0,22	-0,20	0,02	5,37E-18	MGC45491	76973	VEGFA	137144	INTRONIC	0,96	1

rs910610	6	44059418	A	0,23	-0,21	0,03	2,50E-17	MGC45491	16898	MRPL14	129931	INTERGENIC	0,79	1
rs1418898	6	44027285	A	0,01	-2,60	0,31	7,27E-17	MGC45491	49031	MRPL14	162064	INTERGENIC	0,14	1
rs1418897	6	44027365	G	0,01	-2,60	0,31	7,37E-17	MGC45491	48951	MRPL14	161984	INTERGENIC	0,14	1
rs9296424	6	44043864	C	0,18	-0,49	0,06	9,98E-17	MGC45491	32452	MRPL14	145485	INTERGENIC	0,18	1
rs1631938	6	44060024	A	0,23	-0,20	0,02	1,74E-16	MGC45491	16292	MRPL14	129325	INTERGENIC	0,83	1
rs7761865	6	43976194	G	0,36	-0,51	0,06	2,21E-16	MGC45491	100122	VEGFA	113995	INTRONIC	0,10	1
rs6993770	8	106650704	T	0,32	-0,17	0,02	2,50E-16	ZFPM2	0	LRP12	980360	INTRONIC	0,97	1
rs9367181	6	44064593	G	0,20	-0,19	0,02	3,61E-16	MGC45491	11723	MRPL14	124756	INTERGENIC	0,98	0
rs1740074	6	44055768	G	0,06	-0,46	0,06	8,17E-16	MGC45491	20548	MRPL14	133581	INTERGENIC	0,44	1
rs11968152	6	43944908	A	0,04	1,16	0,14	1,18E-15	VEGFA	82709	MGC45491	131408	INTERGENIC	0,11	1
rs3932536	6	43944278	A	0,04	1,15	0,14	1,43E-15	VEGFA	82079	MGC45491	132038	INTERGENIC	0,11	1
rs6936668	6	43944048	C	0,04	1,15	0,14	1,45E-15	VEGFA	81849	MGC45491	132268	INTERGENIC	0,11	1
rs7832219	8	106648153	C	0,30	-0,16	0,02	1,72E-15	ZFPM2	0	LRP12	977809	INTRONIC	0,98	1
rs4734879	8	106652300	G	0,30	-0,16	0,02	1,78E-15	ZFPM2	0	LRP12	981956	INTRONIC	0,98	1
rs2343592	8	106641446	G	0,30	-0,16	0,02	1,98E-15	ZFPM2	0	LRP12	971102	INTRONIC	0,97	1
rs4734875	8	106623913	C	0,26	-0,16	0,02	9,46E-15	ZFPM2	0	LRP12	953569	INTRONIC	1,00	0
rs16873346	8	106618657	C	0,26	-0,16	0,02	1,07E-14	ZFPM2	0	LRP12	948313	INTRONIC	0,99	1
rs844294	6	44008685	C	0,51	-0,15	0,02	1,19E-14	MGC45491	67631	VEGFA	146486	INTRONIC	0,97	1
rs4236085	6	43971742	C	0,38	-0,43	0,06	1,27E-14	MGC45491	104574	VEGFA	109543	INTRONIC	0,12	1
rs16873402	8	106658423	T	0,33	-0,15	0,02	1,97E-14	ZFPM2	0	LRP12	988079	INTRONIC	0,99	1
rs910604	6	44061649	A	0,20	-0,18	0,02	2,15E-14	MGC45491	14667	MRPL14	127700	INTERGENIC	0,98	0
rs16873418	8	106661321	G	0,33	-0,15	0,02	2,28E-14	ZFPM2	0	LRP12	990977	INTRONIC	0,99	1
rs6931378	6	43982797	A	0,50	0,37	0,05	7,27E-14	MGC45491	93519	VEGFA	120598	INTRONIC	0,14	1
rs4416670	6	44058431	T	0,55	0,13	0,02	1,47E-12	MGC45491	17885	MRPL14	130918	INTERGENIC	0,99	0
rs1740077	6	44057885	C	0,56	0,13	0,02	5,21E-12	MGC45491	18431	MRPL14	131464	INTERGENIC	0,96	1
rs1776704	6	44057919	G	0,56	0,13	0,02	5,38E-12	MGC45491	18397	MRPL14	131430	INTERGENIC	0,96	1
rs16873365	8	106627411	T	0,21	-0,16	0,02	5,65E-12	ZFPM2	0	LRP12	957067	INTRONIC	0,92	1
rs1776706	6	44057965	C	0,56	0,13	0,02	5,79E-12	MGC45491	18351	MRPL14	131384	INTERGENIC	0,97	1
rs4320369	6	44058040	C	0,56	0,13	0,02	5,98E-12	MGC45491	18276	MRPL14	131309	INTERGENIC	0,97	1
rs1740079	6	44059217	T	0,56	0,13	0,02	6,02E-12	MGC45491	17099	MRPL14	130132	INTERGENIC	0,96	1
rs910612	6	44058684	T	0,44	-0,13	0,02	6,07E-12	MGC45491	17632	MRPL14	130665	INTERGENIC	0,97	1
rs910613	6	44058614	T	0,56	0,13	0,02	6,10E-12	MGC45491	17702	MRPL14	130735	INTERGENIC	0,97	1

rs910614	6	44058596	T	0,44	-0,13	0,02	6,12E-12	MGC45491	17720	MRPL14	130753	INTERGENIC	0,97	1
rs7013321	8	106662734	A	0,48	-0,14	0,02	6,75E-12	ZFPM2	0	LRP12	992390	INTRONIC	0,83	1
rs6993696	8	106650460	A	0,46	-0,13	0,02	8,54E-12	ZFPM2	0	LRP12	980116	INTRONIC	0,99	1
rs4741755	9	2657929	C	0,30	-0,23	0,03	2,26E-11	VLDLR	13444	KCNV2	49596	INTERGENIC	0,37	1
rs16873287	8	106597151	G	0,30	-0,13	0,02	5,13E-11	ZFPM2	0	LRP12	926807	INTRONIC	1,02	0
rs16873291	8	106597206	T	0,30	-0,13	0,02	5,30E-11	ZFPM2	0	LRP12	926862	INTRONIC	1,02	0
rs12676726	8	106597884	C	0,30	-0,13	0,02	5,44E-11	ZFPM2	0	LRP12	927540	INTRONIC	1,01	1
rs9472147	6	43944737	A	0,17	-0,49	0,07	5,78E-11	VEGFA	82538	MGC45491	131579	INTERGENIC	0,12	1
rs10105733	8	106595037	G	0,29	-0,14	0,02	6,09E-11	ZFPM2	0	LRP12	924693	INTRONIC	1,01	1
rs4734119	8	106599699	G	0,30	-0,13	0,02	7,33E-11	ZFPM2	0	LRP12	929355	INTRONIC	1,01	0
rs1157141	8	106600486	G	0,30	-0,13	0,02	7,33E-11	ZFPM2	0	LRP12	930142	INTRONIC	1,01	1
rs1157142	8	106600850	A	0,30	-0,13	0,02	7,34E-11	ZFPM2	0	LRP12	930506	INTRONIC	1,01	1
rs6996138	8	106601653	G	0,30	-0,13	0,02	7,35E-11	ZFPM2	0	LRP12	931309	INTRONIC	1,01	1
rs4734873	8	106602506	A	0,30	-0,13	0,02	7,36E-11	ZFPM2	0	LRP12	932162	INTRONIC	1,01	0
rs7001868	8	106602795	G	0,30	-0,13	0,02	7,38E-11	ZFPM2	0	LRP12	932451	INTRONIC	1,01	1
rs10094510	8	106592361	A	0,29	-0,13	0,02	8,33E-11	ZFPM2	0	LRP12	922017	INTRONIC	1,02	0
rs7007968	8	106591021	G	0,29	-0,13	0,02	9,27E-11	ZFPM2	0	LRP12	920677	INTRONIC	1,02	1
rs16873231	8	106587411	G	0,29	-0,13	0,02	1,36E-10	ZFPM2	0	LRP12	917067	INTRONIC	1,01	1
rs6997293	8	106587104	C	0,29	-0,13	0,02	1,47E-10	ZFPM2	0	LRP12	916760	INTRONIC	1,01	1
rs1868650	8	106587088	C	0,29	-0,13	0,02	1,52E-10	ZFPM2	0	LRP12	916744	INTRONIC	1,01	1
rs1868649	8	106587041	A	0,29	-0,13	0,02	1,57E-10	ZFPM2	0	LRP12	916697	INTRONIC	1,01	1
rs2291192	8	106586248	A	0,29	-0,13	0,02	1,77E-10	ZFPM2	0	LRP12	915904	INTRONIC	1,01	1
rs12678719	8	106585230	G	0,29	-0,13	0,02	1,95E-10	ZFPM2	0	LRP12	914886	INTRONIC	1,01	1
rs10093110	8	106634590	A	0,43	-0,12	0,02	2,18E-10	ZFPM2	0	LRP12	964246	INTRONIC	0,99	0
rs6988664	8	106582477	G	0,29	-0,13	0,02	2,33E-10	ZFPM2	0	LRP12	912133	INTRONIC	1,01	1
rs4734869	8	106582004	A	0,29	-0,13	0,02	2,60E-10	ZFPM2	0	LRP12	911660	INTRONIC	1,01	0
rs910611	6	44058829	C	0,08	-0,26	0,04	2,61E-10	MGC45491	17487	MRPL14	130520	INTERGENIC	0,77	1
rs748785	9	2684347	C	0,13	0,24	0,04	1,01E-09	KCNV2	23178	VLDLR	39862	INTERGENIC	0,52	1
rs7767854	6	44065304	T	0,11	-0,22	0,04	1,02E-09	MGC45491	11012	MRPL14	124045	INTERGENIC	0,74	1
rs6995272	8	106662838	T	0,46	0,14	0,02	1,98E-09	ZFPM2	0	LRP12	992494	INTRONIC	0,66	1
rs12675041	8	106598221	A	0,34	-0,12	0,02	2,10E-09	ZFPM2	0	LRP12	927877	INTRONIC	0,96	1
rs1740080	6	44060390	A	0,12	-0,18	0,03	2,76E-09	MGC45491	15926	MRPL14	128959	INTERGENIC	0,93	1

rs910606	6	44061307	A	0,12	-0,18	0,03	2,90E-09	MGC45491	15009	MRPL14	128042	INTERGENIC	0,94	0
rs1631662	6	44061336	T	0,12	-0,18	0,03	2,93E-09	MGC45491	14980	MRPL14	128013	INTERGENIC	0,94	1
rs2051074	6	44056431	T	0,04	-0,41	0,07	5,80E-09	MGC45491	19885	MRPL14	132918	INTERGENIC	0,46	1
rs9381262	6	43983427	A	0,45	-0,29	0,05	6,78E-09	MGC45491	92889	VEGFA	121228	INTRONIC	0,14	1
rs1450163	8	106601298	C	0,37	-0,11	0,02	1,11E-08	ZFPM2	0	LRP12	930954	INTRONIC	1,00	1
rs7836542	8	106603619	T	0,37	-0,11	0,02	1,13E-08	ZFPM2	0	LRP12	933275	INTRONIC	1,01	0
rs16897113	6	44076194	T	0,03	0,62	0,11	1,34E-08	MGC45491	122	MRPL14	113155	UPSTREAM	0,29	1
rs2343595	8	106660383	G	0,45	-0,11	0,02	2,26E-08	ZFPM2	0	LRP12	990039	INTRONIC	1,01	1
rs16873415	8	106660978	G	0,45	-0,11	0,02	2,39E-08	ZFPM2	0	LRP12	990634	INTRONIC	1,01	0
rs1901061	8	106619426	A	0,39	0,11	0,02	3,08E-08	ZFPM2	0	LRP12	949082	INTRONIC	0,94	1
rs1349319	8	106625810	A	0,39	0,11	0,02	3,59E-08	ZFPM2	0	LRP12	955466	INTRONIC	0,94	1
rs9369430	6	43942229	G	0,16	0,24	0,04	3,68E-08	VEGFA	80030	MGC45491	134087	INTERGENIC	0,33	1
rs748786	9	2684436	T	0,12	0,23	0,04	3,91E-08	KCNV2	23089	VLDLR	39951	INTERGENIC	0,52	1
rs4734122	8	106629546	A	0,41	0,11	0,02	4,15E-08	ZFPM2	0	LRP12	959202	INTRONIC	0,99	1
rs1375955	8	106624215	T	0,39	0,11	0,02	4,30E-08	ZFPM2	0	LRP12	953871	INTRONIC	0,94	1
rs12679049	8	106598022	G	0,22	-0,12	0,02	4,75E-08	ZFPM2	0	LRP12	927678	INTRONIC	0,99	0

CA: coded allele; CAF: coded allele frequency; Chr: chromosome; KCNV2: potassium voltage-gated channel subfamily V, member 2; LRP12: low-density lipoprotein receptor-related protein gene; MCG45491: uncharacterized protein, also known as C6orf223; MRPL14: mitochondrial ribosomal protein L14; O/E ratio: observed over expected ratio (measuring imputation accuracy); p: p-value; SE: standard error; SNP: single nucleotide polymorphism; VEGF: vascular endothelial growth factor; VLDLR: very low density lipoprotein receptor; ZFPM2: zinc finger protein, multitype 2

Online Table III: Test of heterogeneity between studies in the meta-analyses combining discovery and replication cohorts

SNP	Chr	$p_{heterogeneity}$	
		Inverse variance weighted meta-analysis (FHS+PIVUS)	Effective sample size weighted meta-analysis (FHS+SFS+PIVUS)
rs6921438	6	1	5.50×10^{-21}
rs4513773	6	0.52	0.19
rs9472159	6	5.70×10^{-3}	6.07×10^{-18}
rs9369434	6	4.32×10^{-15}	7.74×10^{-26}
rs1776717	6	0.18	0.69
rs1776721	6	0.31	0.068
rs1886979	6	0.90	0.20
rs9472155	6	0.20	0.056
rs844294	6	0.71	0.14
rs4416670	6	0.10	0.23
rs910611	6	0.52	0.13
rs6993770	8	0.087	0.099
rs16873402	8	0.033	0.011
rs16873365	8	0.052	0.022
rs7013321	8	1	0.41
rs6993696	8	0.67	0.39
rs16873291	8	0.069	0.098
rs1349319	8	0.66	0.70
rs10738760	9	0.14	2.20×10^{-3}
rs6475920	9	0.78	7.20×10^{-3}
rs4741756	9	0.11	9.84×10^{-4}
rs2375980	9	0.44	0.018
rs10122587	9	1	0.015
rs10967492	9	1	5.79×10^{-3}
rs10967470	9	1	0.016

$p_{heterogeneity}$: p-value for Cochran's Q-statistic for heterogeneity

Online Table IV: Secondary genetic association analysis adjusting for clinical covariates previously found to be associated with VEGF levels

SNPID	Chr	Position	p (FHS)	p (PIVUS)	p (SFS)	Meta-p (FHS+PIVUS) *	Meta-p (all) †
Model B							
rs6921438	6	44033585	$1.72x10^{-506}$	NA	$1.84x10^{-39}$	$1.72x10^{-506}$	$1.06x10^{-524}$
rs4513773	6	44033504	$1.58x10^{-482}$	$7.98x10^{-139}$	NA	$4.41x10^{-619}$	$1.28x10^{-584}$
rs9472159	6	44027673	$2.89x10^{-452}$	$2.90x10^{-109}$	$3.58x10^{-35}$	$8.76x10^{-558}$	$7.76x10^{-553}$
rs9369434	6	44026385	$8.70x10^{-443}$	$3.02x10^{-63}$	$5.81x10^{-28}$	$1.54x10^{-490}$	$2.23x10^{-496}$
rs1776717	6	44059314	$1.23x10^{-19}$	$2.27x10^{-4}$	$8.96x10^{-6}$	$2.59x10^{-22}$	$8.45x10^{-27}$
rs1776721	6	43998961	$1.17x10^{-19}$	$4.34x10^{-8}$	0.018	$5.02x10^{-26}$	$3.12x10^{-26}$
rs1886979	6	44012879	$3.23x10^{-19}$	$2.71x10^{-6}$	0.013	$4.82x10^{-24}$	$1.29x10^{-24}$
rs9472155	6	44005705	$4.98x10^{-19}$	$4.83x10^{-9}$	0.014	$3.39x10^{-26}$	$1.68x10^{-26}$
rs844294	6	44008685	$1.34x10^{-14}$	$1.95x10^{-5}$	0.087	$1.41x10^{-18}$	$2.50x10^{-18}$
rs4416670	6	44058431	$2.04x10^{-12}$	0.1	$2.79x10^{-4}$	$1.95x10^{-12}$	$2.81x10^{-15}$
rs910611	6	44058829	$4.77x10^{-10}$	$4.72x10^{-6}$	0.11	$1.43x10^{-14}$	$2.99x10^{-14}$
rs6993770	8	106650704	$2.03x10^{-16}$	$3.61x10^{-8}$	0.016	$1.94x10^{-22}$	$2.96x10^{-23}$
rs16873402	8	106658423	$1.45x10^{-14}$	$1.16x10^{-8}$	0.14	$9.09x10^{-21}$	$3.86x10^{-20}$
rs16873365	8	106627411	$6.93x10^{-12}$	$2.10x10^{-6}$	0.37	$5.14x10^{-16}$	$2.91x10^{-15}$
rs7013321	8	106662734	$4.66x10^{-12}$	NA	0.013	$4.66x10^{-12}$	$2.84x10^{-13}$
rs6993696	8	106650460	$8.83x10^{-12}$	$1.57x10^{-4}$	0.040	$6.67x10^{-15}$	$1.82x10^{-15}$
rs16873291	8	106597206	$5.43x10^{-11}$	$1.42x10^{-6}$	0.061	$1.76x10^{-15}$	$6.60x10^{-16}$
rs1349319	8	106625810	$2.93x10^{-8}$	$1.73x10^{-3}$	0.040	$2.03x10^{-10}$	$3.05x10^{-11}$
rs10738760	9	2681186	$1.17x10^{-34}$	$3.24x10^{-9}$	0.035	$6.36x10^{-42}$	$2.26x10^{-40}$
rs6475920	9	2663933	$2.23x10^{-32}$	$1.46x10^{-8}$	0.022	$2.21x10^{-39}$	$3.30x10^{-38}$
rs4741756	9	2658187	$2.29x10^{-31}$	$7.05x10^{-5}$	0.097	$2.73x10^{-34}$	$2.65x10^{-32}$
rs2375980	9	2682622	$1.53x10^{-27}$	$8.92x10^{-9}$	0.022	$1.10x10^{-34}$	$5.61x10^{-34}$
rs10122587	9	2681951	$3.89x10^{-24}$	NA	0.026	$3.89x10^{-24}$	$6.74x10^{-24}$
rs10967492	9	2671175	$1.86x10^{-21}$	NA	0.11	$1.86x10^{-21}$	$2.36x10^{-20}$
rs10967470	9	2665698	$2.19x10^{-21}$	NA	0.044	$2.19x10^{-21}$	$4.96x10^{-21}$
Model C							
rs6921438	6	44033585	$3.73x10^{-507}$	NA	$3.54x10^{-32}$	$3.73x10^{-507}$	$1.22x10^{-521}$
rs4513773	6	44033504	$4.54x10^{-484}$	$1.13x10^{-143}$	NA	$1.93x10^{-625}$	$3.75x10^{-589}$
rs9472159	6	44027673	$5.87x10^{-454}$	$6.67x10^{-113}$	$3.49x10^{-30}$	$3.65x10^{-563}$	$7.09x10^{-556}$
rs9369434	6	44026385	$4.64x10^{-445}$	$3.27x10^{-65}$	$1.76x10^{-26}$	$8.50x10^{-495}$	$1.18x10^{-504}$
rs1776717	6	44059314	$8.01x10^{-20}$	$5.20x10^{-4}$	$2.77x10^{-5}$	$5.03x10^{-22}$	$4.48x10^{-26}$
rs1776721	6	43998961	$1.05x10^{-19}$	$5.89x10^{-9}$	0.025	$8.84x10^{-27}$	$4.80x10^{-27}$
rs1886979	6	44012879	$2.65x10^{-19}$	$2.12x10^{-6}$	$2.84x10^{-3}$	$3.12x10^{-24}$	$6.44x10^{-26}$
rs9472155	6	44005705	$5.96x10^{-19}$	$1.33x10^{-9}$	0.010	$1.42x10^{-26}$	$2.46x10^{-27}$
rs844294	6	44008685	$1.63x10^{-14}$	$1.95x10^{-5}$	0.041	$1.71x10^{-18}$	$5.41x10^{-19}$
rs4416670	6	44058431	$1.16x10^{-12}$	0.11	$1.49x10^{-4}$	$1.38x10^{-12}$	$1.48x10^{-15}$
rs910611	6	44058829	$3.37x10^{-10}$	$7.04x10^{-6}$	0.076	$1.39x10^{-14}$	$1.07x10^{-14}$
rs6993770	8	106650704	$2.11x10^{-16}$	$6.87x10^{-8}$	$2.67x10^{-3}$	$3.12x10^{-22}$	$2.95x10^{-24}$
rs16873402	8	106658423	$1.26x10^{-14}$	$1.75x10^{-8}$	0.043	$9.95x10^{-21}$	$2.63x10^{-21}$
rs16873365	8	106627411	$7.36x10^{-12}$	$2.02x10^{-6}$	0.26	$5.26x10^{-16}$	$6.72x10^{-16}$
rs7013321	8	106662734	$3.41x10^{-12}$	NA	$2.21x10^{-3}$	$3.41x10^{-12}$	$2.90x10^{-14}$

rs6993696	8	106650460	$6.23x10^{-12}$	$2.09x10^{-4}$	$2.57x10^{-3}$	$6.00x10^{-15}$	$6.22x10^{-17}$
rs16873291	8	106597206	$4.25x10^{-11}$	$9.77x10^{-7}$	0.031	$1.00x10^{-15}$	$1.04x10^{-16}$
rs1349319	8	106625810	$2.33x10^{-8}$	$1.61x10^{-3}$	$7.71x10^{-3}$	$1.51x10^{-10}$	$4.18x10^{-12}$
rs10738760	9	2681186	$1.91x10^{-34}$	$5.53x10^{-8}$	$9.24x10^{-3}$	$2.94x10^{-40}$	$7.37x10^{-41}$
rs6475920	9	2663933	$3.43x10^{-32}$	$9.54x10^{-8}$	$6.55x10^{-3}$	$2.33x10^{-38}$	$8.21x10^{-39}$
rs4741756	9	2658187	$5.22x10^{-31}$	$2.27x10^{-4}$	0.049	$2.71x10^{-33}$	$1.28x10^{-32}$
rs2375980	9	2682622	$1.15x10^{-27}$	$8.63x10^{-8}$	0.021	$1.01x10^{-33}$	$7.71x10^{-34}$
rs10122587	9	2681951	$4.10x10^{-24}$	NA	0.027	$4.11x10^{-24}$	$2.79x10^{-24}$
rs10967492	9	2671175	$2.39x10^{-21}$	NA	0.19	$2.39x10^{-21}$	$3.15x10^{-20}$
rs10967470	9	2665698	$2.66x10^{-21}$	NA	0.096	$2.66x10^{-21}$	$9.16x10^{-21}$
Model D							
rs6921438	6	44033585	$1.53x10^{-508}$	NA	$1.40x10^{-40}$	$1.53x10^{-508}$	$1.51x10^{-529}$
rs4513773	6	44033504	$2.35x10^{-485}$	$6.36x10^{-136}$	NA	$4.72x10^{-619}$	$1.67x10^{-585}$
rs9472159	6	44027673	$1.61x10^{-454}$	$4.22x10^{-108}$	$1.52x10^{-36}$	$8.63x10^{-559}$	$3.15x10^{-557}$
rs9369434	6	44026385	$6.46x10^{-445}$	$7.63x10^{-63}$	$2.70x10^{-29}$	$3.63x10^{-492}$	$3.43x10^{-501}$
rs1776717	6	44059314	$2.43x10^{-19}$	$2.96x10^{-4}$	$7.08x10^{-6}$	$6.53x10^{-22}$	$1.76x10^{-26}$
rs1776721	6	43998961	$7.12x10^{-20}$	$4.46x10^{-8}$	0.013	$3.12x10^{-26}$	$1.14x10^{-26}$
rs1886979	6	44012879	$1.54x10^{-19}$	$2.15x10^{-6}$	$7.09x10^{-3}$	$1.85x10^{-24}$	$2.03x10^{-25}$
rs9472155	6	44005705	$1.37x10^{-19}$	$4.11x10^{-9}$	$7.43x10^{-3}$	$7.78x10^{-27}$	$1.49x10^{-27}$
rs844294	6	44008685	$2.77x10^{-15}$	$1.48x10^{-5}$	0.048	$2.23x10^{-19}$	$1.65x10^{-19}$
rs4416670	6	44058431	$1.95x10^{-12}$	0.088	$2.05x10^{-4}$	$1.55x10^{-12}$	$1.74x10^{-15}$
rs910611	6	44058829	$3.19x10^{-10}$	$3.45x10^{-6}$	0.11	$7.40x10^{-15}$	$1.70x10^{-14}$
rs6993770	8	106650704	$5.19x10^{-16}$	$5.30x10^{-8}$	0.016	$7.22x10^{-22}$	$9.86x10^{-23}$
rs16873402	8	106658423	$3.72x10^{-14}$	$1.34x10^{-8}$	0.15	$2.91x10^{-20}$	$1.10x10^{-19}$
rs16873365	8	106627411	$2.38x10^{-11}$	$3.87x10^{-6}$	0.36	$2.99x10^{-15}$	$1.14x10^{-14}$
rs7013321	8	106662734	$8.07x10^{-12}$	NA	0.013	$8.07x10^{-12}$	$4.55x10^{-13}$
rs6993696	8	106650460	$1.36x10^{-11}$	$2.41x10^{-4}$	0.040	$1.52x10^{-14}$	$3.72x10^{-15}$
rs16873291	8	106597206	$9.39x10^{-11}$	$1.37x10^{-6}$	0.071	$3.23x10^{-15}$	$1.30x10^{-15}$
rs1349319	8	106625810	$3.87x10^{-8}$	$2.46x10^{-3}$	0.057	$3.66x10^{-10}$	$7.71x10^{-11}$
rs10738760	9	2681186	$1.36x10^{-35}$	$1.06x10^{-8}$	0.046	$3.32x10^{-42}$	$1.35x10^{-40}$
rs6475920	9	2663933	$9.18x10^{-33}$	$2.67x10^{-8}$	0.018	$1.68x10^{-39}$	$1.36x10^{-38}$
rs4741756	9	2658187	$9.04x10^{-32}$	$1.28x10^{-4}$	0.071	$2.35x10^{-34}$	$9.51x10^{-33}$
rs2375980	9	2682622	$3.07x10^{-28}$	$3.55x10^{-8}$	0.015	$1.02x10^{-34}$	$1.70x10^{-34}$
rs10122587	9	2681951	$5.73x10^{-25}$	NA	0.020	$5.73x10^{-25}$	$6.91x10^{-25}$
rs10967492	9	2671175	$3.72x10^{-22}$	NA	0.091	$3.72x10^{-22}$	$3.50x10^{-21}$
rs10967470	9	2665698	$5.48x10^{-22}$	NA	0.045	$5.48x10^{-22}$	$1.30x10^{-21}$
Model E							
rs6921438	6	44033585	$6.58x10^{-506}$	NA	$2.91x10^{-39}$	$6.58x10^{-506}$	$7.66x10^{-524}$
rs4513773	6	44033504	$6.46x10^{-482}$	$3.04x10^{-138}$	NA	$6.76x10^{-618}$	$1.66x10^{-583}$
rs9472159	6	44027673	$9.84x10^{-452}$	$4.86x10^{-110}$	$4.30x10^{-35}$	$4.42x10^{-558}$	$1.30x10^{-552}$
rs9369434	6	44026385	$2.44x10^{-442}$	$3.05x10^{-65}$	$5.33x10^{-28}$	$1.10x10^{-492}$	$6.10x10^{-498}$
rs1776717	6	44059314	$9.92x10^{-20}$	$2.53x10^{-4}$	$1.00x10^{-5}$	$2.40x10^{-22}$	$8.47x10^{-27}$
rs1776721	6	43998961	$5.41x10^{-20}$	$2.57x10^{-8}$	0.020	$1.47x10^{-26}$	$1.30x10^{-26}$
rs1886979	6	44012879	$2.55x10^{-19}$	$1.66x10^{-6}$	0.013	$2.41x10^{-24}$	$7.07x10^{-25}$
rs9472155	6	44005705	$1.74x10^{-19}$	$3.04x10^{-9}$	0.015	$7.84x10^{-27}$	$4.93x10^{-27}$
rs844294	6	44008685	$8.15x10^{-15}$	$1.39x10^{-5}$	0.086	$6.33x10^{-19}$	$1.20x10^{-18}$
rs4416670	6	44058431	$1.86x10^{-12}$	0.11	$2.89x10^{-4}$	$2.16x10^{-12}$	$3.14x10^{-15}$

rs910611	6	44058829	$2.35x10^{-10}$	$1.99x10^{-6}$	0.11	$3.34x10^{-15}$	$8.62x10^{-15}$
rs6993770	8	106650704	$1.34x10^{-16}$	$3.63x10^{-8}$	0.018	$1.27x10^{-22}$	$2.52x10^{-23}$
rs16873402	8	106658423	$1.24x10^{-14}$	$8.58x10^{-9}$	0.16	$6.38x10^{-21}$	$3.39x10^{-20}$
rs16873365	8	106627411	$8.10x10^{-12}$	$4.01x10^{-6}$	0.37	$9.32x10^{-16}$	$5.17x10^{-15}$
rs7013321	8	106662734	$3.64x10^{-12}$	NA	0.014	$3.64x10^{-12}$	$2.48x10^{-13}$
rs6993696	8	106650460	$5.82x10^{-12}$	$1.73x10^{-4}$	0.047	$4.78x10^{-15}$	$1.67x10^{-15}$
rs16873291	8	106597206	$3.14x10^{-11}$	$7.92x10^{-7}$	0.066	$6.56x10^{-16}$	$2.99x10^{-16}$
rs1349319	8	106625810	$3.53x10^{-8}$	$1.84x10^{-3}$	0.046	$2.60x10^{-10}$	$4.55x10^{-11}$
rs10738760	9	2681186	$6.77x10^{-35}$	$3.90x10^{-9}$	0.034	$4.65x10^{-42}$	$1.61x10^{-40}$
rs6475920	9	2663933	$1.85x10^{-32}$	$9.61x10^{-9}$	0.022	$1.20x10^{-39}$	$1.94x10^{-38}$
rs4741756	9	2658187	$1.32x10^{-31}$	$6.02x10^{-5}$	0.092	$1.26x10^{-34}$	$1.22x10^{-32}$
rs2375980	9	2682622	$7.94x10^{-28}$	$1.45x10^{-8}$	0.022	$9.69x10^{-35}$	$4.67x10^{-34}$
rs10122587	9	2681951	$1.47x10^{-24}$	NA	0.024	$1.47x10^{-24}$	$2.56x10^{-24}$
rs10967492	9	2671175	$6.75x10^{-22}$	NA	0.10	$6.76x10^{-22}$	$9.22x10^{-21}$
rs10967470	9	2665698	$9.41x10^{-22}$	NA	0.045	$9.41x10^{-22}$	$2.39x10^{-21}$

All analyses were adjusted for age, sex, and principal components. Model B was additionally adjusted for hypertension; model C for smoking; model D for central obesity; model E for the presence of a metabolic syndrome; *inverse variance meta-analysis; †effective sample size weighted meta-analysis

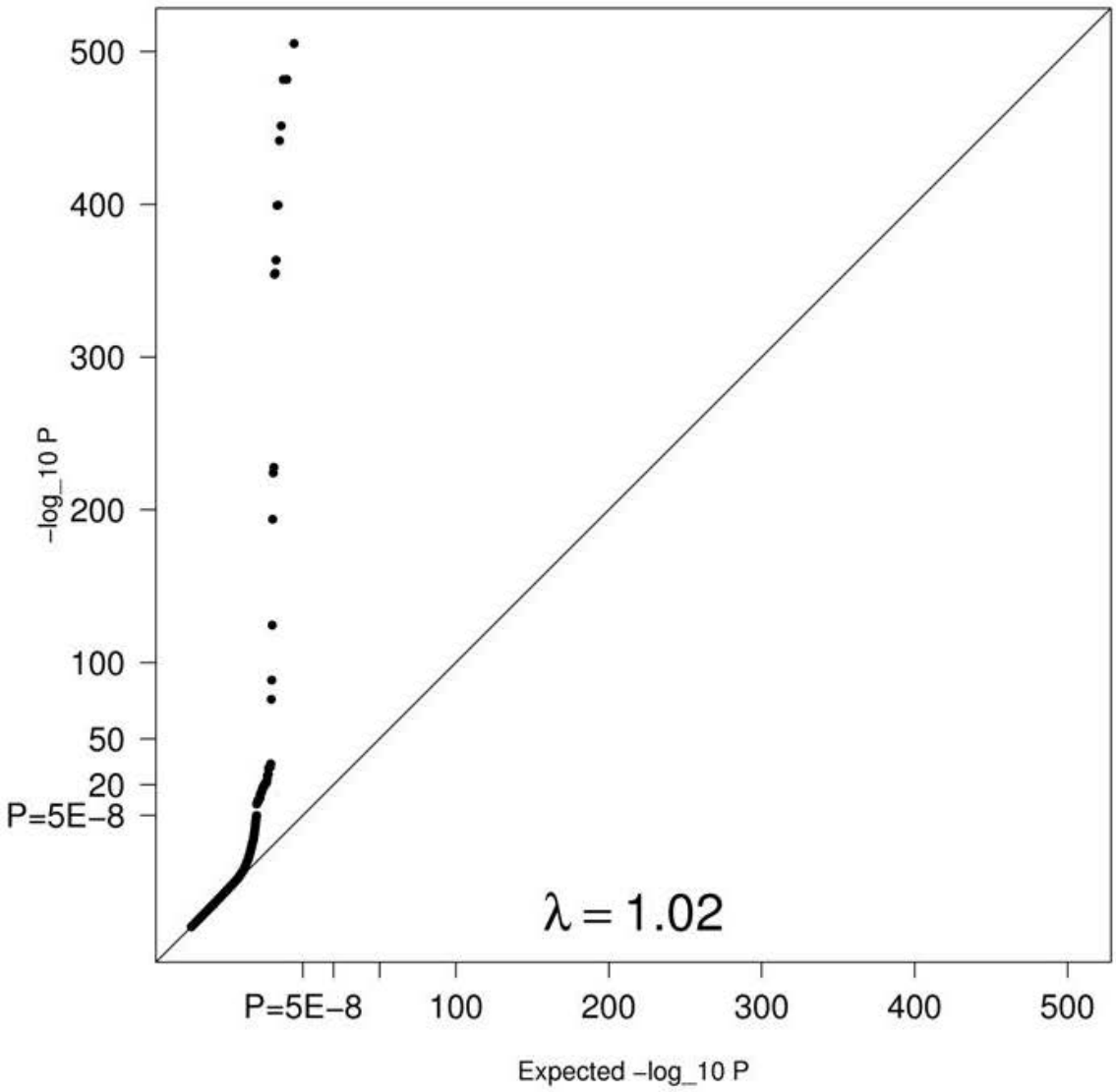
Online Table V: Candidate gene association studies of VEGF variants and circulating VEGF levels

Author	Population	N	Sample	SNP					
				rs699947	rs1570360*	rs833061	rs2010963*	rs3025039*	-2549 18bp I/D
Steffensen [22]	ovarian cancer patients	143	serum	C ↑ VEGF	NS	T ↑ VEGF	C ↑ VEGF	NS	-
Costa [16]	post-menopausal women	252	serum	-	-	-	NS	NS	-
Medford [17]	patients with (and at risk of) ARDS	70	plasma	-	-	-	-	NS	-
Kamoun [23]	Behcet patients and healthy controls	135	serum	-	-	-	NS	NS	D ↑ VEGF
Langsenlehner [18]	healthy persons	81	plasma	NS	-	NS	NS	NS	-
Petrovic [29]	PDR patients	104	serum	-	-	-	CC ↑ VEGF	-	-
Mateo [19]	AD patients and healthy controls	117	serum	NS	-	-	NS	-	-
Balasubramanian, [20]	post-menopausal women	62	serum and plasma	-	-	NS	NS	NS	-
Zhai [24]	ARDS patients	71	plasma	-	-	NS	NS	T ↓ VEGF	-
Berrahmoune [21]	community sample	647	plasma	-	-	NS	NS	NS	-
Ferrante [25]	IBD patients and healthy controls	1,142	serum	NS	GG ↑ VEGF	-	NS	NS	-
Krippl [26]	post-menopausal women	21	plasma	-	-	-	-	T ↓ VEGF	-
Awata [27]	healthy persons	64	serum	-	NS	NS	CC ↑ VEGF	NS	-
Renner [28]	healthy persons	23	plasma	-	-	-	-	T ↓ VEGF	-
Present study	**community sample**	**3,527**	**serum**	**C ↑ VEGF $p=2.33x10^{-7}$ (beta±SE= 0.13±0.02)**	**NA**	**T ↑ VEGF $p=3.05x10^{-7}$ (beta±SE= 0.12±0.02)**	**NA**	**NA**	**NA**

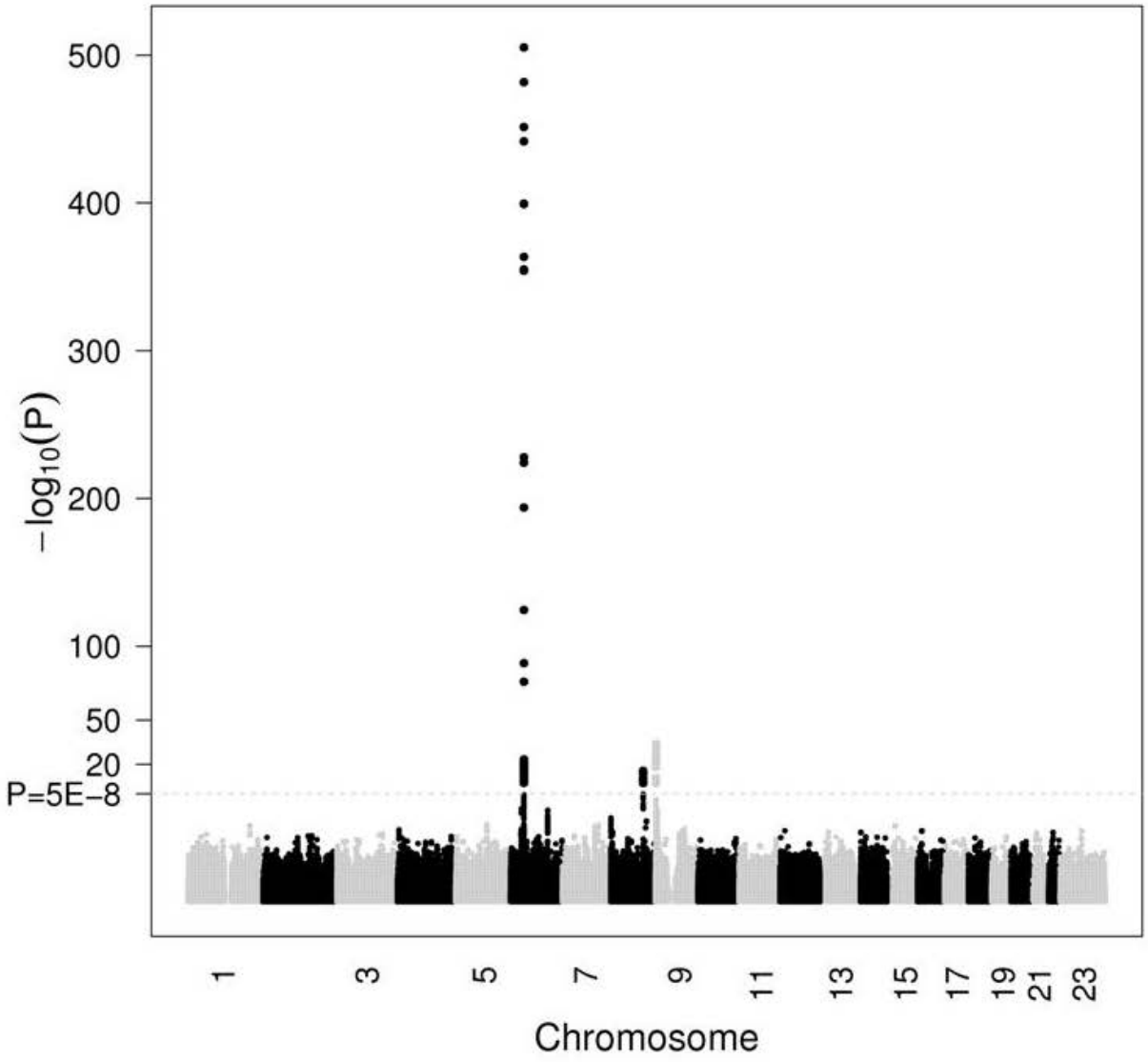
AD: Alzheimer Disease; ARDS: Acute Respiratory Distress Syndrome; IBD: Inflammatory Bowel Disease; HAM/TSP: HTLV-I- associated myelopathy/tropical spastic paraparesis; NS: non significant (no association); NA: not available; PDR = Proliferative Diabetic Retinopathy;
*these SNPs were not available in our GWAS and were nota in linkage disequilibrium with the VEGF SNPs yielding a genome-wide significant association with VEGF levels ($r^2 < 0.095$)

C. Supplemental Figures

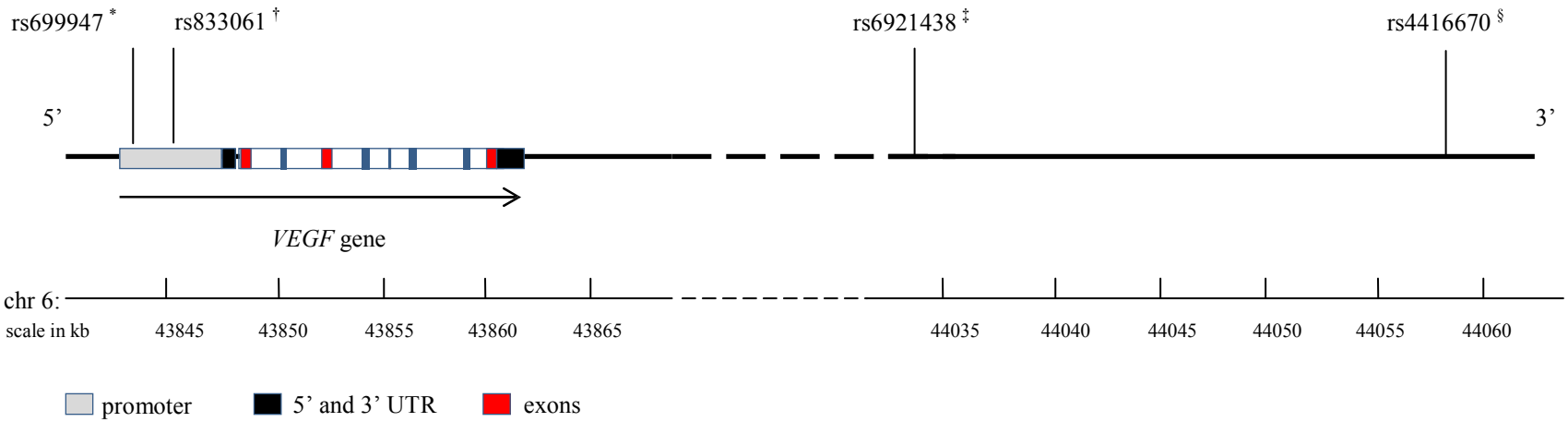
Online Figure I: Quantile-quantile QQ-plot showing the observed versus the expected p-values after meta-analysis for serum VEGF levels (the solid line shows the distribution under the null-hypothesis)



Online Figure II: Manhattan plot showing individual p-values against their genomic position for GWAS of serum VEGF levels. Within each chromosome (x-axis), results are plotted left to right from p-terminal end. Dashed line indicates preset threshold for genome-wide significance, p=5.0x10-8; solid line threshold for suggestive associations, $p=4.0x10^{-7}$



Online Figure III: Genomic organization of the VEGF gene and localization of the SNPs identified in the genetic association and transcriptomic studies



*chr6:43844367, p=2.33x10^{-7} replicates findings from previous association studies (Supplementary Table IV)
†chr6:43845464, p=3.05x10^{-7}, replicates findings from previous association studies (Supplementary Table IV)
‡ chr6:44033585, p=6.11x10^{-506}, top SNP, explains 41.2% of the phenotypic variance in the FHS
§ chr6:44058431, p=1.47x10^{-12}, associated with serum VEGF levels independently of rs6921438 in conditional GWAS, explains 1.5% of the phenotypic variance in the FHS

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Publication 4

Associations of vascular endothelial growth factor (VEGF) with adhesion and inflammation molecules in a healthy population.

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Running title: VEGF and adhesion and inflammation molecules

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Key words: VEGF, adhesion molecules, CRP, IL-6, TNF-α, PBMCs

Background: The relationships between VEGF and adhesion molecules and inflammatory markers have not been assessed in the healthy population.
Results: VEGF was associated with ICAM-1, Eselsectin, L-selectin, IL-6, TNF-α and CRP

Conclusion: There is a biological connection between VEGF and adhesion/inflammation molecules.

Significance: Common genetic and transcriptional mechanisms may link these molecules and control their effect in healthy conditions.

SUMMARY

Vascular endothelial growth factor (VEGF) is implicated in numerous pathologies through complex relationships with cellular adhesion molecules (CAM) and inflammation markers. These have not been assessed in non-pathological conditions. Our aim was the evaluation of associations between VEGF and CAM/inflammation molecules in a healthy population, and of possible genomic interplays in order to better apprehend the underlying mechanisms leading to the pathology. We examined the associations between VEGF and ICAM-1, VCAM-1, E-, L-, P-selectins, TNF-α, CRP and IL-6 plasma levels in 403 healthy individuals. Gene expression of CAM/inflammation molecules and VEGF isoforms (121, 145, 165, and 189) were quantified in peripheral blood mononuclear cells (PBMCs).The effect of 4 genetic variants (explaining ~50% of VEGF heritability) and of their interactions on plasma and mRNA levels of CAM/inflammation molecules was examined. VEGF was associated with ICAM-1 and E-selectin in plasma. In PBMCs, $VEGF_{145}$mRNA was associated with ICAM-1, L-selectin and TNF-α expression. Interactions of the genetic variants were shown to affect ICAM-1, E-selectin, IL-6 and TNF-α plasma levels, while rs4416670 was associated with L-selectin expression. These findings propose a biological connection between VEGF and CAM/inflammation markers. Common genetic and transcriptional mechanisms may link these molecules and control their effect in healthy conditions.

Cellular adhesion molecules (CAM) are cell membrane receptors that mediate several interactions known to play a key role in a variety of physiological and pathological conditions related to traffic and interactions between cells, cell-matrix contact and in determining the specificity of cell-cell binding (1). Their participation in the development of the atherogenic plaque is well described (2). Furthermore, atherosclerosis is characterized by chronic inflammation and inflammation markers have been associated with cardiovascular pathologies (3).

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that shows distinguished functions in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis (4,5). VEGF is a highly conserved, disulfide-bonded dimeric glycoprotein of 34 to 45 kDa and it is produced by several cell types including fibroblasts, neutrophils, endothelial cells and peripheral blood mononuclear cells (PBMCs) particularly T lymphocytes and macrophages (6). Six isoforms of human VEGF, which range from 121 to 206 amino acid residues, have been identified. Unlike $VEGF_{145}$ and $VEGF_{206}$ that are comparatively rare and seem restricted to placental cells, most VEGF-producing cells express the $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ isoforms. $VEGF_{183}$ also has a broad tissue expression and may have not been easily detected earlier due to confusion with $VEGF_{189}$ (4).
VEGF has been linked with a number of vascular pathologies including cardiovascular diseases (ischemic heart disease, heart failure, stroke). Elevated circulating VEGF levels have been also observed in several types of cancer and in various other disorders, including diabetes, cognitive decline and dementia, reproductive, immune-inflammatory disorders (7). Especially $VEGF_{145}$, has a key role during the vascularization of the human ovarian follicle and corpus luteum, in placentation and

embryonic period as well as in bone and wound healings (5, 8, 9).
What could be the cause of this wide diversity of VEGF functions throughout life? The existence of common molecular pathway(s) interplaying between VEGF and CAM/inflammation molecules could probably explain this broad range of VEGF functions.
Indeed, complex relationships have been observed between VEGF, CAM and inflammation markers in many diseases and especially in cardiovascular-related phenotypes. For instance, some studies reported the involvement of E-, P- and L-selectin, vascular cell adhesion molecule 1 (VCAM), intercellular adhesion molecule 1 (ICAM-1) and interleukin 6 (IL-6) in angiogenesis (10). Also, hypoglycemia has been shown to increase circulating levels of VEGF, IL-6, E-selectin and ICAM-1 in healthy individuals and in patients with type 1 diabetes (11). Moreover, it is noteworthy that several reports linked angiogenesis and inflammation, highlighting a key role of TNF-α (tumor necrosis factor- α) and CRP (12, 13). IL-6, that mediates inflammation, is a primary determinant of the hepatic production of CRP(14). Recent studies indicated the simultaneous increasing of IL-6 and VEGF circulating levels in some pathological conditions such as ovarian hyperstimulation syndrome induced ascites, diabetes, visceral obesity, cancer and hypertension (15-19). Furthermore, IL-6 seems to induce *VEGF* expression in diabetic nephropathy and rheumatoid arthritis (20, 21). Serum levels of soluble adhesion molecules have also been correlated with serum levels of some acute phase proteins such as C-reactive protein (CRP) in individuals with breast cancer (22). In addition, in patients with hepatic cancer, the levels of soluble P-selectin have been associated with plasma levels of $VEGF_{165}$ (23).

The assessment of these relationships in different pathologies is progressing; however, due to the complexity of the pathophysiological mechanisms implicated in a disease, the origin of these biological connexions is difficult to be interpreted. Therefore, their investigation in the healthy state, where there is no implication of evident pathology, could offer important information concerning the physiological connexions between these molecules before the development of a specific disease. This could also be the key for the better understanding of the VEGF biological roles. However, to our knowledge, there is no published study of the link between VEGF and both CAM and inflammation markers in a healthy population.

Therefore, the aim of this study was to examine the associations between VEGF and ICAM-1, VCAM-1, E-, L- and P-selectins, TNF-α, IL-6 and CRP in plasma of supposed healthy individuals. Furthermore, we seek to investigate whether there is a relationship in expression profile of these molecules in peripheral blood mononuclear cells (PBMCs). Finally, we thought to assess possible genetic links between these molecules. We have recently identified four single nucleotide polymorphisms (SNPs) (rs6921438, rs4416670, rs6993770 and rs10738760) explaining ~50% of VEGF circulating levels heritability [7]. Thus, we aimed to determine possible associations of these SNPs with plasma levels of CAM/inflammation markers, as well as their effect on gene expression in PBMCs.

Materials and Methods:

Study population: The STANISLAS Family Study (SFS) is a 10-year longitudinal survey involving 1,006 volunteer families from Vandoeuvre-lès-Nancy, France between 1993-1995 (24). Individuals with chronic disorders (cardiovascular diseases or cancer) or having a personal history of cardiovascular disease were not included, as the aim of the study was the assessment of genetic susceptibility factors on the variability of intermediate phenotypes in physiological conditions without the influence of any long term medication and disease. The study protocol was approved by the Local Ethics Committee of Nancy and all subjects gave written informed consent for their participation in the study. Four hundred and three unrelated adults collected during the second examination of the SFS were involved in the present study for which all data were available.

Laboratory measurements: Blood samples were collected after overnight fasting. Serum and plasma samples were separated by centrifugation at 2000×g for 15 min.

Circulating plasma levels quantification was performed by Randox Ltd (Crumlin, UK) using a biochip array analyser (Evidence ®) (25).
Plasma IL-6, TNF-α, ICAM-1, E-, L- and P-selectins were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK) according to the manufacturer's instructions. Concentrations of plasma CRP were determined using the BN II nephelometer (Germany).
Before the quantification of CAM and inflammation molecules, we tested the effect of the procedures of storage and sample collection on their concentrations. Fresh and frozen state had no significant effect on the concentration of the different molecules except from VCAM-1, which was less stable. Thus it was not included in the analyses of the present study.

Gene expression analysis: Total RNAs were extracted from PBMCs with a MagNaPure automate, using the MagNA Pure LC RNA HP isolation kit and RNA HP Blood External lysis protocol [Roche Diagnostics, France]. Reverse transcription of total RNAs were performed using 200 units of M-MuLV Reverse Transcriptase with 0.25 µg of oligos(dT) (Promega, France) according to a previously described protocol (26). Quantification of the transcripts coding for the VEGF isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$), ICAM-1, E-, L-, P-selectins, TNF-α, IL-6 and the beta 2 microglobulin (β2M) control gene were performed using TaqMan® and LightCycler technologies (LC TaqMan Master Kit, Roche Diagnostics, France). All experiments were performed in duplicate. RT-PCR optimization and specificity of Real Time-PCR products were conducted using SYBR® Green technology (LC FastStart DNA Master^{PLUS} SYBR Green I kit, Roche Diagnostic, France), melting curves analysis and agarose gel electrophoresis of the PCR amplicons, as previously described [26]. $VEGF_{183}$, $VEGF_{206}$, E-selectin and CRP expression in PBMCs were not quantified due to their insufficient expression in this cell type. In total, available mRNA data existed for a subsample of 65 subjects. All mRNA levels were normalized to the mRNA levels of β2M gene.

Genotyping: SNPs rs6921438, rs4416670, rs6993770, and rs10738760 were genotyped by Genoscreen (http://genoscreen.fr), using a Sequenom iPLEX Gold assay–Medium Throughput Genotyping Technology (27).

Statistical analysis: Continuous variables are presented as mean value ± standard deviation and categorical variables are given in percentages. Hardy-Weinberg equilibrium was tested using the chi-square test. All molecules blood concentrations were log-transformed to normalize their distribution. Similar transformation was performed for mRNA values for all variables with exception of $VEGF_{165}$ and $VEGF_{189}$ whose values were normally distributed and IL-6 whose values were ln-transformed to achieve normal distribution.

Linear regression models adjusted for age, gender and body mass index (BMI) were used to test possible associations between VEGF related SNPs and plasma levels of the adhesion/inflammation molecules. Similar linear regression models adjusted for age and gender and BMI were applied for the assessment of associations between VEGF mRNA levels and mRNA levels of these molecules and between the four SNPS and blood levels and mRNA levels of the assessed markers.
For the SNPs associations analyses an additive model was used. Results are presented using the minor allele as reference allele.
The assessment of gene*gene epistatic interactions was tested using all possible 2 × 2 combinations between the four SNPS. In the regression models adjusted for age, gender and BMI, two SNPS and their interactions term were added in order to test the association of these interactions with adhesion/inflammation molecules plasma levels only. Epistatic interactions were not assessed for associations with mRNA levels due to the small sample size with available transcriptomic data and the low statistical power to assess this type of interactions.
All analyses were performed using PLINK 1.07
(http://pngu.mgh.harvard.edu/purcell/plink)
(28) and the SPSS 16.0 (SPSS, Inc, Chicago, Illinois) statistical software. Significance was determined at a two-tailed P=0.05 level.

Results

The general characteristics of the studied population and of the four VEGF-related SNPs are presented in Tables 1 and 2 respectively.

Associations of VEGF plasma levels with plasma levels of CAM and inflammation molecules: VEGF plasma levels were associated with ICAM-1 levels in regression models adjusted for age, gender and BMI (beta=0.043, *P*=0.023) and with E-selectin (beta=0.085, *P*=0.039) (Table 3).

Associations of VEGF isoforms mRNA and gene expression of CAM and inflammation molecules: We then assessed the association between four VEGF isoforms and gene expression of ICAM-1, P- and L-selectin, TNF-α, and IL-6 in PBMCs. In regression models adjusted for age, gender and BMI, $VEGF_{145}$ isoform was significantly associated with ICAM-1 mRNA (beta=0.353, *P*=0.021), L-selectin mRNA (beta=0.611, *P*=0.001) and TNF-α mRNA (beta=0.616, *P*=0.001) (Table 3). The other VEGF isoforms were not significantly associated with gene expression of the assessed molecules.

Associations of rs6921438, rs4416670, rs6993770, rs10738760 and their epistatic interactions with plasma levels and transcripts of CAM and inflammation molecules and epistatic interactions: Significant epistatic interactions were identified for E-selectin, TNF-α, ICAM-1 and IL-6 levels (Table 4, Supplementary Table 1). A significant association was observed between rs4416670 and mRNA levels of L-selectin (beta=0.196, *P*=0.039). No other significant association of the genetic variants with mRNA levels was observed.

Discussion

In the present study, we assessed the relationship between VEGF and CAM and inflammation molecules in healthy state conditions and by a multi-dimensional approach: associations in plasma, in gene expression in PBMCs, common genetic determinants and possible functional effects of these determinants on gene expression. In all these levels, significant results that may explain the physiological relationships between these molecules were observed.

VEGF plasma levels were positively associated with ICAM-1 and E-selectin. To the best of our knowledge, this is the first study to report a relationship between VEGF, the main angiogenic factor, ICAM-1, the mediator of leukocytes firmed-adhesion to endothelial cells and E-selectin, the mediator of leukocytes rolling on vascular endothelium (29). CAM have a pivotal role in angiogenesis (1). They regulate angiogenesis both indirectly and directly [30]. Among the soluble adhesion molecules, E-selectin and ICAM-1, have been shown to be directly angiogenic [31]. Therefore, the observed association of these adhesion molecules with VEGF could be explained by common angiogenic activities present already in healthy conditions.

In contrary, no association was found with L- and P-selectins and with any of the inflammatory markers and VEGF. Unlike E-selectin which is transcriptionally regulated in endothelial cells, P-selectin is translocated within minutes after the inflammation stimuli from intracellular granules and promotes the immediate reattachment and quick rolling of leukocytes over vascular surfaces (32). Also, L-selectin is cleaved rapidly from the surface of activated leukocytes (33). Therefore, in a healthy state, where inflammation process is not evident, these two molecules may not have an important role in the physiological interconnections, with VEGF. The absence of associations of VEGF with inflammation markers, further support this hypothesis. CRP has been previously shown not to be associated with VEGF levels in a healthy population (34). In the current study we confirmed this finding and extent it to other inflammation molecules as well. These results could be explained by the fact that the study population is healthy, thus, the relationship between VEGF and inflammation markers could be consequence of the pathology, such as vascular dysfunction (e.g. in individuals with diabetes mellitus) (35).

Apart from the associations in the level of plasma concentrations, we examined possible similarities in the expression profile of CAM/inflammation molecules in PBMCs with VEGF. This cell type is easily accessible and contain cell types (lymphocytes and monocytes) that interact with different tissues, thereby probably acting as ‘sensors’

differentiating individuals with CVDs risk, especially for the pro-inflammatory state (36, 37). We demonstrated that $VEGF_{145}$ isoform mRNA is positively associated with ICAM-1, L-selectin and TNF-α expression. These associations could indicate the existence of common transcriptional factors or other molecular mechanisms that regulate the expression of these molecules in PBMCs in non-pathological situations.

The regulation of VEGF levels occurs at the gene transcriptional, translational, and post-translation levels. Upon binding of VEGF to its corresponding receptor (VEGFR2) in endothelial cells, three main pathways promote cell proliferation, migration, survival and nitric oxide production (29). These pathways included the PI 3'-kinase (phosphoinositide) /Akt (protein kinase b) cascade, the tyrosine phosphorylation of PLC gamma (phospholipase c), and the MAPK/ERK (mitogen-activated protein kinase/extracellular-regulated kinase) cascade. Among these regulatory pathways the stimulation of PLC gamma activates the protein kinase C (PKC) (29) and then leads to activation of NF-kB transcription factor [38]. A growing body of evidence indicates that the activation of NF-kB induces expression of ICAM-1 transcripts in endothelial cells (39-41). In agreement with this concept, Kim and colleagues (29) have examined the signal transduction mechanisms by which VEGF induces adhesion molecules in human umbilical vein endothelial cells (HUVECs). Indeed, they have shown that VEGF-induced expression of ICAM-1 is mediated mainly through NF-kB activation. Interestingly, Kiriakidis and colleagues (6) have documented the involvement of the transcriptional factor NF-kB in the up-regulation of VEGF in human macrophages to lipopolysaccharide-induced response. Also, it regulates many of the genes involved in immune and inflammatory responses (42). Concerning TNF-α, it has been shown to induce VEGF production via the stimulation of its corresponding transcription factor, HIF1-α [43]. Westra and colleagues (44) have shown that blocking of TNF-α reduces TNF-α induced VEGF production. Further studies have documented that TNF-α up regulates VEGF production by synovial membrane cells and PBMCs of patients with rheumatoid arthritis (45, 46). It has also been shown that NF-kB is partly responsible for the up-regulation of VEGF mRNA expression in response to TNF-α (47). The study of Zhou and colleagues(48) indicates that trophoblasts produce leukocyte like-L-selectin. Based on this finding, the functional L-selectin of trophoblast stimulates the cytotrophoblast differentiation, induces vasculogenesis via VEGF and facilitates the leukocyte emigration from the blood into tissues. Waddell and colleagues [49] have shown that ligation of L-selectin by different antibodies identifying separate epitopes resulted to increased tyrosine phosphorylation of several cellular proteins with dominant bands at 40-42, 55-60, 70-72, and 105-120 kDa. Surprisingly, according to mentioned study the 42-kDa band, which matches with the range of VEGF bands (5,8), co-migrated with mitogen-activated protein kinase/extracellular-regulated kinase (MAPK). MAP kinase has been directly linked to long term responses, such as proliferation (49). As we previously mentioned, after VEGF binding to VEGFR2in endothelial cells, MAPK/ERK cascade is one of the three main pathways that promote cell proliferation, migration and survival (29). Ancelin and colleagues [50] have speculated the influence of polymorphonuclear neutrophil-drived –VEGF (PMND-VEGF) on L-selectin expression while all VEGF isoforms down regulated L-selectin in this cell type (PMN). L-selectin, as mentioned before, is cleaved rapidly from the surface of activated leukocytes and also undergoes slower constitutive shedding in inactivated cells (33). Based on Ancelin study (50), PMND- VEGF can modulate polymorphonuclear neutrophils migration. In fact, they have proposed an autocrine amplification mechanism that would allow permanent VEGF release to occur at inflammation sites which may contribute to both normal and pathological angiogenesis [50]. These *in vitro* findings and our results in healthy individuals' PBMCs support the hypothesis that connections mechanisms between these molecules are present and act in synergy already in healthy conditions. It is thus important to deeper investigate them, in order to better understand which disregulations are leading to the development of a pathology.

Concerning IL-6, Hao and colleagues [51] have illustrated that VEGF; via vascular endothelial growth factor receptor 2- protein kinase D (VEGFR2-PKD) axis induces the production of some proinflammatory cytokines such as IL-

6 in endothelial cells but not in leukocytes. This could probably explain the lack of association between VEGF and IL-6 expression in PBMCs. Also, as mentioned before, P-selectin's reaction in inflammation is not trancriptionally regulated (32). Thus, the absence of common expression profile between VEGF and P-selectin could be explained in non-pathological state.
It is important also to mention that the expression of a gene in one cell type does not reflect the total concentration in plasma. Therefore, the associations between VEGF, L-selectin and TNF-α mRNA levels are not expected to be necessarily present in plasma. Our transcriptomic approach showed the utility of this epidemiological model in identifying unsuspected until now relationships of these molecules in healthy conditions.
Furthermore, very interestingly, only $VEGF_{145}$ isoform mRNA showed associations with the CAM/inflammation molecules expression. $VEGF_{145}$ binds to heparin but behaves like $VEGF_{121}$ with regard to its receptor recognition ability. Unlike $VEGF_{121}$ and $VEGF_{165}$, it binds to a basement membrane like extracellular matrix in a biologically active form. It also has angiogenic effects similar to $VEGF_{121}$ and $VEGF_{165}$. It appears that $VEGF_{145}$, in contrast to $VEGF_{121}$ and $VEGF_{165}$, induces endothelial cell proliferation (52). Also, among VEGF isoforms, $VEGF_{145}$ is predominantly expressed in reproductive tissues and in several tumor cell lines derived from female reproductive organs as well as in PBMCs of acute myeloid leukemia patients (52, 53). These findings highlight the importance of $VEGF_{145}$ in both vascular and malignant diseases and in combination with our results in healthy population, may give new directions for further research concerning the implication of this isoform in the development of cardiovascular and cancer pathologies.

Finally, as we have previously identified 4 SNPs that explain a large percentage of VEGF plasma levels heritability in healthy individuals, we sought to examine whether these SNPs could have an effect on CAM/inflammation traits and whether they could be associated with their gene expression in PBMCs. Significant epistatic interactions were revealed. In particular, an interaction between rs6993770 (TA genotype and T allele) and rs10738760 (G allele) was associated with increased levels of ICAM-1 and E-selectin. This result further supports the associations of VEGF with these molecules (especially ICAM-1) in plasma and expression levels. We also demonstrated multiple epistatic interactions between the VEGF-related SNPs and IL-6 plasma levels. Specifically, the T allele of rs6993770 interacts with the C allele of rs4416670 and the A allele of rs6921438 to increase IL-6 levels. These two latter SNPs show a similar positive interaction with rs10738760. A complex but common genetic regulation between VEGF and IL-6 is thus speculated. However, as no association was found for IL-6 and VEGF in plasma concentrations and in expression levels, the effect of SNPS on IL-6 is likely to be independent from VEGF. Moreover, a significant interaction was detected between rs4416670 and rs6921438 with a positive effect on TNF-α plasma levels.

It should be mentioned that these SNPs have not been previously associated with any of the CAM/inflammation assessed traits. Therefore, these interactions may indicate novel genetic markers for these molecules. However, replication of these findings is needed before the establishment of a conclusion.

Finally, we examined whether these genetic polymorphisms had a functional effect on the traits in terms of gene expression modification. A positive association was identified only for the C allele of rs4416670 with L-selectin mRNA. Nevertheless, this SNP was not associated with L-selectin levels; therefore a direct effect of the SNP cannot be speculated. rs4416670 could have a functionality in L-selectin gene expression modification, indirectly, possibly through a transcriptional factor or a more complex mechanism. As $VEGF_{145}$ mRNA was positively associated with L-selectin expression, this mechanism could be the common link between the two molecules and rs4416670.

The multi-dimensional approach used in this study can be considered as a strong point of this work in the assessment, validation and explanation of the relations between VEGF and adhesion/inflammation molecules in healthy individuals, thus giving new perspectives and hypotheses for further studies in order to identify the exact molecular

mechanisms of VEGF implication in the development of specific diseases.

In conclusion, we have addressed in this study for the first time the associations of VEGF with adhesion molecules and a number of inflammation cytokines in a healthy population. We have found significant associations of VEGF with ICAM-1 and E-selectin in plasma concentrations and common expression profiles for VEGF and ICAM-1, L-selectin, and TNF-α in PBMCs. Finally, epistatic interactions between VEGF-related SNPs were shown to affect the levels of ICAM-1, E-selectin, IL-6 and TNF-α and the expression of L-selectin. These findings support the existence of complex relationships between angiogenic, adhesion and inflammation molecules that exist even in non-pathological situations, which should be taken into account in future studies concerning the implication of VEGF in related pathologies. Their understanding may promote the knowledge of the molecular mechanisms and the understanding of the processes that mediate complex pathologies such as cardiovascular diseases and cancer.

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Disclosures

None.

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TABLES

Table 1: Characteristics of study population (n=403)

Variable	**Mean***	**SD**
Age (years)	44.460	4.885
Gender (%) Male	50.4	
Body mass index (Kg/m^2)	24.9	3.9
Vascular endothelial growth factor (ng/l)	42.748	43.330
Interleukin 6 (pg/ml)	1.376	1.695
Tumor necrosis factor α (pg/ml)	2.320	4.535
E-selectin (mg/l)	53.159	25.882
L-selectin (mg/l)	1060.693	407.889
P-selectin (mg/l)	138.917	42.891
C-reactive protein (mg/l)	1.794	3.426
Intercellular adhesion molecule-1 (mg/l)	263.023	69.486

* mean values for continuous variables and percentages for categorical variables

Table 2: SNPs characteristics.

Chromosome	**SNP**	**Function**	**Closed to/on gene**	**Minor allele**	**Common allele**	**Minor allele frequency**
6	rs6921438	Intergenic	MGC45491 and MRPL14 (near VEGF)	A	G	0.445
6	rs4416670	Intergenic	MGC45491 and MRPL14 (near VEGF)	C	T	0.476
8	rs6993770	Intronic	ZFPM2	T	A	0.283
9	rs10738760	Intergenic	VLDLR and KCNV2	A	G	0.491

Table 3: Association between plasma VEGF and its isoforms transcripts and adhesion and inflammation molecules, adjusted for age, gender, and BMI (beta coefficient is presented)

VEGF	ICAM-1		L-selectin		E-selectin		TNF-α	
	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein
mRNA isoforms								
$VEGF_{121}$	-	-	-	-		-	-	-
$VEGF_{145}$	0.353*	-	0.611***	-		-	0.616***	-
$VEGF_{165}$	-	-	-	-		-	-	-
$VEGF_{189}$	-	-	-	-		-	-	-
Plasma levels								
VEGF	-	0.043*	-	-		0.085*	-	-

*: P≤0.05, **: 0.001<P≤0.01, ***: P≤0.001

Table 4: Epistatic interactions of SNPs on blood levels of VEGF, E-selectin, TNF-α, ICAM-1 and IL6 (regression models adjusted for age, gender and BMI)

SNPs	rs6921438	rs 4416670	rs 6993770	rs 10738760
rs 6921438				
rs 4416670	TNF-α (P=0.035)			
rs 6993770	IL-6 (P=0.004)	IL-6 (P=0.0008)		E-selectin (P=0.041)
rs 10738760	IL-6 (P=0.050)	IL-6 (P=0.004)	ICAM-1 (P=0.021)	

Publication 5

Association of vascular endothelial growth factor trans-acting genetic variant with metabolic syndrome

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Background and aim: Metabolic syndrome refers to a cluster of metabolic risk factors associated with impaired angiogenesis. Vascular endothelial growth factor (VEGF) is known to be associated with metabolic syndrome and some of its related components. In a previous genome-wide association study, we identified four polymorphisms that explain ~50% of VEGF circulating levels heritability. The aim of this study is the evaluation of the possible effect of VEGF and VEGF-associated variants on metabolic syndrome and /or on its related risk components in a supposed healthy population.

Methods and results: We examined the associations of rs6921438, rs4416670, rs6993770, rs10738760 with metabolic syndrome and its related components in 403 supposed healthy unrelated adults. Metabolic syndrome was defined according to the International Diabetes Federation criteria. We found a significant association between metabolic syndrome and rs10738760 [intergenic SNP located between the very low-density lipoprotein receptor (VLDLR) and potassium voltage-gated channel subfamily V, member 2 (KCNV2) genes] and also a trend for triglycerides levels.

Conclusions: The inverse association of a VEGF regulatory variant, rs10738760, with metabolic syndrome might lead us to propose a probable protective role for VEGF in metabolic syndrome via the regulation of VLDLR and Kncv2. Further research is needed to determine the possible molecular mechanisms of the function of this polymorphism in metabolic syndrome as well as its possible effect on other cardiovascular risk factors like type 2 diabetes.

Key words: Metabolic syndrome, VEGF genetic variants, VLDLR, *Kcnv2*

Introduction

Metabolic syndrome (MetS) refers to a cluster of metabolic risk factors linked with impaired angiogenesis and narrowly associated with insulin resistance [1]. In its advanced form, clinical fasting hyperglycemia or even type 2 diabetes is present [2]. The syndrome has been broadened to encompass features as central obesity, glucose abnormalities, dyslipidemia, elevated blood pressure, and low grade inflammation state with a pro-thrombotic circumstance [3;4]. The prevalence of MetS, mainly due to emerging of "obesity epidemic", varies between 35-40% of adults in developed countries [5]. In circumstances of metabolic burden such as obesity and diabetes mellitus, coordinated regulation between oxygen supply and consumption has been reported to be interrupted and subsequently cause tissue hypoxia [6].

The risk for atherosclerotic cardiovascular disease and type 2 diabetes is approximately doubled and fivefold respectively in patients with MetS [7]. It is noteworthy that several studies have revealed that ischemic tissues neovascularization is impaired in type 2 diabetes with obesity, hypercholesterolemia, or hypertension [1;8].

Vascular endothelial growth factor (VEGF), as a multifunctional cytokine has distinguished functions in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis [9]. Adipocytes produce VEGF, which may act as an angiogenic and vascular survival factor for the omental vasculature [10].

Elevated circulating levels of VEGF have been observed in ischemic heart disease, heart failure and stroke and in various other disorders such as type 2 diabetes and polycystic ovary disease [9;11]. Recent evidence indicates that VEGF could be an adaptive response to obesity induced hypoxia [12]. However, the implication of VEGF on MetS is not extensively studied

[1;13;14]. Few recent publications have shown a positive association of VEGF circulating levels with MetS as well as with a number of its components [13;15].

Furthermore, a significant number of genetic variants have been shown to be associated with MetS and its components [16-18]. We have recently identified four single nucleotide polymorphisms (SNPs) (rs6921438, rs4416670, rs6993770, rs10738760) explaining ~50% of VEGF heritability using a genome-wide association study (GWAS) [11]. As a relationship between VEGF and MetS has been previously reported, these SNPs could be considered as candidate genes for MetS and its components as well. Therefore, in the present study, we aimed to assess the possible associations of MetS and its related components with VEGF plasma levels and with VEGF-related SNPs, in a healthy population. In addition to the major components of MetS including waist circumference (WC), systolic blood pressure (SBP), diastolic blood pressure (DBP), central obesity and fasting plasma glucose, a number of linked factors to cardiovascular diseases such as waist to hip ratio, waist to height ratio, pulse pressure, hypertension, general obesity, insulin levels, apolipoprotein E, apolipoprotein A-I, apolipoprotein B and apolipoprotein CIII were tested.

Materials and Methods:

Study population

This study is based on the STANISLAS Family study (SFS), a 10-year longitudinal survey involving 1,006 volunteer families from Vandoeuvre-lès-Nancy (in the east of France) [19]. Individuals with known acute or chronic diseases such as stroke, myocardial infarctions, hypertension, dyslipidemia or cancer were not included, as the aim of the study was the assessment of genetic susceptibility factors on the variability of intermediate phenotypes in physiological conditions without the influence of any long term medication and disease. The study protocol was approved by the Local Ethics Committee of Nancy and all subjects gave written informed consent for their participation in the study. A total of 403 unrelated adults were involved in the current study. All data were collected during the second examination of the SFS.

Anthropometric indices, medical history and lifestyle data

Weight and height were measured in standing position with light clothing without shoes. Weight was recorded with digital scales to the nearest 200 grams using a weight scale. Height was measured to the nearest 0.1 cm using wall-mounted stadiometer, with the subjects' shoulders in a normal position. Body mass index (BMI) was calculated according to the Quetelet's formula: weight (kg)/height (m^2). Waist circumference was taken at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest (hip bone), and hip circumference was measured at the maximum level over light clothing, using a standard tape measure, without any pressure on the body surface. Measurements were recorded to the nearest 0.1 cm. All measurements were taken by trained nurses according to standard

procedures, and the reliability of the measuring devices was periodically checked during the study period. SBP and DBP were calculated as the mean of three measurements taken under standardized conditions with a sphygmomanometer, with the subject in a supine position [20]. Pulse pressure was calculated as the difference between SBP and DBP. Also, data were collected using standard questionnaire including information about lifestyle such as smoking and personal medical history. Hypertension was defined as SBP >140mmHg and/or DBP >90mmHg. Central obesity was defined as a WC >102 cm in men and >9 cm in women, while general obesity was defined as BMI ≥30Kg/m^2 [20].

MetS was defined based on the International Diabetes Federation (IDF) criteria [21]: WC ≥94 cm in men or ≥80 cm in women plus any two of the four following criteria (1) triglyceride ≥1.7 mmol/l or drug treatment for elevated triglyceride, (2) high-density lipoprotein cholesterol (HDL-C) < 1.03 mmol/l in men or <1.3 in women or having medication for reduced HDL-C, (3) SBP ≥ 130 mmHg or DBP ≥ 85 mmHg or anti-hypertensive medication, (4) fasting blood glucose ≥ 5.6 mmol/l or drug treatment for increased serum glucose.

Laboratory measurements

Blood samples were collected after overnight fast between 8:00 and 9:00 am. or 11:00 and 12:00 a.m. Serum and plasma samples were separated by centrifugation at 2000×*g* for 15 min. Serum fasting blood glucose, total cholesterol and triglycerides levels were measured using standard enzymatic methods (Merck, Germany) on an automated analyzer AU5021 (Olympus, Japan). Insulin, apolipoprotein A-I, apolipoprotein B, apolipoprotein CIII were determined by immunonephelometry on Behring Nephelometer analyzed with Behring reagents (France). Apolipoprotein E and HDL-C were measured by turbidimetry and precipitation by phosphotungstate respectively, on a Cobas-Mira analyzer (Roche). VEGF plasma levels

quantification was performed by Randox Ltd (Crumlin, UK) using a biochip array analyser (Evidence ®) [19].

Genotyping

The SNPs rs6921438, rs4416670, rs6993770, rs10738760 were genotyped by Genoscreen (http://genoscreen.fr), using a Sequenom iPLEX Gold assay–Medium Throughput Genotyping Technology [22].

Statistical analysis

Continuous variables are presented as mean value ± standard deviation and categorical variables are given in percentages. Hardy-Weinberg equilibrium was tested using the chi-square test. All continuous variables were log-transformed to normalize their distribution. For the SNPs associations analyses an additive model was used. Results are presented using the minor allele as reference allele.

Logistic regression models adjusted for age, gender and BMI were used to test possible associations between VEGF-related SNPs and VEGF plasma levels with MetS. Subsequent analyses adjusted for age, gender and smoking were performed. Similar models adjusted for age and gender were used for general obesity and central obesity and models adjusted for age, gender and BMI were applied for the associations with hypertension.

Concerning the quantitative traits including waist circumference, waist to hip ratio, waist to height ratio BMI, SBP, DBP, fasting blood glucose, insulin and lipids linear regression models adjusted for age, gender and BMI were applied for the assessment of possible effects of the VEGF-related SNPs (the model for the assessment of association with BMI was adjusted for age and gender only).

All analyses were performed using PLINK 1.07 software (http://pngu.mgh.harvard.edu/purcell/plink) (23) and the SPSS statistical software version 16.0 (SPSS, Inc, Chicago, Illinois). Significance was assessed at a two-tailed *P*=0.05 level. The significant results of this study were compared to previous findings in the literature (previous GWAS), by imputation analyses using Plink. SNPs with a correlation coefficient ≥ 80% were considered in linkage disequilibrium (LD). The GWAS investigator of HuGENavigator engine [24] and the NHGRI Catalog of published GWAS (http://www.genome.gov/gwasstudies) (25) were used in order to assess previous GWAS concerning blood lipid levels.

Results

The general characteristics of the studied population and polymorphisms are presented in Tables 1 and 2 respectively.

Regarding the effect of the assessed variants, rs10738760 was significantly associated with MetS (table 3). The presence of the minor allele A was associated with decreased risk for MetS. The result remained significant after adjustment for smoking (data not shown). We also observed a trend for association between rs10738760 and triglycerides levels (beta=-0.028, P-value=0.056). There was no association of this SNP with other lipid profile molecules. No statistically significant associations were observed between VEGF plasma levels and MetS as well as with its related components in this population.

Among three previous GWAS [16-18] performed on MetS and related components, rs10738760 was not included in the significant SNPs (genotyped or in LD with another SNP). Therefore, this is considered as a novel polymorphism for MetS.

Discussion

In this study, we examined the associations of MetS, its related components and some of linked factors to cardiovascular diseases with VEGF levels and cis and trans-acting VEGF genetic variants. We report a significant association of rs10738760 with metabolic syndrome, independently of its components.

Epidemiological studies show that the visceral fat accumulation, the predominant driving force behind the MetS [2], is the most important determinant factor for VEGF circulating levels [10]. Very recent evidence indicates that an increase in adipose tissue VEGF-induced angiogenesis is a protective phenomenon against obesity-induced hypoxia and, consequently, insulin resistance [12]. In the present study, we did not obtain a significant statistical relationship between MetS or its related components and VEGF. Lieb and colleagues [15] have reported a significant association of circulating VEGF with MetS. It appears that a number of differences between our and their populations such as sample size and obesity indices could explain these diversities. Tarantino and colleagues [14] in a case control study have found a higher circulating VEGF levels in individuals with MetS compared to lean subjects. Based on a Japanese report, there is an association between VEGF and MetS, however, it seems that they have used the National Cholesterol Education Program's Adult Treatment Panel III (NCEP-ATP III) definition of MetS [1]. In the present study we used the IDF definition as it is more sensitive than NCEP-ATP III for predicting diabetes and cardiovascular disease [26] Also, the ethnic variations between the two populations may explain the different results. Therefore, it is not clear whether the elevated VEGF level is a deleterious factor or a physiologic/negative feedback response to prevent the progress of ischemia in metabolic linked ischemic status [8;27;28] as well as in MetS [1;14;15;27].

The most known and unifying theory to explain the pathophysiology of the MetS is insulin resistance [3]. Insulin resistance has been characterized with a glucocentric prospective, where failure of insulin function results in fasting hyperinsulinemia to maintain euglycaemia. Nevertheless, even prior to fasting hyperinsulinaemia, postprandial hyperinsulinaemia exist [3]. Insulin resistance, indeed, is a key element associated with clustering atherogenic abnormalities including the elevated levels of triglyceride, apolipoprotein B, an increased proportion of small dense low-density lipoprotein particles and a reduced concentration of HDL-C, a state of inflammation and a prothrombotic profile. Moreover, insulin resistance could also contribute to hypertension and dysglycemia, finally leading, among genetically susceptible individuals, to type 2 diabetes [29]. Després and colleagues [29] have proposed that the coincident presence of fasting hypertriglyceridemia and of an increased waist circumference (hypertriglyceridemic waist) could stand for a clinical phenotype to identify patients at risk of insulin resistance.

Plasma VEGF levels are augmented in individuals with hyperlipidemia and in obese type 2 diabetes patients with or without established atherosclerosis, and lipid as well as glucose lowering with either fluvastatin or fenofibrate and metformin results in a significant reduction of VEGF levels [30;31] . Here, we have found a trend for association between a trans-acting genetic variant of VEGF and triglycerides levels in healthy circumstance. Our results may suggest that the presence of the corresponding polymorphism may decrease the triglyceride levels in individuals with metabolic syndrome.

The polymorphism, rs10738760, a novel polymorphism for MetS, is located on 9p24.2, and it is an intergenic SNP close to the very low-density lipoprotein receptor (*VLDLR*) and potassium voltage-gated channel subfamily V, member 2 (*KCNV2*) genes [11]. Recently, we have

reported that rs10738760 up-regulates and explains around 5% of variation of circulating VEGF [11]. Furthermore, our ingenuity pathway analyses in the mentioned study found plausible biological links between VEGF and VLDLR gene in related loci. In the present study, we have found a trend for association between rs10738760 and triglycerides in addition to its relationship with MetS. The VLDLR gene encodes a lipoprotein receptor that is a member of the low density lipoprotein receptor (LDLR) family and plays important roles in VLDL-triglyceride metabolism and the reelin signaling pathway [32]. This receptor is expressed abundantly in adipose tissue, skeletal muscle and heart, which all participate actively in lipid metabolism. The expression of VLDLR in macrophages and monocytes has been shown in human atherosclerotic lesions by Argraves and Takahashi [33;34]. As VLDLR knockout mice remained lean and did not show insulin resistance after high fat, high calorie diet [35], it seems that VLDLR may play a role in MetS. Based on an animal study, the knockout mice in VLDLR gene (VLDLR $^{-/-}$) showed improved survival and decreased infarct area after an induced myocardial infarction [36]. Zenimaru and colleagues [37] have reported that VLDLR activates mitogen-activated protein kinase (MAPK) during the uptake of triglycerides-rich lipoprotein in glucose shortfall condition in skeletal muscle cells. The evidences of Pedrini and Tatarczyk [38;39] indicate that triglycerides-rich lipoprotein particles cause insulin resistance in hepatocytes and cultured skeletal muscle cells. Impairment of muscle and adipocyte glucose uptake and oxidation is a consequence of insulin resistance in skeletal muscle cells as well as in adipose tissue [40]. In insulin resistance and in hyperglycemia, the MAPK pathway remains functional and even hypersensitive, whereas the pathways leading to activation of phosphatidylinositol 3-kinases (PI-3K), which is " the normal pathway of insulin functions and VEGF", is blocked [4]. However, VEGF induces its

anti-apoptotic and proliferative functions via the MAPK pathway [41]. Interestingly, based on Miyokawa-Gorin evidence, chronic MAPK activation results to VEGF expression in adipose tissue [10;42]. Furthermore, Karakelides and colleagues [43] have documented that insulin deficiency increases VEGF gene expression in muscle cells.

Voltage-gated potassium (Kv) channels represent the most complex class of voltage-gated ion channels from both functional and structural point of views. Their diverse functions include regulating neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and cell volume.

Kcnv2 encodes the voltage-gated potassium channel subunit Kv8.2, which also called Kv11.1 and RCD3B, and is a silent subunit when expressed as a homotetramer [44]. However, when co-formed as a heterotetramer with Kv2 family members, Kv8.2 influences membrane translocation and biophysical properties of these channels [44]. In other words, Kv8.2 and Kv2.1 form a heterotetramer which adapt the functions of Kv2 channels. Insulin secreting cells express mRNA transcripts for a number of Kv channels and Kv2.1 protein. The mRNA of Kv8.2 is strongly expressed in pancreas and testis and has a weaker expression in several other tissues such as lung, liver, kidney, spleen, thymus, prostate, and ovary [44]. A growing body of evidences indicates that there is an impairment of coronary ion channels including Kv and ca^{2+} channels in Mets which play an important role in the alterations in the control of coronary blood flow [45;46]. The Kv2.1 has a negative regulatory effect on insulin secretion while its inhibition in mouse pancreatic beta cells enhances glucose-dependent insulin secretion [47]. Furthermore VEGF has been shown to act as a survival factor for human pancreatic islets and improve their functions of insulin secretion[48].

Cytosolic free ca^{2+} rises in pancreatic beta-cells in response to glucose stimulation and is part of the coupling to insulin secretion [49]. It has been shown that VEGF stimulates increase in cytosolic calcium concentration [50]. Muoio and colleagues have well described that obese and insulin resistant individuals can remain in a state of beta cell compensation that protects them from diabetes for long periods of time before beta cell failure [51]). Indeed, the onset of type 2 diabetes has been determined by the failure of compensatory response of beta cells for insulin resistance [48]. Watada et al [48] in an animal study have proposed possible VEGF-induced compensatory beta cells activation, nevertheless they have not discussed how the proposed model may affect insulin secretion. So far, based on our knowledge, it seems that there is no evidence to precise the molecular pathways which explain the mechanism of VEGF-induced insulin secretion. Therefore, our study for the first time, could propose a possible link between VEGF, Kv2, insulin secretion and MetS. Thus, it seems that elevated levels of VEGF in MetS, which have been described by some reports [14;15;26], may be a compensatory procedure which contributes; independently or along with other possible factors, to maintenance of the effective levels of insulin. Further studies are needed to reveal these possible molecular mechanisms. It is noteworthy to mention that impairment of coronary vasodilatation in the MetS is mediated by reductions in the functional contribution of voltage-dependent K+ channels to the dilator response [52]. Interestingly, K_V channels are considered as possible end-effectors in reactive hyperemia while decreased functional expression of K+ channels such as voltage-gated potassium channels which leads to decrease coronary blood flow is a critical mechanism underlying increased morbidity and mortality to coronary and cardiac events in patients with MetS [52]. Further studies should examine the reactive stimulatory effects of

VEGF on K+ channels expression in coronary microcirculation of individuals with MetS as well.

Therefore, VEGF may be bonded with MetS and insulin metabolism through VLDLR, Kv8.2 and Kv2.1. via the rs10738670. However, further studies are needed to define the effect of this SNP on gene expression and the production of the functional protein products. Moreover, a study on the probable effects of VEGF on heterotetramerization of Kv8.2 with Kv2.1 and the impact of the resulted complex on insulin secretion is recommended. The elucidation of these mechanisms could finally lead to the identification of a new potential therapeutic target in MetS.

In conclusion, the up-regulatory effects of rs10738760 on VEGF in healthy population and its inverse associations with MetS and possibly with triglyceride levels might lead us to propose a probable protective role for VEGF in MetS via the regulation of VLDLR and Kncv2 genes. Future studies will reveal whether the elevated VEGF levels in MetS is a compensatory phenomenon against switching of pre-diabetic condition to advanced type 2 diabetes / cardiovascular disease.

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Disclosures

None.

Reference List

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Tables

Table 1: Characteristics of STANISLAS Family Study population

Variable	**Adults (n=403)**	
	Mean	**SD**
Age (years)	44.460	4.885
Gender (%) Male Female	50.4 49.6	
Smoking (%) No Yes Ex-smoker	45 25.4 29.6	
BMI (Kg/m^2)	24.904	3.880
Waist circumference (cm)	82.433	11.018
Waist/Hip ratio	0.844	0.089
Waist/Height ratio	48.962	5.850
Systolic blood pressure (mmHg)	123.29	13.79
Diastolic blood pressure (mmHg)	79.93	10.130
Pulse pressure (mmHg)	49.35	8.75
Fasting blood glucose (mmol/l)	5.033	0.682
Insulin (IU/ml)	6.210	5.644
Triglycerides (mmol/l)	1.343	1.960
Low-density lipoprotein-C (mmol/l)	3.558	0.898
High-density lipoprotein-C (mmol/l)	1.606	0.465
Total cholesterol (mmol/l)	5.744	1.001
Apolipoprotein A1 (g/l)	1.630	0.259
Apolipoprotein B (g/l)	1.013	0.233
Apolipoprotein E (mg/l)	41.816	17.574
Apolipoprotein CIII (mg/l)	98.330	37.193
Vascular endothelial growth factor (ng/l)	42.748	43.330

Table 2: Genetic variants' characteristics of STANISLAS cohort study population

Chromosome	**SNP**	**Function**	**Closed to/on gene**	**Minor allele**	**Common allele**	**Minor allele frequency**
6	rs6921438	Intergenic	near of VEGF	A	G	0.445
6	rs4416670	Intergenic	near of VEGF	C	T	0.476
8	rs6993770	Intronic	between ZFPM2 and LRP12 genes	T	A	0.283
9	rs10738760	Intergenic	between VLDLR and KCNV2	A	G	0.491

Table 3: Associations of the 4 VEGF single nucleotide polymorphisms with metabolic syndrome in adults from the STANISLAS population (n=403) (model adjusted for age, gender and BMI)

	Rs6921438		**RS4416670**		**Rs6993770**		**RS10738760**	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Metabolic syndrome	1.363 (0.78-2.37)	0.272	0.7545 (0.43-1.31)	0.320	0.7938 (0.43-1.45)	0.454	0.4755 (0.27-0.84)	**0.010**

OR: Odds ratio

95% CI: 95% Confidence Interval

Publication 6

Contribution of VEGF and related common genetic variants to the variation of blood lipids

Abbreviated title: VEGF related SNPs and plasma lipids levels

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Abbreviations: APO-E, apolipoprotein E; BMI, body mass index; BRC IGE-PCV, Biological Resources Bank “Interactions Gène-Environnement en Physiopathologie CardioVasculaire”; CVDs, cardiovascular diseases; GWAS, genome-wide association study; *MRPL14,* mitochondrial ribosomal protein L14 gene; SNPs, single nucleotide polymorphisms; TC, total cholesterol; VEGF, vascular endothelial growth factor

Abstract

The vascular endothelial growth factor (VEGF) is among the most significant stimulators of angiogenesis and its effect on cardiovascular diseases is considered important, although unclear yet. Recently, our team identified four polymorphisms (rs6921438, rs4416670, rs6993770 and rs10738760) explaining up to ~ 50% of the heritability of serum VEGF levels. In this study we tested the hypothesis whether these SNPs contribute to the variation of blood lipid levels in healthy subjects. The effect of these SNPs on apolipoprotein E (APO-E), triglycerides, total cholesterol, low and high-density lipoproteins (LDL and HDL) was evaluated using linear regression in a discovery and a replication sample (n=1,006 and 1,145 healthy unrelated adults from European origin respectively) followed by a meta-analysis. Their gene×gene and gene×environment interactions were also assessed. SNP rs6921438 was significantly associated with HDL ($P_{overall}$=1.2×10^{-7}) and LDL ($P_{overall}$=1.5×10^{-4}). A significant interaction between rs4416670 and hypertension for APO-E was also identified ($P_{overall}$=1.7×10^{-5}) as well as an epistasis between rs6921438 and rs6993770 ($P_{overall}$=2.6×10^{-3}). Common genetic determinants were identified for VEGF and HDL and LDL, while epistatic and environmental interactions seem to play a role. These results offer new insights in the genetic regulation of cardiovascular diseases and investigation of possible associations between other cardiovascular risk factors traits and these SNPs would be of interest.

Supplementary key words: VEGF polymorphisms, lipid metabolism, cardiovascular diseases, HDL, LDL, epistatic interactions, gene×environment interactions

Introduction

The vascular endothelial growth factor (VEGF) family is one of the most important regulators of vascular biology. Especially, VEGF-A stimulates angiogenesis in a wide range of normal and pathological processes (1). Due to this significant position on blood vessel homeostasis, the role of VEGF on cardiovascular diseases (CVDs) and atherogenesis has been among the objectives of cardiovascular research during the last years (2). However, VEGF's effect on CVDs is still unclear.

The use of anti-VEGF agents in cancer therapy has shown significant cardiovascular side effects such as hypertension, cardiomyopathy and hemorrhagic events (3). Furthermore, VEGF protein or gene therapy has been tested in randomized clinical trials, particularly in patients with coronary artery disease, with results showing small clinical importance (2). Many studies have identified higher VEGF levels in patients with vascular diseases; nevertheless, it is not clear whether the up-regulation of VEGF is an adaptation to ischemia or if it is rather causal in the onset of these diseases (4-11). Concerning the involvement of VEGF in atherosclerosis, results seem to be conflicting. A possible neovascularization and proinflammatory effect of VEGF has been identified in animal models and *in vitro* studies, which leads to progression of atherosclerosis and plaque instability (12-17). In contrary, clinical trials using VEGF protein or gene therapy in humans (2) and in animal studies (18, 19) do not support a positive effect of VEGF on atherosclerosis progression. The complexity of CVDs combined with the pleiotropic effects of VEGF could partially explain the differences between studies.

An interesting point would be the assessment of the effect of VEGF on known cardiovascular risk factors in supposed healthy populations, such as lipids levels (20), as this would probably support possible implications of VEGF in the physiopathology of CVDs. Especially high-density and low-density lipoproteins (HDL, LDL respectively) are considered as independent risk factors for the development of CVD (20). Increased levels of circulating VEGF have been found in subjects with uncomplicated hyperlipidemia in a small sample-size study (21) and similar finding has been shown in a pilot study in patients with hypercholesterolemia (22). Significant associations were found between HDL and VEGF levels in a supposed healthy population from Japan (23), while in a supposed healthy population of SAPHIR study, VEGF was negatively correlated with LDL, total cholesterol (TC) and apolipoprotein B only in women (24). However, a study in end stage renal disease patients has shown no change in VEGF, sVEGFR1 and sVEGFR2 between primary and secondary prevention patients. VEGF was only associated with LDL levels. Although the relations between lipids profile and VEGF levels are not yet clearly defined, these observations suggest an eventual implication for this molecule in lipids metabolism.

The genetic research can be used for the assessment of VEGF role in CVDs, through the identification of new possible molecular mechanisms. Via a recent genome-wide association study (GWAS), we identified four single nucleotide polymorphisms (SNPs) that explained up to ~ 50% of the heritability of serum VEGF levels (25). The investigation of the effect of these new genetic variants, as well as their interactions between them and with environmental factors, on blood lipids levels might give some insight in the relation between VEGF and blood lipids. Therefore, the aim of the present

study is the assessment of genetic determinants of blood lipids levels using these four novel VEGF related SNPs, in supposed healthy discovery and replication populations.

Methods

Subjects

Discovery and replication population

Discovery (*n*=1,006) and replication (*n*=1,145) samples belong to two independent and non-overlapping populations extracted from the Biological Resources Bank (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) in Nancy, East of France. They consist of supposed healthy unrelated adults of European origin (discovery population: Portugal, France; and replication population: Ireland, Greece). Individuals with chronic disorders (cardiovascular or cancer) or having a personal history of CVD have not been included. Subjects taking medication with blood lowering effect or having an effect on cardiovascular function were also excluded. The study protocols have been approved by the Local Ethics Committee of each recruitment centre and all subjects gave written informed consent for their participation in the study.

Data collection

For both populations, biological and clinical measurements, health and lifestyle information have been collected using appropriate, validated questionnaires and procedures as described previously (26, 27). Hypertension was defined as systolic blood pressure ≥140mmHg, diastolic blood pressure ≥90mmHg and smokers were identified based on current smoking status. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. Obesity for adults was defined as BMI ≥30kg/m^2.

Apolipoprotein E (APO-E), triglycerides, TC, and HDL were measured in plasma (26, 27) and VEGF levels were measured also in plasma in a subsample of 403 individuals from the discovery population as previously described (25). LDL was calculated using the Friedewald formula (28).

Genotyping

DNA has been extracted from all participants and relative biobanks have been constructed in the BRC IGE-PCV. The SNPs rs6921438, rs4416670, rs6993770, and rs10738760 were genotyped by Genoscreen© (http://genoscreen.fr) using a Sequenom® iPLEX Gold assay – Medium Throughput Genotyping Technology (29) and in Kbioscience (http://www.kbioscience.co.uk) using the competitive allele specific PCR (KASP) chemistry coupled with a FRET-based genotyping system (http://www.kbioscience.co.uk/reagents /KASP/KASP.html) in the replication population.

Statistical analysis

Continuous variables are presented as mean value ± standard deviation and categorical variables are given in percentages. Hardy-Weinberg equilibrium was tested using the chi-square test. VEGF concentrations were natural log-transformed to normalize their distribution in a subsample of the discovery population. Correlations were evaluated by calculating the Pearson coefficient (r). Linear regression models adjusted for age, gender and BMI were used to test possible associations between VEGF plasma levels and the levels of the assessed lipid traits. Significance was assessed at a two-tailed P=0.05 level.

Genetic analyses were performed under the assumption of an additive model.

For the discovery population and the replication populations, linear regression models adjusted for age, gender and BMI were used for the assessment of the effect of each SNP (independent variable) in blood lipids concentrations (dependent variables). Further adjustments were performed in both populations for smoking and hypertension. Significance was assessed at a two-tailed P=0.0125 level (adjustment for multiple testing). In the case where more than one SNP are associated with one trait, a conditional analysis assessing the main effect of all significant SNPs in the same model of linear regression (adjusted for age, gender and BMI) was performed in order to clarify the independent determinants of the trait.

The environmental factors used for the gene×environment interactions assessment included BMI, smoking and hypertension. In the previous regression modelsthe environmental factor and an additional interaction term (SNP×environmental factors) was added. Significant results were considered those with P=0.004. For the significant SNPs implicated in gene×environment interactions, separate regression models using the environmental factor as dependent variable were performed in order to control for a direct association between the SNPs and the factor.

The assessment of gene×gene interactions was tested using all possible pair-wise combinations between the four SNPs in both discovery and replication populations. In the regression models adjusted for age, gender and BMI, two SNPs and their interaction term were added. In order to adjust for multiple testing, significance was set at P=0.008.

All analyses were performed using PLINK 1.07 software (http://pngu.mgh.harvard.edu/purcell/plink) (30) and the SPSS statistical software version 16.0 (SPSS, Inc, Chicago, Illinois).

Meta-analysis for each quantitative trait was performed using a weighted inverse normal method via the function "metagen", with a fixed effect, in the "META" R 2.15.1 package.

The significant results of this study were compared to previous findings in the literature (previous GWAS) by imputation analyses using Plink. SNPs with a correlation coefficient ≥ 80% were considered in linkage disequilibrium (LD). The GWAS investigator of HuGENavigator engine (31) and the NHGRI Catalog of published GWAS (http://www.genome.gov/gwasstudies) (32) were used in order to assess previous GWAS concerning blood lipid levels.

Results

Participants' data are presented in table 1. The characteristics of the genotyped SNPs are shown in table 2. All SNPs in both populations are in agreement with the Hardy-Weinberg equilibrium.

In the discovery population, significant associations were observed between rs6921438 and rs6993770, and the levels of HDL (β=-0.09mmol/l, $P=1.2\times10^{-4}$ and β=-0.01 mmol/l, $P=8.3\times10^{-3}$ respectively; table 3) and between rs6921438 with plasma LDL concentrations (β=0.14mmol/, $P=6.7\times10^{-3}$, table 3). Specifically, the presence of the minor allele A of rs6921438 is associated with decreased HDL and increased LDL values, while the minor allele T of rs6993770 is associated with increased levels. These significant associations were only confirmed for SNP rs6921438 in the replication population (β=-0.07mmol/l, $P=2.7\times10^{-4}$ and β=0.12mmol/l, $P=8.4\times10^{-3}$ for HDL and LDL respectively; table 3). Conditional analysis including both rs6993770 and rs6921438 revealed that rs6921438 was the only SNP with significant direct effect on HDL levels (β=-0.09mmol/l, $P=1.2\times10^{-4}$; β=-0.07mmol/l, $P=2.7\times10^{-4}$). Of note, these results remained significant in both populations after adjustments for both smoking and hypertension. SNP rs6921438 explained 1% of the variability of HDL and 0.2% for LDL in both populations. Meta-analysis of the results of both populations gave highly significant associations between rs6921438 and both HDL and LDL levels (β=-0.08mmol/l, $P=1.2\times10^{-7}$ and β=0.13mmol/l, $P=1.5\times10^{-4}$ for HDL and LDL respectively; table 3). SNP rs6993770 was not significantly associated with HDL levels in joint analysis of the two populations. Furthermore, meta-analysis of the conditional analysis

results including rs6993770 and rs6921438 verified that rs6921438 is the only independent determinant of HDL levels (β=-0.08mmol/l, P=1.2×10^{-7}).

Among nine previously published GWAS studies concerning lipids levels, rs6921438 was not reported in the lists of statistically significant SNPs (genotyped or imputed) (33-41). Therefore, it is a novel SNP associated with both HDL and LDL levels.

The gene×environment interactions assessment revealed that rs4416670 was interacting with hypertension for APO-E (P= 3.5×10^{-3}, 1.6×10^{-3}, and 1.7×10^{-5} respectively in the discovery, replication populations and meta-analysis; table 4). The minor allele of the polymorphism was associated with lower levels of APO-E in hypertensive participants (β=-0.71mg/l, -0.75mg/l, and -0.73mg/l respectively; table 4). In order to test for a possible direct association of rs4416670 on hypertension, further analyses were performed in both populations. No significant associations were observed between the same SNP and hypertension.

Regarding epistatic interactions in the discovery set, we found that the SNP rs6921438 interacted with rs6993770 for HDL levels (β=0.05mmol/l, P=2.4×10^{-3}, table 4). Although this finding was not significant in the replication population (β=0.02mmol/l, P=0.035, table 4), it was significant in the meta-analysis of the results (β=0.03mmol/l, P =2.6×10^{-3}, table 4).

In a subsample of the discovery population (n=403) with VEGF plasma levels measurements, no correlation was found between VEGF plasma levels and any of the assessed lipid traits. Furthermore, VEGF levels were not associated with lipids levels in regression models.

Discussion

The present study assessed the effect of VEGF-related SNPs on blood lipid traits and found significant associations and gene×environment interactions for HDL, LDL and APO-E levels.

In the discovery population, rs6921438 and rs6993770 were associated with HDL and rs6921438 were associated with LDL plasma levels. The minor alleles of rs6921438 had a negative effect, while rs6993770 seems to be protective through increase of HDL levels. Conditional analyses showed that only rs6921438 is significantly associated with HDL in the discovery population. Furthermore, the association of rs6921438 with HDL and LDL was observed also in the replication sample, while similar results were observed when meta-analysing the results of both populations.

The rs6921438 is an intergenic SNP in chromosome 6, close to *VEGFA* gene and located between the mitochondrial ribosomal protein L14 gene (*MRPL14*) and the *MCG45491* gene (C6orf223). We negatively associated this SNP with VEGF levels in a previous GWAS (25). Consequently, it appears that rs6921438 could have a negative effect in cardiovascular system through decrease of HDL, increase of LDL levels, while decreasing VEGF levels.

It should be mentioned, that to our knowledge, this is the first study that investigates the effects of VEGF-related SNPs with blood lipid traits. Also, rs6921438 has not been identified in any previous GWAS study concerning HDL and LDL levels (33-41). Furthermore, as the existing GWAS have managed to explain a small percentage of the blood lipid traits variance (e.g for HDL it ranges from 0.6 to 10%), other genetic variants

remain to be found. Nevertheless, in the present study, rs6921438 explained 1% of HDL variability and 0.2% of LDL variability (in both populations). In this study, a candidate-gene approach was used based on SNPs identified from a GWAS concerning VEGF levels heritability. The use of GWAS-identified SNPs as candidate genes for other traits could help the elucidation of genetic relationships between phenotypes and new biological mechanisms associated with pathologies. Hence, the present study that suggests a common genetic regulation of blood lipid traits and VEGF could support this aim.

In order to assess the effect of this common regulation, we tested the association between VEGF and blood lipid levels, though the results were non significant. Therefore, plasmatic levels of VEGF have no direct implication in lipid metabolism, at least in supposed healthy individuals. However, Blann et al (21) demonstrated that subjects with hyperlipidemia have increased levels of VEGF compared with healthy controls even if authors found no correlation of VEGF with blood lipids levels. Additionally, similar results were observed in a small pilot study of hypercholesterolemia patients by Belgore et al (22). These studies have been performed in small sample sizes and they have included pathological populations in a case-control design. Thus, these results can not be directly compared with the present study, where healthy populations have been used. In the study of Kimura et al (23), serum VEGF levels were negatively correlated with HDL levels in healthy adults, however, this correlation was observed only in male population. Moreover, in this study measurements have been performed in serum samples, which demonstrate higher levels of VEGF compared with plasma levels that were measured in the present study (23, 25). Finally, Sandhofer et al (24) have shown that plasma VEGF

levels were negatively associated with TC and LDL in a healthy female sample. Although the population of this study is larger than our discovery cohort, the female sample is significantly older compared to ours. As previously observed, VEGF levels increase with age, especially in women (42). In the abovementioned study, VEGF plasma levels in the female sample are higher than the levels of our study, and this could probably explain the different results between them. It should be mentioned that in our sample there were no significant differences between genders (data not shown). Taken together, it seems that in supposed healthy populations, VEGF is only marginally or not associated with blood lipid levels. Thus, although a common genetic background between VEGF and blood lipids, especially HDL and LDL, may exist, a clinical manifestation can not be detected in physiological situations. However, the functionality of these SNPs and the clarification of molecular pathways that are implicated should be determined in other functional studies including transcriptomic analysis for the expression of key proteins.

Another interesting finding is the interactions of the SNPs between them and with environmental factors. It is currently widely accepted that gene×gene and gene×environmental interactions can explain a significant amount of genetic heritability (43-45). SNP rs4416670 was interacting with hypertension for decreasing APO-E levels. This SNP×hypertension interaction is not due to a direct relationship between the two factors. Hypertension is among the major risk factors for CVDs (46). Presence of altered blood lipid levels and high blood pressure are very common manifestations in subjects with CVDs. Thus, the identification and explanation of these types of interactions between risk factors and genes could be important for the understanding of the complex

mechanisms that define the phenotypes in CVDs. Also, these interactions, if functionally validated, could improve the prevention and prognosis of the diseases.

In addition, an rs6921438×rs6993770 interaction for HDL was detected in the discovery cohort. In the same set of individuals, these two SNPs have a direct effect on HDL levels. Conditional analysis showed that when both SNPs were assessed in the same model only rs6921438 was significantly and independently associated with HDL levels in the discovery population. This finding may also be the reason for the lack of epistatic interaction verification in the replication sample. Additionally, the meta-analysis gave significant results.

As the polymorphisms that were genotyped in this study have not been studied before in relation to blood lipid traits, there are no previous reports on possible gene×gene or gene×environment interactions.

This study was the first designed to assess common genetic regulation between VEGF and blood lipids. The selection of healthy populations is important for the understanding of these relationships as in pathological situations, like CVDs, the clinical profile of both blood lipids and VEGF is affected by many disease-related factors that complicate the situation and do not allow the demonstration of comprehensive results. Another strong point of the study is the replication of the most significant results in an independent population, as well as the use of meta-analyses, that ensured the validity of findings. We acknowledge however the limited number of individuals with VEGF plasma levels measurements.

In conclusion, common genetic variants were identified for VEGF and HDL and LDL, through independent mechanisms. Also, APO-E was associated with interactions between one SNP and environmental factors that are known to be related with CVDs (hypertension). The assessment of other CVDs risk factors associations with these polymorphisms could assist in the understanding of the normal regulation and the pathophysiological mechanisms that underlie these complex diseases.

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Tables

Table 1. Characteristics of the studies participants

Variable	**Discovery (*n*=1,006)**		**Subsample with VEGF plasma levels measurements (*n*=403)**		**Replication (*n*=1,145)**	
	Mean[a]	**SD**	**Mean**[a]	**SD**	**Mean**[a]	**SD**
Age (years)	43.17	9.08	44.52	4.91	41.98	9.24
Gender (male %)	43.00		50.40		82.00	
Body mass index (kg/m^2)	25.19	4.15	24.92	3.94	26.70	3.91
Hypertension (%)	25.80		14.30		27.80	
Obesity (%)	10.12		7.93		15.90	
Smoking (%)	23.33		25.40		27.30	
High-density lipoprotein (mmol/l)	1.47	0.44	1.60	0.47	1.61	0.62

Low-density lipoprotein (mmol/l)	3.27	1.09	3.55	0.89	3.57	1.17
Total cholesterol (mmol/l)	5.51	1.09	5.73	1.03	5.92	1.18
Triglycerides (mmol/l)	1.24	1.36	1.30	1.91	1.63	1.30
Apolipoprotein E (mg/l)	42.68	15.14	41.84	17.63	47.39	18.33
Vascular endothelial growth factor (ng/l)			42.71	43.33		

[a] mean value for continuous variables and percentage for categorical variables

Table 2. Characteristics of the four studied genetic variants

Chromosome	SNP	Discovery		Replication	
		Minor allele	**MAF** [a]	**Minor allele**	**MAF**
6	rs6921438	A	0.42	A	0.40
6	rs4416670	C	0.47	C	0.45
8	rs6993770	T	0.30	T	0.31
9	rs10738760	A	0.48	G	0.45

[a] ***MAF***, minor allele frequency

Table 3. Significant associations of SNPs with plasma lipids

SNP	Minor allele	Phenotype	Discovery		Replication		Meta-analyses	
			β [SE] [a] (mmol/l)	*P*	β [SE] [a] (mmol/l)	*P*	β [SE] [a] (mmol/l)	*P*
rs6921438	A	HDL [b]	-0.09 [0.02]	1.2×10^{-4}	-0.07 [0.02]	2.7×10^{-4}	-0.08 [0.01]	1.2×10^{-7}
rs6921438	A	LDL [c]	0.14 [0.05]	6.7×10^{-3}	0.12 [0.04]	8.4×10^{-3}	0.13 [0.03]	1.5×10^{-4}
rs6993770	T	HDL [b]	0.01 [0.02]	8.3×10^{-3}	-0.02 [0.02]	0.318	0.01 [0.01]	0.298

[a] ***β***, effect size; ***SE***, standard error

[b] ***HDL***, High-density lipoprotein;

[c] ***LDL***, Low-density lipoprotein;

Table 4. Significant gene×environment and gene×gene interactions with plasma lipids

SNP	Phenotype	Discovery		Replication		Meta-analyses	
		β [SE] [a]	*P*	β [SE] [a]	*P*	β [SE] [a]	*P*
rs4416670× hypertension	Apolipoprotein E	-0.71 [0.24]	3.5×10^{-3}	-0.75 [0.23]	1.6×10^{-3}	-0.73 [0.17]	1.7×10^{-5}
rs6921438× rs6993770	High-density lipoprotein	0.05 [0.01]	2.4×10^{-3}	0.02 [0.01]	0.035	0.03 [0.01]	2.6×10^{-3}

[a] ***β***, effect size; ***SE***, standard error; units for β coefficient are: mg/l for apolipoprotein E and mmol/l for high-density lipoprotein

Publication 7

High Prevalence of Metabolic Syndrome in Iran in Comparison with France: What Are the Components That Explain This?

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Abstract

Background: The aim of this study was to investigate the difference in the prevalence of metabolic syndrome and its components between an Iranian and a French population.

Methods: The prevalence of metabolic syndrome, defined according to the Adult Treatment Panel III (ATP III), and of related abnormalities, was estimated in 1,386 French and 1,194 Iranian adults.

Results: The prevalence of metabolic syndrome was significantly higher in Iranian women (55.0%), followed by Iranian men (30.1%), than in French men (13.7%) and French women (6.6%). Iranian women were characterized by high rates of abdominal obesity (65.0%), hypertension (52.1%), hypertriglyceridemia (43.1%), and low high-density lipoprotein cholesterol (HDL-C; 92.7%). Iranian men were characterized by high rates of hypertension (48.9%), hypertriglyceridemia (42.8%), and low HDL-C (81.8%). French men had high rates of hypertension (44.7%) and mild rates of hypertriglyceridemia (28.6%) and hyperglycemia (23.9%). There was a relationship between waist circumference and the lipid components of metabolic syndrome in both countries.

Conclusion: The main finding of this study is the high prevalence of low HDL-C concentrations in the Iranian population, especially in Iranian women, compared with French women. Explanation of this observation could help in establishing prevention strategies.

Introduction

Metabolic syndrome is a cluster of four major cardiovascular risk factors: Obesity, insulin resistance (hyperglycemia), dyslipidemia, and arterial hypertension (HT).[1] The prevalence of metabolic syndrome varies greatly among countries and ethnic groups[2]; it fluctuates between 20% and 30% among Europeans and white Americans, with a similar distribution for men and women.[3] However, in Asian countries, particularly in Middle Eastern societies, metabolic syndrome seems to be more prevalent, with a different pattern of related components and a different distribution for the sexes.[4,5] Certain questions arise about the nature of these east–west differences, although population-based studies and comparative scientific reports are scarce. The aim of this study was to compare the prevalence of metabolic syndrome and its components in a French population, the STANISLAS cohort, with an Iranian population, to analyze the eventual differences observed.

Materials and Methods

Study populations

The French population was part of the STANISLAS cohort, a 10-year longitudinal study conducted since 1994 on 1,006 families recruited at the Centre for Preventive Medicine of Vandoeuvre-lès-Nancy, in the east of France.[6] These apparently healthy individuals were identified from the files of the State Health Insurance Fund and invited to this health examination center for routine checkups. In this study, we present data from a random subsample of 678 men and 708 women between 35 and 55 years old who attended the second checkup in 1999–2000. Each subject gave written informed consent for participating in this study, which was

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approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine (France). In addition, we certify that all applicable governmental regulations concerning the ethical use of human volunteers were followed during this research. The Iranian population had participated in a national survey on noncommunicable disease that started in 2003. Using a multistage sampling method, 5,000 subjects were recruited from the Greater Khorasan province, in northeastern Iran.[7] In this study, we present data from a random subsample of 589 men and 605 women in the same age range as our French subsample, who participated in the first recruitment.

Data collection and definition of metabolic syndrome

Lifestyle information, such as socioeconomic status, tobacco, alcohol and drug consumption, physical activity, and personal medical history, was collected in both countries using appropriate questionnaires. Physical examinations and functional tests were performed and basic blood constituents were measured as described previously.[6,7] Weight and height were measured while the participants were standing in light clothing without shoes. Weight was recorded with digital scales to the nearest 200 grams. Height was measured to the nearest 0.1 cm using wall-mounted stadiometers, with the subjects' shoulders in a normal position. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference was taken at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest (hip bone), and hip circumference was measured at the maximum level over light clothing, using a standard tape measure, without any pressure on the body surface. Measurements were recorded to the nearest 0.1 cm. All measurements were taken by trained nurses according to standard procedures, and the reliability of the measuring devices was periodically checked during the study period. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated as the mean of three measurements taken under standardized conditions with a sphygmomanometer, with the subject in a supine position. In both centers, blood samples were collected in the early morning after an overnight fast.

In France, concentrations of fasting glucose, total cholesterol and triglycerides (TG) were measured in fresh aliquots within 2 h with commercially available kits on an AU5021 apparatus (all from Merck, Darmstadt, Germany). High-density lipoprotein cholesterol (HDL-C) was measured after precipitation with phosphotungstate on a Cobas-Mira analyzer (Roche). All of the blood measurements were performed in the same laboratory (Centre of Preventive Medicine of Vandoeuvre-lès-Nancy, France) and monitored by the French national quality control scheme for biomedical samples.

In Iran, serum glucose, total cholesterol, HDL-C, and TG were assayed using standard techniques with the Cobas autoanalyzer system (ABX Diagnostics, Montpellier, France). Blood measurements were performed in the laboratories under the scrutiny of the state health center and were monitored by the Iranian national quality reference control scheme. Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated for both populations according to the Friedewald formula [LDL-C = total cholesterol – HDL-C – (TG/2.2) when TG was < 4.52 mmol/L, all analytes being expressed in mmol/L].

Metabolic syndrome was confirmed, according to the definition by the National Cholesterol Education Program Adult Treatment Panel (NECP ATP III NC),[3] if a subject met more than three of the following criteria: Waist circumference ‡ 88 cm in women or ‡ 102 cm in men; TG ‡ 1.7 mmol/L or drug treatment for elevated TG; HDL-C £ 1.03 mmol/L in men or £ 1.3 mmol/L in women or drug treatment for reduced HDL-C; SBP ‡ 130 mmHg or DBP ‡ 85 mmHg or antihypertensive drug treatment; fasting glucose ‡ 5.6 mmol/L or drug treatment for elevated glucose.

Data analysis

Statistical analyses were performed using the SAS software package version 9.2 (SAS Institute Inc., Cary, NC). For continuous variables, differences in characteristics between countries or age were tested using analyses of variance with interaction terms (ANOVA). Student–Newman–Keuls multiple-range tests were performed to compare the four population groups. For categorical variables, differences between countries or ages were analyzed using chi-squared tests. Participants were placed in four age groups. Pearson correlation coefficients were calculated between the components of metabolic syndrome in each population group, and statistical significance for group interaction was estimated. Factor analyses were then undertaken and followed by orthogonal (varimax) rotations to assist interpretation of the factors and to ensure that they were uncorrelated. We determined the number of factors to retain using the Scree test. The Scree plot is a plot of the eigenvalues of derived factors. Only variables with factor loading having absolute values greater than 0.20 were shown (see Table 4, below). For all tests, statistical significance was set at P £ 0.05.

Results

The general characteristics and components of metabolic syndrome according to sex and age are presented in Table 1. In men, weight, height, BMI, waist circumference, and concentrations of total cholesterol, HDL-C, and fasting glucose were significantly lower in the Iranians than in the French, whereas DBP, TG concentration, the level of LDL-C, and the number of ATP III criteria were significantly higher. SBP in men was not significantly different between the countries. In women, differences between Iran and France were significant for all the variables of interest, except for weight and fasting glucose concentration. Height, total cholesterol, and HDL-C were lower in Iran in comparison with France. Conversely, BMI, waist circumference, DBP and SBP, TG, LDL-C, and the number of ATP III criteria were significantly higher in Iranian women.

In both countries, for men and for women, waist circumference, DBP and SBP, the level of LDL-C (except for Iranian men), and the number of ATP III criteria significantly increased with age. All lipid profile components to HDL-C ratios, including LDL-C/HDL-C, TG/HDL-C, and total cholesterol/HDL-C, were higher in Iranian men and women than in their French counterparts. Levels of LDL-C were higher in Iranian people than in French individuals.

Height was significantly and inversely related to age only in men of both countries, whereas weight did not differ, whatever the population group, and BMI slightly increased with age only in French men. In women of both countries and in French

Table 1. General Characteristics and Components of Metabolic Syndrome in Iranian and French Samples Aged 36–55 Years[a]

		Men				P ANOVA[b]			Women				P ANOVA[b]		
		36–40 years	41–45 years	46–50 years	51–55 years	Age	Country	Interaction	36–40 years	41–45 years	46–50 years	51–55 years	Age	Country	Interaction
Number	Iran	135	161	148	145				146	157	167	134			
	France	50	240	269	119				108	311	203	86			
Age (years)	Iran	38.1 – 1.4	43.3 – 1.4	47.7 – 1.3	52.9 – 1.5	—	—	—	38.1 – 1.5	43.0 – 1.5	48.0 – 1.5	53.0 – 1.5	—	—	—
	France	39.0 – 0.9	43.2 – 1.3	47.7 – 1.4	52.5 – 1.4				38.4 – 1.4	43.0 – 1.4	47.8 – 1.5	52.4 – 1.5			
Weight (kg)	Iran	69.1 – 13.1	70.7 – 12.1	70.1 – 12.1	68.7 – 12.7	0.729	£ 0.001	0.392	64.1 – 13.4	65.4 – 12.8	66.1 – 13.6	63.6 – 13.4	0.881	0.365	0.332
	France	79.3 – 8.9	79.1 – 11.2	79.6 – 11.4	80.4 – 12.2	0.519			63.9 – 10.1	63.2 – 11.9	64.3 – 11.4	64.8 – 9.9	0.556		
Height (m)	Iran	1.68 – 0.06	1.69 – 0.07	1.67 – 0.06	1.67 – 0.07	0.006	£ 0.001	0.612	1.54 – 0.06	1.55 – 0.06	1.55 – 0.06	1.53 – 0.05	0.079	£ 0.001	0.282
	France	1.75 – 0.06	1.74 – 0.06	1.73 – 0.07	1.73 – 0.06	0.006			1.61 – 0.06	1.61 – 0.06	1.60 – 0.06	1.60 – 0.06	0.158		
Body mass index (kg/m^2)	Iran	24.1 – 3.9	24.6 – 3.9	25.1 – 3.7	24.4 – 3.8	0.289	£ 0.001	0.224	26.7 – 5.3	27.1 – 4.8	27.5 – 5.5	26.9 – 5.2	0.594	£ 0.001	0.558
	France	25.7 – 2.9	25.8 – 3.3	26.4 – 3.3	26.7 – 3.6	0.033			24.4 – 3.8	24.3 – 4.6	25.0 – 4.4	25.1 – 4.3	0.190		
Waist circumference (cm)	Iran	86.8 – 14.2	87.6 – 13.4	90.4 – 12.7	89.8 – 12.7	0.016	0.012	0.602	90.7 – 13.2	93.5 – 14.2	93.8 – 14.6	94.8 – 13.5	0.015	£ 0.001	0.457
	France	88.4 – 7.7	89.3 – 8.9	91.0 – 9.3	92.8 – 9.2	0.002			76.2 – 9.0	76.1 – 10.3	77.8 – 10.0	79.2 – 10.1	0.027		
Diastolic blood pressure (mmHg)	Iran	80.8 – 10.1	80.1 – 10.3	82.7 – 10.9	82.6 – 10.6	0.041	£ 0.001	0.099	75.4 – 12.9	79.4 – 12.7	81.7 – 14.7	84.1 – 15.3	£ 0.001	£ 0.001	0.309
	France	72.8 – 8.3	76.5 – 9.3	77.6 – 10.2	79.5 – 11.5	£ 0.001			69.4 – 9.8	70.1 – 9.9	73.4 – 9.5	77.4 – 8.9	£ 0.001		
Systolic blood pressure (mmHg)	Iran	122.6 – 14.3	122.2 – 13.4	128.6 – 18.8	130.6 – 17.4	£ 0.001	0.089	0.205	119.6 – 16.1	123.5 – 17.7	129.8 – 19.8	137.4 – 23.7	£ 0.001	£ 0.001	0.074
	France	123.1 – 11.1	126.0 – 11.5	128.1 – 12.5	132.7 – 16.0	£ 0.001			117.0 – 11.4	118.1 – 13.2	121.9 – 13.6	127.7 – 15.2	£ 0.001		
Total cholesterol (mmol/L)	Iran	4.88 – 0.93	4.88 – 1.01	4.88 – 0.81	4.96 – 1.00	0.513	£ 0.001	0.104	4.77 – 0.90	4.87 – 1.05	5.18 – 1.21	5.43 – 0.91	£ 0.001	£ 0.001	0.285
	France	5.64 – 0.84	5.89 – 1.01	6.09 – 1.07	6.06 – 0.98	0.005			5.55 – 1.12	5.46 – 0.83	5.68 – 0.85	6.14 – 0.93	£ 0.001		
HDL-C (mmol/L)	Iran	0.80 – 0.31	0.79 – 0.36	0.83 – 0.38	0.80 – 0.28	0.861	£ 0.001	0.573	0.89 – 0.30	0.87 – 0.27	0.88 – 0.39	0.87 – 0.27	0.625	£ 0.001	0.220
	France	1.35 – 0.34	1.43 – 0.43	1.40 – 0.38	1.40 – 0.35	0.537			1.69 – 0.42	1.72 – 0.44	1.79 – 0.43	1.79 – 0.50	0.049		
LDL-C (mmol/L)	Iran	3.40 – 0.89	3.42 – 1.00	3.40 – 0.76	3.47 – 0.93	0.900	£ 0.001	0.133	3.24 – 0.79	3.43 – 0.98	3.61 – 1.06	3.81 – 0.88	£ 0.001	£ 0.001	0.206
	France	3.76 – 0.77	3.96 – 0.95	4.14 – 0.91	4.15 – 0.88	0.009			3.45 – 0.84	3.41 – 0.83	3.53 – 0.84	3.93 – 0.94	£ 0.001		
LDL-C/HDL-C	Iran	4.65 – 2.01	5.01 – 2.55	4.47 – 1.73	4.67 – 2.11	0.162	£ 0.001	0.076	4.01 – 1.85	4.36 – 1.97	4.58 – 2.17	4.80 – 2.09	0.010	£ 0.001	0.078
	France	2.96 – 0.91	3.07 – 1.36	3.16 – 1.15	3.16 – 1.09	0.633			2.18 – 0.79	2.17 – 0.94	2.12 – 0.79	2.43 – 1.09	0.051		
Cholesterol/HDL-C	Iran	7.19 – 4.69	7.11 – 3.15	6.72 – 2.83	7.61 – 7.20	0.460	£ 0.001	0.309	5.98 – 2.88	6.25 – 2.79	6.68 – 3.04	7.05 – 3.07	0.013	£ 0.001	0.058
	France	4.39 – 1.14	4.51 – 1.62	4.68 – 1.61	4.58 – 1.34	0.484			3.49 – 1.34	3.39 – 1.05	3.34 – 0.89	3.71 – 1.26	0.050		
Triglycerides (TG) (mmol/L)	Iran	1.95 – 1.24	1.78 – 0.97	1.85 – 1.28	1.97 – 1.23	0.753	£ 0.001	0.567	1.70 – 1.13	1.57 – 0.92	1.89 – 1.10	2.07 – 1.25	0.001	£ 0.001	0.109
	France	1.42 – 0.83	1.42 – 1.01	1.47 – 1.07	1.39 – 0.98	0.907			1.25 – 3.58	0.90 – 0.42	0.98 – 0.57	1.15 – 0.56	0.701		
TG/HDL-C	Iran	3.44 – 5.14	2.89 – 2.54	3.07 – 3.83	3.27 – 4.23	0.659	£ 0.001	0.663	2.34 – 2.51	2.31 – 2.76	2.70 – 2.73	3.06 – 3.21	0.084	£ 0.001	0.828
	France	1.18 – 0.85	1.19 – 1.11	1.28 – 1.67	1.14 – 1.10	0.752			1.00 – 4.22	0.59 – 0.42	0.60 – 0.43	0.75 – 0.59	0.167		
Fasting glucose (mmol/L)	Iran	4.76 – 0.86	4.92 – 1.57	5.06 – 1.94	4.96 – 1.47	0.201	£ 0.001	0.872	4.61 – 0.97	4.73 – 1.04	5.22 – 1.84	5.25 – 1.88	£ 0.001	0.489	0.046
	France	5.14 – 0.43	5.25 – 0.70	5.40 – 1.01	5.43 – 0.56	0.014			4.85 – 0.42	4.92 – 0.47	5.06 – 0.54	5.16 – 0.56	£ 0.001		
Number of criteria[c]	Iran	1.8 – 1.1	1.9 – 1.1	2.0 – 1.2	2.2 – 1.2	0.005	£ 0.001	0.958	2.3 – 1.0	2.5 – 1.1	2.8 – 1.2	3.0 – 1.1	£ 0.001	£ 0.001	0.248
	France	1.0 – 1.1	1.1 – 1.1	1.2 – 1.1	1.4 – 1.1	0.019			0.5 – 0.8	0.6 – 1.0	0.7 – 0.9	1.0 – 1.1	0.003		

[a]Values are expressed as the frequencies for number of individuals and mean – standard deviation for the rest of variables.
[b]P ANOVA, age (P for trend), country differences, and age$country interaction.
[c]ATP III criteria (AHA/NHLBI statement from 2005) are three out of the five of the following: (1) WC ‡ 88 cm (women) or ‡ 102 cm (men); (2) triglycerides ‡ 1.7 mmol/L or drug treatment for elevated triglycerides; (3) HDL-C £ 1.3 mmol/L (women) or < 1.03 mmol/L (men) or drug treatment for reduced HDL-C; (4) systolic blood pressure ‡ 130 mmHg or diastolic blood pressure ‡ 85 mmHg or antihypertensive drug treatment; and (5) fasting glucose ‡ 5.6 mmol/L or drug treatment for elevated glucose.

ANOVA, analysis of variance; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ATP III, Adult Treatment Panel III; AHA/NHLBI, American Heart Association/National Heart, Lung and Blood Institute; WC, waist circumference.

men, cholesterol and fasting glucose significantly increased with increasing age. Age was significantly related to TG in Iranian women and to HDL-C in French women.

Consequently, the prevalence of metabolic syndrome was significantly higher in Iranians than in the French of either sex (Table 2). Student–Newman–Keuls multiple range tests were performed to compare the four population groups, after adjustment for age. Metabolic syndrome prevalence was significantly higher in Iranian women (55.0%), followed by Iranian men (30.1%), French men (13.7%), and French women (6.6%). Iranian women were characterized by high rates of abnormality in abdominal obesity (65.0%), HT (52.1%), hypertriglyceridemia (43.1%), and low HDL-C (92.7%). Iranian men were characterized by high rates of HT (48.9%), hypertriglyceridemia (42.8%), and low HDL-C (81.8%). French men had high rates of HT (44.7%) and mild rates of hypertriglyceridemia (28.6%) and hyperglycemia (23.9%).

Metabolic syndrome component rates increased steadily with age in the four sex/country groups (Table 2). In men, the prevalence of the individual components of metabolic syndrome, hypertriglyceridemia and low HDL-C was significantly higher in Iran than in France, whereas high fasting glucose was more prevalent in French men. In women, high waist circumference, high blood pressure, hypertriglyceridemia, and low HDL-C were significantly more prevalent in Iran than in France. In men only, the prevalence of high waist circumference increased with age, whereas hypertriglyceridemia increased with age in women only.

Correlation coefficients between the components of metabolic syndrome according to sex and country are presented in Table 3. All coefficients were significantly different from 0 (P £ 0.001). Relationships between all of the metabolic syndrome components that correlated significantly were stronger in French individuals than in Iranians.

Factor analysis identified two major factors—''blood pressure'' and ''lipids'' (Table 4). In the two groups of Iranians and in French men, the ''blood pressure'' factor included DBP, SBP, and waist girth; the ''lipids'' factor included TG, fasting glucose, waist circumference, and HDL-C. In French women, fasting glucose aggregated with components of ''blood pressure'' factors.

Discussion

This is the first study conducted in French and Iranian populations to investigate common points as well as differences in metabolic syndrome between the two countries. The main findings of this study are a high prevalence of metabolic syndrome and multiple and questionable prevalence of low HDL-C and high waist circumference in Iranian women compared with French women. Although these wide differences may be derived from different laboratory methods used in the two countries, these findings agree with previous studies that advised screening for the progression of metabolic syndrome in Iran, including the measurement of waist circumference, TG, and HDL-C.[8–10] In addition, HDL-C was associated with waist circumference in our analysis, which suggests a relationship between increased waist circumference and decreased HDL-C concentrations.

Several studies have shown a high prevalence of metabolic syndrome, low HDL-C, and waist circumference in Iranian adults and adolescents[11–26] as well as in other Middle Eastern populations.[27–29] These studies also indicate that there is a higher prevalence of metabolic syndrome among Iranian women than among Iranian men. There is a variation in the prevalence of metabolic syndrome in adults between the different geographical parts of Iran,[4,8,23,25] from 23.7% in the west to 50% in the south.[23,25] Although these studies note small differences in the prevalence of metabolic syndrome and related abnormalities around Iran,[23] low HDL-C is mentioned as the predominant abnormality in several geographic parts of this country,[11–26] consistent with our results. Sharifi et al.[25] found that low HDL-C was the most prevalent abnormality in 2,941 Iranians over 20 years of age (prevalence of low HDL-C was 73%, with 63% for men and 93.3% for women). Azizi et al. also showed that among people affected by metabolic syndrome, low HDL-C was the most common abnormality (91%).[9] Evidence indicates a higher prevalence of low levels of HDL-C in Iranian adults and youths than in American, Swedish, or German populations.[30,31] The high prevalence of low HDL-C, even in nonobese and normo-triglyceridaemic individuals, supports ethnic predisposition. The findings of Koochek et al.[32] illustrated the significant association between migration from Iran to Sweden and the prevalence of HT and smoking, but not dyslipidemia, and consequently provide further confirmatory evidence of such ethnic predisposition to low HDL-C.[30,32]

The prevalence of metabolic syndrome in France is lower than in North America and in other European countries.[33] The Monica study[34] highlighted a French north–south gradient for metabolic syndrome, with higher prevalence in Lille than in Toulouse. The prevalence of metabolic syndrome in France varies from 11.7% in men to 7.5% in women, according to the NCEP ATP III.[33–42] Our results showed that the prevalence of metabolic syndrome in the STANISLAS subsample is in this range. Our study also revealed that high blood pressure was the most prevalent abnormality in French individuals. This finding is consistent with previous studies.[37,38]

Why is metabolic syndrome more prevalent among Iranian people, particularly women, than among the French population? Should we expect a higher cardiovascular mortality rate in Iranian women in the next decades? One reason that might explain these differences could be that the French participants in this study were apparently healthy, but when compared with other published reports,[35,37,38] there are no noteworthy differences between our study population and other populations.

The Iranian dietary pattern is significantly associated with dyslipidemia, particularly with a high prevalence of low HDL-C and high waist circumference.[43] In line with concepts concerning nutritional effects, recent meta-analysis documents have shown the effect of the Mediterranean dietary pattern in increasing levels of HDL-C in developed societies as it does in southern European countries.[44] Richard et al. also recently reported that even without weight loss, the Mediterranean diet leads to significant changes in the blood lipid profile.[45] Consistent with the effect of environmental factors on HDL-C levels, this is more marked in Iranian women because they have a sedentary lifestyle, use labor-saving devices, have physically inactive leisure activities, and consume high-calorie diets rich in fat.[8] Interestingly, Esmaillzadeh et al.[46] found an independent association between major Iranian dietary patterns and plasma concentrations of inflammatory markers such as interleukin 6 and C-reactive protein (CRP). It has also been reported that CRP is strongly associated with stable coronary artery disease and

Table 2. Prevalence of Metabolic Syndrome, Its Components According to ATP III Criteria (AHA/NHLBI Statement)[a]

Metabolic syndrome criteria[b]		Men				P			Women				P		
		36–40 years	41–45 years	46–50 years	51–55 years	Age[c]	Country[d]	Interaction[e]	36–40 years	41–45 years	46–50 years	51–55 years	Age	Country	Interaction[e]
High waist circumference	Iran	0.103	0.130	0.189	0.172	0.048	0.123	0.865	0.609	0.668	0.648	0.671	0.361	£ 0.001	0.701
	France	0.080	0.088	0.134	0.160	0.024			0.150	0.140	0.163	0.186	0.417		
High blood pressure	Iran	0.423	0.428	0.540	0.565	0.003	0.104	0.455	0.349	0.445	0.607	0.686	£ 0.001	£ 0.001	0.455
	France	0.320	0.408	0.446	0.580	£ 0.001			0.178	0.198	0.281	0.337	0.001		
High triglyceride	Iran	0.422	0.409	0.405	0.475	0.387	£ 0.001	0.574	0.404	0.343	0.458	0.529	0.008	£ 0.001	0.851
	France	0.280	0.271	0.309	0.269	0.822			0.065	0.068	0.089	0.163	0.023		
Low HDL-C	Iran	0.822	0.819	0.777	0.855	0.633	£ 0.001	0.260	0.911	0.942	0.934	0.917	0.874	£ 0.001	0.484
	France	0.160	0.142	0.152	0.109	0.458			0.168	0.179	0.113	0.160	0.113		
High glucose	Iran	0.081	0.093	0.114	0.137	0.049	£ 0.001	0.987	0.041	0.076	0.142	0.186	£ 0.001	0.488	0.725
	France	0.160	0.217	0.249	0.294	0.033			0.009	0.084	0.128	0.198	£ 0.001		
Metabolic syndrome	Iran	0.259	0.254	0.297	0.393	0.009	£ 0.001	0.975	0.431	0.496	0.631	0.641	£ 0.001	£ 0.001	0.705
	France	0.100	0.108	0.145	0.193	0.022			0.028	0.065	0.069	0.105	0.039		

Values are expressed as the frequency.

[a]Frequency.

[b]ATP III criteria (AHA/NHLBI statement from 2005) are three of the five following: (1) WC ‡ 88 cm (women) or ‡ 102 cm (men); (2) triglycerides ‡ 1.7 mmol/L or drug treatment for elevated triglycerides; (3) HDL-C < 1.3 mmol/L (women) or < 1.03 mmol/L (men) or drug treatment for reduced HDL-C; (4) SBP ‡ 130 mmHg or DBP ‡ 85 mmHg or antihypertensive drug treatment; and (5) fasting glucose ‡ 5.6 mmol/L or drug treatment for elevated glucose.

[c]Cochran–Armitage trend test.

[d]Chi-squared test.

[e]Test for age$country interaction by using logistic regression model.

HDL-C, high-density lipoprotein cholesterol; ATP III, Adult Treatment Panel III; AHA/NHLBI, American Heart Association/National Heart, Lung and Blood Institute.

Table 3. Pearson Correlation Coefficients Between the Components of the Metabolic Syndrome According to Sex and Countries

	Components	SBP	Interaction P value	DBP	Interaction P value	TG	Interaction P value	Waist girth	Interaction P value	Glucose	Interaction P value
DBP U	Iran men	0.711***	£ 0.001[a]	—		—		—		—	
	Iran women	0.762***		—		—		—		—	
	France men	0.718***		—		—		—		—	
	France women	0.777***		—		—		—		—	
TG U	Iran men	0.124**	0.366	0.039[b]	0.048	—				—	
	— Iran women	0.155***		0.122**		—				—	
	— France men	0.158***		0.157***		—				—	
	— France women	0.141***		0.144***		—				—	
	—										
Waist circumference	Iran men	0.251***	0.019	0.217***	0.505	0.232***	£ 0.001	—		—	
	Iran women	0.241***		0.235***		0.146***		—		—	
	France men	0.326***		0.298***		0.333***		—		—	
	France women	0.385***		0.309***		0.142***		—		—	
Fasting U glucose	Iran men	0.049	0.057	0.072[b]	0.951	0.124***	£ 0.001	0.204***	0.242	—	
	Iran women	0.194***		0.120**		0.143***		0.203***		—	
	France men	0.151***		0.145***		0.253***		0.267***		—	
	France women	0.254***		0.195***		0.002		0.266***		—	
HDL-C U	Iran men	- 0.100*	0.609	- 0.032[b]	0.328	- 0.466***	£ 0.001	- 0.145***	£ 0.001	- 0.077[b]	0.194
	Iran women	- 0.069		- 0.108**		- 0.358***		- 0.133***		- 0.084*	
	France men	- 0.076*		- 0.119**		- 0.433***		- 0.297***		- 0.121**	
	France women	- 0.082*		- 0.081*		- 0.184***		- 0.369***		- 0.040[b]	

[a]Significance for group interactions.
[b]NS, Not significant.
* £ 0.05, ** £ 0.01, *** £ 0.001: test to 0 for correlation coefficients.
U DBP, diastolic blood pressure; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

that its measurement may thus improve coronary risk assessment in Iranian patients with coronary artery disease.[47] Interestingly, serum high-sensitivity CRP is an independent predictor of coronary artery disease defined by angiography in the Iranian population.[48]

For both countries, our analysis showed correlation between waist circumference and TG, and an inverse relationship with HDL-C. These findings agree with the study by Chateau-Degat et al.,[49] who reported that the presence of abdominal obesity, increased levels of TG, and low levels of HDL-C are more prevalent in Canadian women than in men of different ethnicities. Interestingly, studies by Nabipour et al.[23] in Iran and Menotti et al.[50] in Italy have shown that, of the components of metabolic syndrome, low HDL-C is significantly and independently associated with nonfatal ischemic heart disease in the Iranian population, and its presence is also a predictor of coronary heart disease and cardiovascular disease.[50] In a meta-analysis, Hagdoost et al.[51] reported greater prevalence and risk of HT, as a component of metabolic syndrome, in Iranian women in comparison with men. Another Iranian study[52] indicated that the hazard ratio of metabolic syndrome components of ischemic heart disease is higher in Iranian women at 1.72 (1.08–2.74) than in men, at 1.58 (1.06–2.35).

In addition to the environmental factors and ethnic differences above, Kathireasan et al.[53] proposed a genetic risk score using 14 genome-wide significant single-nucleotide polymorphisms for HDL-C. In their opinion, genetic risk scores are strongly correlated with HDL-C levels. However, the difference in HDL-C levels between the most 'deleterious' and the most 'favorable' genetic score was 0.306 mmol/L. Previous studies have reported great differences between Middle Eastern countries and other countries regarding the prevalence of low HDL-C and hypertriglyceridemia.[5,9,25,28] For

Table 4. Results of Factor Analysis with Metabolic Syndrome Components and Factor Loadings in the Four Groups[a]

	Iranian men		Iranian women		French men		French women	
Components	Factor 1: BP	Factor 2: Lipids	Factor 1: BP	Factor 2: Lipids	Factor 1: BP	Factor 2: Lipids	Factor 1: BP	Factor 2: Lipids
SBP	0.775	—[b]	0.816	—	0.782	—	0.832	—
DBP	0.780	—	0.806	—	0.769	—	0.810	—
TG	—	0.611	—	0.505	—	0.607	—	0.251
Waist girth	0.259	0.332	0.251	0.279	0.295	0.490	0.310	0.547
Fasting glucose	—	0.201	—	0.252	—	0.304	0.254	—
HDL-C	—	- 0.562	—	- 0.482	—	- 0.549	—	- 0.506

[a]Factor loadings represent the correlations between the variables and the factors.
[b]Factor loading < 0.20.
BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

instance, 37% of U.S. adults[54] and 69% of Middle Eastern populations have low HDL-C levels.[9] These differences are greater than could be explained by genetic factors alone.

As an answer, although the difference in the prevalence of metabolic syndrome in the two countries supports the het- erogeneity of metabolic syndrome,[49,54] we might suppose that Iranian women are more at risk of cardiovascular dis- ease than their European counterparts. Although it seems that environmental factors, including diet and drink patterns as well as physical activity, may play a considerable role in explaining the main differences in the prevalence of meta- bolic syndrome, particularly in women of the two countries, the contribution to these differences made by genetic factors should ultimately be considered.

Study limitations and strengths

Analytical variations in laboratory data and anthropo- metric measurements may have some impact on the differ- ences that we observed in the two populations. However, our findings are consistent with ethnic differences previously highlighted among children and the adolescent population. The main strength of our study is its novelty in comparing two populations, one from Europe and one from Asia. Our results provide confirmatory evidence that Asians are ethnically predisposed to the HDL-C disorder and metabolic syndrome documented in populations of children and adolescents.

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Author Disclosure Statement

We declare that there are no competing financial interests.

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