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(54) **Title:** ASSOCIATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR GENETIC VARIANT WITH METABOLIC SYNDROME

(57) **Abstract:** Methods and kits for use in patient diagnosis are described. The presence of wild type rs10738760 in patient samples is associated with increased risk of metabolic syndrome.

Association of Vascular Endothelial Growth Factor Genetic Variant with Metabolic Syndrome

Background of the Invention

Metabolic syndrome (MetS) refers to a cluster of metabolic risk factors linked with impaired angiogenesis and often insulin resistance. In its advanced form, clinical fasting hyperglycemia or even type 2 diabetes is present. The syndrome has been broadened to encompass features such as central obesity, glucose abnormalities, dyslipidemia, elevated blood pressure, and low grade inflammation state with a pro-thrombotic circumstance ('components' of MetS). The risk of atherosclerotic cardiovascular disease and type 2 diabetes is greatly increased in patients with MetS. The prevalence of MetS in adults in developed countries, mainly due to the emerging "obesity epidemic", is as high as 35-40%. Several studies have revealed that ischemic tissues neovascularization is impaired in type 2 diabetes with obesity, hypercholesterolemia, or hypertension. Vascular endothelial growth factor (VEGF) is a multifunctional cytokine and is functional in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis. Adipocytes produce VEGF, which may act as an angiogenic and vascular survival factor for the omental vasculature. 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. A positive association of VEGF circulating levels with MetS as well as with a number of its components has been shown (Lieb et al. 2009). Four single nucleotide polymorphisms (SNPs), explaining ~50% of VEGF heritability, were recently identified using a genome-wide association study (GWAS) (Debette et al. 2011). Furthermore, a significant number of genetic variants have been shown to be associated with MetS and its components (Kraja et al. 2011; Kristiansson et al. 2012). There are numerous criteria used by various international bodies to classify MetS (e.g. Grundy et al. 2004 and 2005) including abdominal obesity (usually waist circumference, various cut-offs), BMI (various cut-offs), systolic blood pressure (various cut-offs), diastolic blood pressure (various cut-offs), antihypertensive medication, type 2 diabetes, family history of type 2 diabetes/CVD/hypertension, impaired fasting glucose, impaired glucose tolerance, 2-hour post glucose challenge, triglyceride levels and HDL cholesterol levels (various cut-offs). This lack of an internationally agreed criteria has purportedly contributed to the varied prevalence of MetS that has been reported in various studies (Cai et al. 2012). Thus MetS diagnosis is

based on varied criteria each involving several individual test measurements; a more simplistic and definitive testing method for MetS diagnosis is, therefore, desirable.

FIGURES

Figure 1 nucleotide sequence of the wild-type mutant rs10738760

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SUMMARY OF THE INVENTION

The pathological outcomes of metabolic syndrome are type 2 diabetes and cardiovascular disease. Currently, its diagnosis is dependent upon non-standardized criteria and requires physical examination and several biochemical assays. Following the identification of four SNPs which explained the heritibality of serum VEGF levels (rs6993770, rs6921438, rs4416670 and rs10738760 - Debette *et al.* 2011), the association of MetS and its components with genetic variants, and the possibility of a relationship between VEGF and MetS, it was decided to test whether one or more of the four previously discovered SNPs was associated with MetS and its components. The invention describes the identification of a single nucleotide polymorphism, rs10738760, whose absence is predictive of increased risk of metabolic syndrome. The identification of this genetic component to metabolic syndrome aids the implementation of standardized criteria for recognizing the condition and enables a simple, cost-effective method and kit to help predict the presence or absence of MetS.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure describes a method of aiding the diagnosis of metabolic syndrome in a patient comprising detecting the presence or absence of the single nucleotide polymorphism rs10738760 in an *in vitro* sample taken from the patient. rs10738760, first identified in a GWAS VEGF heritability study, is located on chromosome 9p24.2 and is identified herein as base 201 of SEQ. ID No. 1. The mutant nucleotide at this position is adenine (A) and its occurrence in an *in vitro* patient sample is not associated with an increased or decreased likelihood that the patient has MetS; the presence of guanine (G), the wild type nucleotide, in an *in vitro* patient sample, is associated with an increased likelihood that the patient has MetS. The sample is preferably a nucleic acid sample, the original patient sample, as is standard in the art, having undergone any necessary preparatory steps prior to analysis.

Also described are kits for detecting mutant and/or wild type rs10738760 which include either a single probe to wild type or mutant rs10738760 or two probes, one probe specific to mutant rs10738760 and one probe specific to wild type rs10738760. The kits are preferably used to help diagnose metabolic syndrome.

As used herein, rs10738760 refers to the mutant rs10738760 (nucleotide at base 201 SEQ. ID No. 1= adenine), unless otherwise qualified; for example, reference to wild type rs10738760 implies the nucleotide guanine (G) is present at base 201 of SEQ. ID No. 1. As used herein, an 'allelic variation' refers to a variation in the nucleic acid and typically primary amino acid sequence of a gene in one or more alleles in a subject, such as a human patient. Allelic variations include single or multiple nucleic acid and amino acid substitutions, additons or deletions that have any one of a number of effects on protein expression, including: promoter activity that regulates transcription, frame-shrift, early protein termination, protein mis-folding, altered protein processing, destruction (or enhancement) of active sites or binding sites of a protein, mis-splicing of a mRNA or any other property of a nucleic acid or protein that effects the expression and/or function of the final gene products.

The source of the sequence referenced herein is the dbSNP database of the NCBI (http://www.ncbi.nim.nih.gov/snp). Detailed methods used to detect the rs10738760 SNP are described in *Debette et al.* (2011) and the Supplementary Material (Data Supplement at http://circres.ahajournals.org/content/ 109/5/554/suppl/DC1). A brief overview of these methods follows; reference to example(s) are not to be construed as being limited to only the described example(s).

A large number of methods, including high throughput methods, are available for detection of SNPs and /or other allelic variations, for example the PCR and restriction fragment length polymorphisms methods. DNA from a sample is sequenced (re-sequenced) by any method to identify a SNP or small allelic variation. A large variety of re-sequencing methods are known in the art, including high-throughput methods. Amplification-based methods are also available to identify allelic variations, such as SNPs, including: PCR, reverse transcriptase PCR (RT-PCR), isothermic amplification, nucleic acid sequence based amplification (NASBA), 5' fluorescence nuclease assay (for example TAQMAN assay), molecular beacon assay and rolling circle amplification. Other methods such as Restriction Fragment Length Polymorphisms RFLP, also may be employed – as is appropriate to identify variant allele(s). Assays may be multiplexed, meaning two or more reactions are conducted simultaneously in the same physical location such as in the same tube or on the same substrate such as a

biochip, ensuring the reaction products of the multiplexed reactions can be distinguished. For example, TAQMAN or molecular beacon assays can be multiplexed by use of and by monitoring of accumulation or depletion of two different flurochromes corresponding to two different sequence-specific probes. In most cases, the appropriate method depends upon personal experience, available equipment and reagents, the need for high throughput and/or multiplexed methods, cost, required accuracy, and the skill levels of the technicians responsible for assay implementation. Design and implementation of these techniques are broadly-known and readily applicable by the skilled person in the art. In the implementation of the methods described herein, an array may be utilized. Arrays are particularly useful for high-throughput assays. The array typically comprises one or more reagents, for example, nucleic acid primers and/or probes, for identifying in a nucleic acid sample from a subject the occurrence of an allelic variation corresponding to one or more SNPs. As used herein, the term 'array' refers to reagents facilitating identification of allelic variations ina gene located at two or more identifiable locations. In one embodiment, an array is an apparatus at two or more discrete, identifiable reaction chambers, such as a 96 well dish, in which reactions comprising identified constituents are performed. In an exemplary embodiment, two or more nucleic acid primers or probes are immobilized onto a substrate in a spatially addressablemanner so that each individual primer or probe is located at a different and (addressable) identifiable location on the substrate. Substrates include multi-well plates, ceramic chips and beads. In one embodiment, the array comprises two or more sets of beads, with each bead having an identifiable marker, such as a quantum dot or fluorescent tag, so that the beads are individually identifiable using, for example, a flow cytometer. In one embodiment, in the context of the present disclosre, an array may be a multi-well plate containing two or more well reaction chambers with primers for amplifying DNA to identify SNPs or probes for binding specific sequences. As such, reagents, such as probes and primers may be bound or otherwise deposited onto or into specific locations on an array. Reagents may be in any suitable form, including: in solution, dried, lyophilized or glassified. Useful array technologies include, for example, Affymetrix GeneChip® Array, GenChip® CustomSeq® Resequencing Arrays (commercially available from Affymetrix Inc. of Santa Clara, California). Informatics and/or statistical software or other computer-implemented processes for analyzing array data and/or identifying genetic risk factors from data obtained from a patient sample, are known in the art.

Materials and Methods

<u>Study population</u>

This study is based on the STANISLAS Family study (SFS), a 10-year longitudinal survey involving 1,006 volunteer families from Vandoeuvre-lès-Nancy, France (Herbeth *et al.* 2010). 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.

<u>Anthropometric indices, medical history and lifestyle data</u>

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 (Azimi-Nezhad *et al.* 2012). 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 (Azimi-Nezhad *et al.* 2012). MetS was defined based on the International Diabetes Federation (IDF) criteria (Nezhad *et al.* 2008): WC ≥94 cm in men or ≥80 cm in women plus any two of the four

following criteria i) triglyceride ≥1.7 mmol/l or drug treatment for elevated triglyceride, ii) high-density lipoprotein cholesterol (HDL-C) < 1.03 mmol/l in men or <1.3 in women or having medication for reduced HDL-C, iii) SBP ≥ 130 mmHg or DBP ≥ 85 mmHg or anti-hypertensive medication, iv) 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 ®) (Herbeth et al. 2010).

Genotyping

The SNPs rs6921438, rs4416670, rs6993770, rs10738760 were genotyped by Genoscreen (http://genoscreen.fr), using a Sequenom iPLEX Gold assay–Medium Throughput Genotyping Technology (Ehrich et al. 2005).

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) (Purcell *et al.* 2007) 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 $\geq$ 80% were considered in linkage disequilibrium (LD). The GWAS investigator of HuGENavigator engine (Yu *et al.* 2008) and the NHGRI Catalog of published GWAS (http://www.genome.gov/gwasstudies) (Hindorff *et al.* 2009) 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. No statistically significant associations were observed between VEGF plasma levels and MetS as well as with its related components in this population.

Regarding the effect of the assessed SNPs, 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. 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.

The three previous GWAS (Kraja *et al.* 2011; Kristiansson *et al.* 2012; Zabaneh *et al.* 2010) conducted in relation to MetS and its components did not address the rs10738760 SNP (genotyped or in linkage disequilibrium with another SNP). Therefore, this is considered as a novel polymorphism for MetS.

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 (MAF)
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; CI: Confidence Interval

CLAIMS

1. A method of aiding the diagnosis of metabolic syndrome in a patient comprising detecting the presence or absence of wild type or mutant rs10738760 in an *in vitro* sample taken from the patient.

2. The method of Claim 1 in which the sample is a nucleic acid sample.

3. The method according to Claim 1 or Claim 2 in which the presence of wild type rs10738760 or the absence of mutant rs10738760 indicates that the patient has a higher likelihood of being diagnosed with metabolic syndrome.

4. A kit for detecting rs10738760 which includes either one or two probes; the single probe kit includes a probe which specifically binds to either mutant rs10738760 or wild type rs10738760, the two probe kit includes a probe which specifically binds to wild type rs10738760 and a probe which specifically binds to mutant rs10738760.

5. A kit according to Claim 4 for use in diagnosing metabolic syndrome.

Figure 1

SEQ. ID No. 1 – mutant rs10738760

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CGGGGCTGTT ATAACCAAGT ACCACAGACT GAGTGGCTTA CACAGCAGAA ATGTATTTCT
TCACAGTTCT GCAGGCTAGA AATCCAGAAT CAAGGTATCA GCAGAGTTTA TTTCTTTTCA
GGTCTCTCTC CTTTGTTTGT AGATTGCCAC CTTCTCCCTA TGTTTTCATA TGTTCTTCCT
TCTCTGTGTG TCTGTATCCT AATCTCTTCT TATAAGGATA CCAGTCATAG ATTGGATTTG
GGTTCACCCA AATGACCTCA TTTACCTTAA TGACTTCTTT AAAGACTATC TCTCCAAATA
TAGCCACATT CTGAGGGATT AGGGATTACG ACTTCAGCAC ATGAATTTTG AAGGTACACA
GTTCATCCCA TGACAGGGAC CTGTAGTAGT TTCTGGAGAG GGCACAAAGC AGATGGCAGC
TAATATCCAA CGAGAGTCCA CATGGAAAAT GCAATTTGTT TCTGTGAGAT GCAGCACCTC
CTGATGGAAG GAAGTTGGGA

AAATGAGCAT TTTGAAGACG CACTTTCCAC TTACCTTGGC ACCTGAAAAT CAACTAGGGC
TCATCTTTTC CAAATACCTC TAATCAACGG CAGCAAGGGA GAAGGGTGAA CTTTGGGCAT
AATGAAGATG ACAAAAGAAT TTTACTGACA ATGACTATGA TGAAAACTGT GAACAATTTA
TGATTTTATC CTCAGTACAA TGAGTGATAA AGGACAGGCA CACAGTGACT CACAATAGCT
ATGTAATTCC ATCATCACTA AGGCAGAGGT AAGAACACAC ACAGTGGGAC TGGGAATAGT
CTTCTGACCT TACCTTCACT CTCTTCCATA GGAGCCACAT TTTCTGGCTG ACATGTCCAA
ATCTCCTC TCTGTTCTAG TTACTTAACA GAAGAAGTTC ATAGACTTTG CTAACATACC
ATCTCAATAT CTTTAACTGG GAATGCATCA GTTGAATTCT GTAAAAGCAG GCTTTGCTTA
CTCTCAATAT TCCCTGTTTA
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A. CLASSIFICATION OF SUBJECT MATTER

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ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/024189 A2 (DAVID GLADSTONE INST [US]; VERDIN ERIC M [US]; HIRSCHEY MATTHEW D [US]) 23 February 2012 (2012-02-23) the whole document -----	1-5
X	A. T. KRAJA ET AL: "A Bivariate Genome-Wide Approach to Metabolic Syndrome: STAMPEED Consortium", DIABETES, vol. 60, no. 4, 8 March 2011 (2011-03-08), pages 1329-1339, XP55074098, ISSN: 0012-1797, DOI: 10.2337/db10-1011 cited in the application the whole document ----- -/--	1-5

X	Further documents are listed in the continuation of Box C.		X	See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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C(Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/04351 A2 (UNIV MANCHESTER [GB]; BRENCHLEY PAUL ERNEST CHARLES [GB]; WATSON CAROL) 18 January 2001 (2001-01-18) the whole document -----	1-5
X	S. DEBETTE ET AL: "Identification of cis- and trans-Acting Genetic Variants Explaining Up to Half the Variation in Circulating Vascular Endothelial Growth Factor Levels", CIRCULATION RESEARCH, vol. 109, no. 5, 14 July 2011 (2011-07-14) , pages 554-563, XP55074095, ISSN: 0009-7330, DOI: 10.1161/CIRCRESAHA.111.243790 cited in the application the whole document -----	1-5

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INTERNATIONAL SEARCH REPORT
Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 2012024189	A2	23-02-2012	NONE		
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