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(54) **Title**: METHOD FOR PREDICTING HYPERTENSION

(57) **Abstract**: The invention relates to materials and methods for predicting the onset of hypertension, in particular isolated systolic hypertension. The methods comprise measuring the concentration of bacterial 16S rDNA in a biological sample from a subject and comparing to a predetermined value.

Method for predicting hypertension

The present invention concerns methods and materials for predicting hypertension, in particular isolated systolic hypertension.

Hypertension is a leading cause of mortality and a major cause of severe disability, via its causative role in stroke, vascular dementia and end stage renal disease. Preventing or delaying the onset of hypertension is a major public health issue. However, little is known about the causative factors of essential hypertension.

Hypertension may be manifest as elevated systolic and diastolic blood pressure, or as elevated systolic pressure with diastolic pressure at or near normal levels ("isolated systolic hypertension" or ISH). Isolated systolic hypertension is common in the elderly, and may be due to an increase in the stiffness of the aorta with age. This stiffness increases the load on the ventricle and reduces the flow of blood to the heart, which can in time lead to coronary ischaemia, heart failure and other consequences of hypertension.

A complex interaction between genes and environmental factors is suspected to contribute to the onset of hypertension, but the causes of hypertension are in most cases still unclear. In the general population, hypertension appears to be associated with inflammation and arterial stiffness, and vascular stiffening appears to play a role in the onset of isolated systolic hypertension (Smulyan and Safar (2000) *Ann Intern Med.* **132**(3):233-237; Mahmud and Feely (2005) *Hypertension.* **46**(5):1118-1122). Patients with chronic inflammatory diseases such as lupus or arthritis rheumatoid exhibit some alterations in vascular mechanical wall properties. It has also been shown that changes in C-reactive protein (CRP) level predict a reduction in pulse pressure in a post hoc analysis of a randomized trial (Amar *et al.* (2005) *Hypertension* **46**:151-5). The role of inflammation induced by innate immunity on aortic stiffness has been explored. The inventors previously showed a correlation between soluble CD14 blood level and aortic stiffness in a cross sectional population-based study (Amar *et al.* (2003) *J Hypertens.* **21**:1869-77). Nevertheless, these studies did not identify any early markers of hypertension, including isolated systolic hypertension.

Isolated systolic hypertension (ISH) starts to become prevalent in subjects over the age of 50 years, as systolic pressure continues to rise and diastolic pressure tends to fall. Isolated systolic hypertension occurs in almost all subjects over the age of 80.

A large majority of patients with ISH are uncontrolled (Burt *et al.* (1995) *Hypertension* **25**:305-313). Overall, 42% of adults over 65 have uncontrolled isolated systolic hypertension. Furthermore, ISH accounts for 88.4% of undiagnosed hypertension in this population (McDonald *et al.* (2009) *J. Gerontol. A. Biol. Sci. Med. Sci.* **64**:256-263). Therefore, ISH accounts for huge number of strokes, myocardial infarction and disability.

There is thus a need in the art for methods for predicting hypertension, and more specifically isolated systolic hypertension.

The present invention is based on the unexpected finding by the inventors that the plasma concentration of bacterial 16S rDNA could be used to predict the onset of hypertension, in particular of isolated systolic hypertension, in a large cohort of apparently healthy subjects.

In its broadest aspect, therefore, the present invention relates to a method of predicting the risk of onset of hypertension in a subject by measuring bacterial 16S rDNA in a sample from the subject.

In one embodiment, the present invention relates to a method for predicting a risk of onset of hypertension, in particular of isolated systolic hypertension, in a subject, which method comprises the steps of:

a1) measuring the concentration of bacterial 16S rDNA in a biological sample from said subject; and

b) based on the result of the measurement in step a1), determining a risk of onset of hypertension, in particular of isolated systolic hypertension, in the subject.

Also provided is a method of identifying a subject at risk of developing hypertension, which method comprises

a1) measuring the concentration of bacterial 16S rDNA in a biological sample from said subject; and

b) based on the result of the measurement in step a1), determining whether the subject is at risk of developing hypertension.

Preferably, the method of the invention is an *in vitro* method.

Detailed description of the invention

Hypertension

As used herein, the term "hypertension" refers to a medical condition in which the blood pressure is chronically elevated.

In hypertension, systolic blood pressure is elevated, as described below. Diastolic blood pressure may also be elevated. An "elevated" blood pressure indicates a blood pressure which is above the accepted normal values for the age group of the subject, and/or which is in a range considered to be associated with adverse health outcomes.

An elevated systolic blood pressure may be a pressure of over 120 mmHg, preferably over 130 mmHg or over, 140 mmHg or over, 150 mmHg or over, or 160 mmHg or over. An elevated diastolic blood pressure may be a pressure of over 80 mmHg, preferably 90 mmHg or over, or 100 mmHg or over.

As used herein, the term "isolated systolic hypertension" or "ISH" refers to a state in which the systolic blood pressure is elevated, as described above, but diastolic blood pressure is not elevated or is within or below the normal range. For example, in ISH, diastolic blood pressure may be below 100 mmHg, below 90 mmHg or below 80 mmHg.

Thus, for example, hypertension may be defined as a condition in which systolic blood pressure is 140 mmHg or over and optionally diastolic blood pressure is 90 mmHg or over. Isolated systolic hypertension may be defined as a condition in which systolic blood pressure is 140 mmHg or over and diastolic blood pressure is under 90 mmHg.

Blood pressure may be measured by any method known in the art. Measurement of blood pressure is a routine procedure which can be carried out by any physician or suitably trained person. The most common method of measuring blood pressure is using a sphygmomanometer or blood pressure cuff. A more reliable, but more invasive and thus less commonly used, method is to place an arterial pressure probe directly into the artery. Preferably, blood pressure is measured using a sphygmomanometer. For example, a mercury sphygmomanometer, an aneroid sphygmomanometer or an electronic sphygmomanometer may be used. The sphygmomanometer may be a random-zero sphygmomanometer, which uses a variable reference level unknown to the user in order to reduce user error.

Subject

In the context of the present invention, a "subject" denotes a human or non-human mammal, such as a rodent (rat, mouse, rabbit), a primate (chimpanzee), a feline (cat), or a canine (dog). Preferably, the subject is human. The subject according to the invention may be in particular a male or a female.

The subject may be in a specific age group. Where the subject is a human, he/she may be for example over 30, over 40, over 50 or over 60 years old. Preferably, a human subject is between 30 and 65 years old.

In some embodiments, the subject does not suffer from hypertension at the time of sampling. Preferably, the subject does not suffer from isolated systolic hypertension at the time of sampling.

In a particular embodiment of the invention, the subject is at risk of hypertension.

As used herein, the expression "subject at risk of hypertension" refers to a subject as defined above who has an increased likelihood of developing hypertension as defined above. In particular, a subject at risk of hypertension according to the invention is a subject who displays at least one known hypertension risk factor.

As used herein, the expression “hypertension risk factor” refers to a biological marker which is associated with the onset of hypertension. Some hypertension risk factors are well-known from the skilled person and include for example age, family history of hypertension, a waist circumference of more than 102 cm in men or of more than 88 cm in women, a body mass index of more than 27 kg/m^2, low activity level, sedentarity, smoking, high sodium diet, low potassium diet, alcohol intake, stress, high cholesterol, a fasting glycaemia equal or superior to 6.1 mmol/L and diabetes.

As used herein, the expression "family history of hypertension" refers to the presence of at least one case of hypertension among the family of the subject, in particular among its ascendants (father, mother) and its siblings.

As used herein, the expression "low activity level" refers to the fact of exercising less than 3 times a week.

As used herein, the expression "sedentarity" or "sedentary life" denotes a type of lifestyle with no or irregular physical activity.

As used herein, the expression "high sodium diet" refers to a diet leading to the intake of a high level of sodium.

As used herein, the expression "low potassium diet" refers to a diet leading to the intake of a low level of potassium.

As used herein, the expression "high cholesterol" or "hypercholesterolemia" refers to the presence of high levels of cholesterol in the blood, in particular to a blood level of cholesterol over 4 mmol/l.

As used herein, the term "diabetes" or "diabetes mellitus" denotes a metabolic disorder in which the pancreas produces insufficient amounts of insulin, or in which the cells of the body fail to respond appropriately to insulin thus preventing cells from absorbing glucose. It includes type 1 diabetes, type 2 diabetes, gestational diabetes and other states that cause hyperglycaemia.

Preferably, the subject according to the invention displays at least one hypertension risk factor selected from the group consisting of:

- a waist circumference of more than 102 cm in men or of more than 88 cm in women;
- a body mass index of more than 27 kg/m²;
- smoking; and
- a fasting glycaemia equal or superior to 6.1 mmol/L.

In other embodiments, the subject is on the contrary free of hypertension risk factors at the time of sampling.

In some embodiments, the subject according to the invention does not have any infection and is free of bacteremia. Accordingly, the subject according to the invention may display a plasma fibrinogen concentration lower than 10 mg/l, in particular lower than 5 mg/l, and/or does not present an abundant leukocyturia and/or does not take antiviral therapy and/or displays a plasma baseline C reactive protein concentration lower than 30 mg/l.

As used herein, the term “C reactive protein” or “CRP” refers to a protein which is a member of the class of acute-phase reactants, as its levels rise dramatically during inflammatory processes occurring in the body. As known to the skilled person, CRP is a 224-residue protein with a monomer molar mass of 25,106 Da, encoded by the *CRP* gene.

As used herein, the term “fibrinogen” refers to a 340,000 Da plasma glycoprotein which is cleaved to fibrin by thrombin during the final stages of the blood coagulation cascade.

As used herein, the term "leukocyturia" refers to the presence of leukocytes in the urine of the subject. In particular, an abundant leukocyturia corresponds to the presence of 100,000 leukocytes per minute in the urine.

Bacterial 16S rDNA

In the context of the invention, the expressions “16S rDNA” or “16S ribosomal DNA” are used indifferently and refer to the gene encoding the 16S ribosomal RNA constituted of about 1500 nucleotides, which is the main component of the small prokaryotic ribosomal subunit (30S). 16S rDNA is highly conserved among bacteria. The reference *Escherichia coli* 16S rDNA gene sequence corresponds to SEQ ID NO: 1 (called *rrsA*). In the context of the invention, 16S rDNA refers to any sequence corresponding to SEQ ID NO: 1 in other bacterial strains.

In vitro method for predicting a risk of onset

The present invention concerns an *in vitro* method for predicting a risk of onset of hypertension, in particular of isolated systolic hypertension, in a subject, which method comprises the steps of:

a1) measuring the concentration of bacterial 16S rDNA in a biological sample of said subject; and

b) based on the result of the measurement of step a1), determining a risk of onset of hypertension, in particular of isolated systolic hypertension, in the subject.

As used herein, a "predictive method" or "method for predicting" refers to a method for determining whether an individual is likely to develop a disease.

As used herein, the expression “risk of onset” of a disease refers to the probability that a disease will appear in a studied subject, in particular within a given period of time.

Preferably, the concentration of bacterial 16S rDNA is measured by polymerase chain reaction (PCR), more preferably by quantitative PCR (qPCR), most preferably by real-time or real-time quantitative PCR (RT-PCR or RT-qPCR).

As used herein, "real-time PCR", "real-time quantitative PCR", "real-time polymerase chain reaction" or "kinetic polymerase chain reaction" refers to a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a sample. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-stranded DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA.

As used herein, the term "biological sample" means a substance of biological origin. Examples of biological samples include, but are not limited to, blood and components thereof such as serum, plasma, platelets, subpopulations of blood cells such as leucocytes, urine, saliva, faecal water and tissues such as adipose tissues, hepatic tissues, pancreatic tissues and the like. Preferably, a biological sample according to the present invention is a blood, serum, plasma, leucocytes, urine, adipose tissue or hepatic tissue sample. More preferably, the biological sample is selected from the group consisting of blood, serum and plasma sample. The biological sample according to the invention may be obtained from the subject by any appropriate means of sampling known to the skilled person.

Specifically, the present inventors demonstrated that the risk of onset of isolated systolic hypertension in a subject was linearly associated with the bacterial 16S rDNA concentration in said subject. Accordingly, the higher the bacterial 16S rDNA concentration, the higher the risk of onset of isolated systolic hypertension.

More particularly, the inventors determined that the adjusted odds ratio (adjusted on sex, baseline age, baseline systolic blood pressure, waist perimeter, smoking status, hypertension, fibrinogen and fasting plasma glucose) for an increase of the logarithm of the standard deviation of 16S rDNA mean concentration (log(0.25)), was of 1.15 (with a 95% confidence interval of 1.04-1.27). Accordingly, typically, in the methods according to the invention, a subject, displaying an increase of log(0.25) of the 16S rDNA concentration, has 1.15 more risk of having isolated systolic hypertension, the 16S rDNA concentration being preferably measured by real-time PCR, preferably using the universal forward and reverse primers eubac-F (5'-TCCTACGGGAGGCAGCAGT-3' SEQ ID NO: 2) and eubac-R (5'-GGACTACCAGGGTATCTAATCCTGTT-3' SEQ ID NO: 3), typically using the following reaction conditions for amplification of DNA : 95°C for 10 min and 35 cycles of 95°C for 15 s and 60°C for 1 min.

In a particular embodiment, the method of predicting a risk of onset of hypertension, in particular of isolated systolic hypertension, as defined above further comprises a step a2) of comparing the measured bacterial 16S rDNA concentration with a predetermined value.

Preferably, the predetermined value corresponds to the normal concentration of bacterial 16S rDNA.

As intended herein a "normal concentration" of bacterial 16S rDNA means that the concentration of 16S rDNA in the biological sample is within the norm cut-off values for that gene. The norm is dependant on the biological sample type and on the method used for measuring the concentration of 16S rDNA in the biological sample. Preferably, the predetermined value is the mean concentration of 16S rDNA in a healthy population.

As used herein, a "healthy population" means a population constituted of subjects who have not previously been diagnosed with hypertension or isolated systolic hypertension or who do not display hypertension risk factors or isolated systolic hypertension risk factors as defined above. Subjects of a healthy population also do not otherwise exhibit symptoms of disease. In other words, such subjects, if examined by a medical professional, would be characterized as healthy and free of symptoms of disease.

Preferably, in the methods of the invention, it is further determined whether the measured concentration of bacterial 16S rDNA is increased compared to the

predetermined value according to the invention. Still preferably, in the methods of the invention, it is further determined the level of increase of the measured concentration of bacterial 16S rDNA compared to the predetermined value according to the invention.

As used herein, the expression "level of increase" means the percentage of increase of the measured concentration of bacterial 16S rDNA compared to the predetermined value according to the invention or the number of fold of increase of the measured concentration of bacterial 16S rDNA compared to the predetermined value according to the invention.

The inventors specifically demonstrated that the increase of concentration of bacterial 16S rDNA in the biological sample of a subject compared to the predetermined value enabled predicting with a very high significance an increase of the risk of onset of isolated systolic hypertension.

Moreover, the inventors demonstrated that the concentration of bacterial 16S rDNA enabled them to predict the onset of hypertension and isolated systolic hypertension as soon as 9 years before the onset of the disease.

Accordingly, in preferred methods according to the invention, a measured concentration of bacterial 16S rDNA in the biological sample of the subject which is higher than the predetermined value is indicative of an increased risk of onset of hypertension or of isolated systolic hypertension within 9 years from the sampling, more particularly within a period of 6 to 9 years from the sampling.

The invention will be further illustrated by the following example and figure.

Description of the figure

The figure is a graphical representation of the relations between quartiles of 16S rDNA and cases of isolated systolic hypertension after nine years follow-up in the overall population, showing on the *y* axis the percentage of cases of ISH (y axis) and on the *x* axis quartiles of 16S rDNA concentration (ng/µl), ISH being defined as systolic blood pressure of ≥ 140 mm Hg and diastolic blood pressure < 90 mm Hg.

Example

The following example demonstrates the predictive value of blood bacterial 16S rDNA on the onset of hypertension, in particular isolated systolic hypertension.

Methods

Population

D.E.S.I.R. is a longitudinal cohort study of 5,212 adults aged 30–65 years at baseline. The primary aim of the study was to describe the natural history of the metabolic syndrome. Participants were recruited in 1994-1996 from ten Social Security Health Examination centres in central-western France, from volunteers insured by the French national social security system (80% of the French population - any employed or retired person and their dependents are offered free periodic health examinations). Equal numbers of men and women were recruited in five-year age groups. All participants gave written informed consent, and the study protocol was approved by the CCPPRB (Comité Consultatif de Protection des Personnes pour la Recherche Biomédicale) of the Hôpital Bicêtre (Paris, France). Participants were clinically and biologically evaluated at inclusion and at 3-, 6-, and 9-yearly follow-up visits. Individuals without hypertension at baseline (where hypertension was defined by treatment for hypertension or blood pressure ≥ 140/90 mmHg) and who had known hypertension status at the year-9 examination were studied with measures of baseline 16S rDNA gene concentrations. Individuals likely to have infection, identified as individuals having blood fibrinogen levels of over 5 mg/l, abundant leucocyturia, or taking antiviral therapy, were excluded from the study.

Parameters Studied

Weight and height were measured in lightly clad participants and body mass index (BMI) was calculated. Waist circumference, the smallest circumference between the lower ribs and the iliac crests, was also measured. Blood pressure was measured in triplicate using a random zero sphygmomanometer. The mean of the three measurements was recorded for the analysis. The examining physician noted the family history of diabetes and treatment for diabetes and hypertension were recorded. Smoking habits were documented in a self-administered questionnaire. Central adiposity was defined by a waist circumference > 102 cm in men and > 88 cm in women, high fasting glucose by ≥ 6.1 mmol /l. Hypertension was defined by systolic/diastolic blood pressure ≥ 140/90 mmHg or being on antihypertensive medications. Isolated systolic hypertension

was defined by systolic blood pressure ≥ 140 mmHg and diastolic blood pressure < 90 mmHg.

Biological Analyses

Blood was drawn after a 12-h fast. All biochemical measurements except insulin and bacterial DNA analysis were from one of four health centre laboratories located in France at Blois, Chartres, La Riche, or Orléans. Fasting plasma glucose measured by the glucose oxidase method, was applied to fluoro-oxalated plasma using a Technicon RA100 (Bayer Diagnostics, Puteaux, France) or a Specific or a Delta device (Konelab, Evry, France). Total cholesterol and triglycerides were measured by enzymatic methods.

16S rDNA gene quantification

Total DNA concentration was determined using the Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen) and a procedure adapted by the genomic platform of the Genopole Toulouse Midi Pyrénées (http://genomique.genotoul.fr). The mean concentration was 121.1 ± 208.3 ng/µl. Each sample was diluted ten-fold in Tris buffer EDTA. The DNA was amplified by realtime PCR (Stepone+; Applied Biosystems) in optical grade 96-well plates. The PCR reaction was performed in a total volume of 25 µL using the Power SYBR® Green PCR master mix (Applied Biosystems), containing 300 nM of each of the universal forward and reverse primers eubac-F (5'-TCCTACGGGAGGCAGCAGT-3' SEQ ID NO: 2) and eubac-R (5'-GGACTACCAGGGTATCTAATCCTGTT-3' SEQ ID NO: 3). The reaction conditions for amplification of DNA were 95°C for 10 min and 35 cycles of 95°C for 15 s and 60°C for 1 min. The amplification step was followed by a melting curve step according to the manufacturer's instructions (from 60°C to 90°C) to determine the specificity of the amplification product obtained. The amount of DNA amplified was compared with a purified 16S rDNA from *E. coli* BL21 standard curve, obtained by real time PCR from DNA dilutions ranging from 0.001 to 10 ng/µL.

Statistical analyses

For statistical analysis, the 16S rDNA gene concentrations were log transformed, as the distribution was skewed, as were the levels of triglycerides. Indeed, while the non-gaussian distribution of 16S rDNA gene concentrations prevented from performing parametric tests, these log transformations, by normalizing the distribution, enabled carrying out such tests.

Characteristics of subjects are shown as means, the standard deviation (SD) being indicated into brackets, or as n, the corresponding percentage in the study population being indicated into brackets. The t test was used to compare blood bacterial gene concentration as a continuous variable (logarithm) between subjects destined to have isolated systolic hypertension and those who did not. Logistic regression was used to calculate the odds ratios and the 95 percent confidence intervals for incident isolated systolic hypertension according to one SD of baseline 16S rDNA gene concentrations, as a continuous variable (logarithm). Adjustments were made for sex, baseline age, baseline systolic blood pressure, BMI, smoking status, fasting plasma glucose. The relation with 16S rDNA gene concentrations (logarithm) was linear, as an additional squared term was not significant. Odds ratios were also calculated over risk-factor strata. The relation between quartiles of 16S rDNA gene concentrations and incident isolated systolic hypertension was shown.

SAS versions 9.1 and 9.2 were used for statistical analysis.

Results

Characteristics of the studied population

At baseline, among the 5212 participants in the D.E.S.I.R. study, 1680 participants had hypertension, 194 presented biological signs of infection or received antiviral therapy, 330 did not undergo 16S rDNA gene concentration determination and for 1126, isolated systolic hypertension status was not known at the end of the nine years, as they did not attend the 9-year examination. Finally, in 14 subjects, smoking status, waist perimeter, fasting blood glucose or fibrinogen levels were not available. These volunteers were excluded from the analysis. The characteristics of study population are shown in **Table 1**.

Table 1: Characteristics of study population

Mean (SD) and n (%)

	Study population n=2350
Age (years)	45.00 (9.61)
Male	1048 (44.60)
Current smokers	502 (21.36)
Body mass index (kg /m^2)	23.80 (3.17)
Weight (kg)	65.72 (11.64)
Waist perimeter (cm) Men Women	 86.97 (8.18) 74.68 (8.51)
Systolic blood pressure (mmHg)	122.91 (8.76)
Diastolic blood pressure (mmHg)	75.61 (6.42)
Fibrinogen (g/l)	2.90 (0.57)

Total cholesterol (mmol/l)	5.60 (0.96)
HDL cholesterol (mmol/l)	1.67 (0.43)
Triglycerides (mmol/l)	1.02 (0.66)
Fasting blood glucose (mmol/l)	5.23 (0.68)
16S rDNA (ng/µl)	0.12 (0.25)

Prediction of isolated systolic hypertension

589 incident cases of isolated systolic hypertension were recorded. The mean 16S rDNA gene concentrations were compared between patients who subsequently developed isolated systolic hypertension and patients who did not.

The inventors demonstrated that the mean 16S rDNA gene concentrations were higher in patients with incident isolated systolic hypertension, relative to patients with no incident isolated systolic hypertension (0.12 ng/µL (0.25) *vs* 0.13 (0.20), standard deviations being indicated into brackets). This difference was statistically significant when comparing the mean log transformed 16S rDNA gene concentrations ($p = 0.003$). A graphical representation of this finding is shown in the **Figure**.

16S rDNA gene concentration predicted the onset of isolated systolic hypertension after adjustment for confounding factors, the odds ratio being 1.15 (95% CI 1.04 to 1.27) (**Table 2**).

While higher 16S rDNA gene concentrations appeared to carry more risk in older subjects (below the median) and in lean subjects, there was no significant interaction for the risk of incident isolated systolic hypertension between bacterial DNA and any of these strata (**Table 2**).

Furthermore when considered hypertension as a whole (both systole-diastolic and isolated systolic hypertension), whereas 16S rDNA concentration level was higher in subjects destined later to become hypertensive, this relation did not reach statistical significance after adjustment for confounders.

Table 2: Adjusted† odds ratios (95% confidence intervals) of incident isolated systolic hypertension for 1 SD of log (16S rDNA gene concentration) in the overall population and in various strata.

	Incident cases of ISH	Odds ratio	95% CI limits		P
Overall	589	1.15	1.04	1.27	0.006
Age					
Below the median <=44	176	1.11	0.94	1.32	0.20
>44	413	1.18	1.04	1.34	0.01

Sex					
Men	322	1.12	0.98	1.28	0.10
Women	267	1.19	1.02	1.39	0.02
Current smokers					
Yes	110	1.25	1.00	1.57	0.05
No	479	1.13	1.01	1.27	0.03
Overweight*					
Yes	137	0.89	0.70	1.13	0.39
No	452	1.22	1.09	1.36	0.0005

* Overweight defined as body mass index ≥ 27 kg/m^2

† Adjusted for sex, baseline age, baseline systolic blood pressure, waist perimeter, smoking status, fibrinogen and fasting plasma glucose, excepting when that parameter is being specifically studied: for example the Odds Ratios for gender are not adjusted for gender, those for age are not adjusted for age, etc.

CLAIMS

1. An *in vitro* method for predicting a risk of onset of hypertension in a subject, which method comprises the steps of:

a1) measuring the concentration of bacterial 16S rDNA in a biological sample of said subject; and

b) based on the result of the measurement in step a1), determining a risk of onset of isolated systolic hypertension in the subject.

2. The *in vitro* method according to claim 1, further comprising a step a2) of comparing the bacterial 16S rDNA concentration measured in step a1) with a predetermined value.

3. The *in vitro* method according to claim 2, wherein a measured concentration of bacterial 16S rDNA higher than the predetermined value is indicative of an increased risk of onset of isolated systolic hypertension

4. The *in vitro* method according to any one of claims 1 to 3, wherein a measured concentration of bacterial 16S rDNA in the biological sample of the subject which is higher than the predetermined value is indicative of an increased risk of onset of hypertension or of isolated systolic hypertension within 9 years from the sampling.

5. The *in vitro* method according to any one of claims 1 to 4, wherein the biological sample is selected from the group consisting in blood, serum and plasma sample.

6. The *in vitro* method according to any one of claims 1 to 5, wherein the concentration of bacterial 16S rDNA is measured by real-time PCR.

7. The *in vitro* method according to any one of claims 1 to 6, wherein the subject does not suffer from hypertension at the time of sampling.

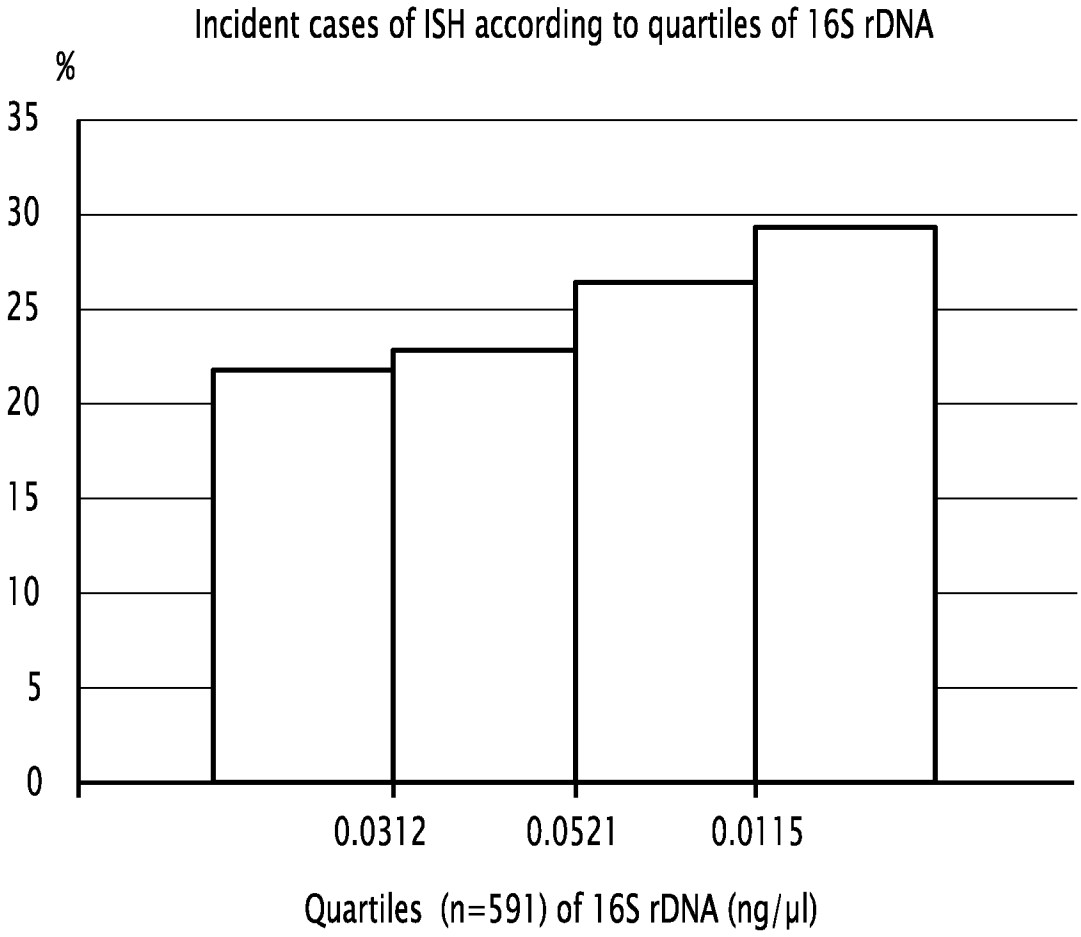
8. The *in vitro* method according to claim 2, wherein the subject is at risk of hypertension.

9. The *in vitro* method according to claim 8, wherein the subject displays at least one hypertension risk factor selected from the group consisting of :

- a waist circumference of more than 102 cm in men or of more than 88 cm in women;
- a body mass index of more than 27 kg/m²;
- smoking; and
- a fasting glycaemia equal or superior to 6.1 mmol/L.

10. The *in vitro* method according to any one of claims 1 to 9, wherein the subject is a human subject aged between 30 and 65 years.

11. The *in vitro* method according to any one of claims 1 to 10, wherein the subject displays a plasma level of fibrinogen lower than 5 mg/l and/or does not present an abundant leukocyturia and/or does not take antiviral therapy.


Incident cases of ISH according to quartiles of 16S rDNA
%
35
30
25
20
15
10
5
0
0.0312
0.0521
0.0115
Quartiles (n=591) of 16S rDNA (ng/µl)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/055935

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
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, EMBASE, BIOSIS, 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 2010/092164 A2 (INST NAT SANTE RECH MED [FR]; CHU DE TOULOUSE [FR]; AMAR JACQUES [FR];) 19 August 2010 (2010-08-19) claims 1, 2, 4; tables 1, 2, 4, 5	1-11

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 August 2012	24/08/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Faber, Cerstin

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MITUSCH ROLF ET AL: "Asymptomatic carotid atherosclerosis is associated with circulating chlamydia pneumoniae DNA in younger normotensive subjects in a general population survey", ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY, HIGHWIRE PRESS, PHILADELPHIA, PA, US, vol. 25, no. 2, 1 February 2005 (2005-02-01), pages 386-391, XP002524570, ISSN: 1524-4636, DOI: 10.1161/01.ATV.0000151284.49967.A7 the whole document -----	1-11
A	VOLDSTEDLUND MARIANNE ET AL: "Broad-range PCR and sequencing in routine diagnosis of infective endocarditis", APMIS, WILEY INTERSCIENCE, DK, vol. 116, no. 3, 1 March 2008 (2008-03-01) , pages 190-198, XP002524573, ISSN: 0903-4641, DOI: 10.1111/J.1600-0463.2008.00942.X the whole document -----	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/055935

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010092164 A2	19-08-2010	EP 2396425 A2	21-12-2011
		US 2012021421 A1	26-01-2012
		WO 2010092164 A2	19-08-2010