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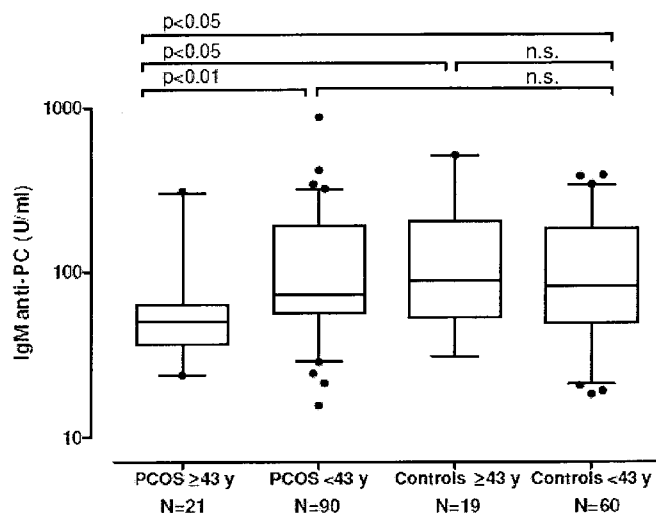
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(54) Title: DIAGNOSTIC AND THERAPEUTIC METHODS AND COMPOSITIONS FOR METABOLIC DISEASE



(57) Abstract: The present invention relates to a method for the immunization or prophylaxis against, or the treatment of, metabolic diseases in a mammal, the method comprising the step of administering to the mammal a pharmaceutical composition comprising at least one PC conjugate, or an antibody preparation with reactivity to PC or a PC conjugate. The metabolic disease may, for example, be a condition selected from the group consisting of metabolic syndrome, insulin resistance, glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS). Also provided is a method for diagnosing metabolic disease, or assessing a patient's risk of developing or progression of metabolic disease, the method comprising the steps of - (a) assessing the patient's level of antibodies with reactivity to PC or a PC conjugate; and (b) diagnosing metabolic disease or determining the patient's level of risk of developing or progression of metabolic disease based on the assessed levels of antibodies with reactivity to PC or a PC conjugate.

DIAGNOSTIC AND THERAPEUTIC METHODS AND COMPOSITIONS FOR METABOLIC DISEASE

FIELD OF THE INVENTION

The present invention relates to the treatment, prevention and diagnosis of
5 metabolic diseases.

BACKGROUND OF THE INVENTION

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

10 The metabolic syndrome is the clustering of a number of symptoms that relates to the consequences of disturbances in energy metabolism, that is the metabolism of lipids, carbohydrates and proteins. Obesity, insulin resistance, diabetes, hypertension and hyperlipidemia are the components of the syndrome. Several definitions of the metabolic syndrome exist. The NECP/ATP III criteria (*Executive Summary of The Third*
15 *Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III)*). JAMA, 2001. **285**(19), 2486-97) is one definition, according to which at least three of the following five criteria should be fulfilled: Blood pressure >130/85 mmHg or antihypertensive treatment, fasting plasma glucose > 6.1 mmol/l,
20 serum triglycerides >1.7 mmol/l, waist circumference > 102 cm in men and >88 cm in women, HDL-cholesterol < 1.0 mmol/l in men and <1.3 in women. The individual components of the syndrome are themselves associated to increased morbidity and mortality, especially for premature cardiovascular disease (CVD), in individuals suffering from metabolic syndrome this risk is greatly increased (Bonora, E., *The*
25 *metabolic syndrome and cardiovascular disease*. Ann Med, 2006. **38**(1), 64-80). All components of the metabolic syndrome have been associated to chronic systemic inflammation (Cirillo, P., Y.Y. Sautin, J. Kanellis, D.H. Kang, L. Gesualdo, T. Nakagawa, and R.J. Johnson, *Systemic inflammation, metabolic syndrome and progressive renal disease*. Nephrol Dial Transplant, 2009. **24**(5) 1384-7).

30 Polycystic ovary syndrome (PCOS) is reported to affect from 5% up to 20 % of women in child-bearing ages (Lindholm, A., L. Andersson, M. Eliasson, M. Bixo, and I. Sundstrom-Poromaa, *Prevalence of symptoms associated with polycystic ovary*

syndrome. Int J Gynaecol Obstet, 2008. **102**(1) 39-43; Teede, H., A. Deeks, and L. Moran, *Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan*. BMC Med. **8**(1) 41). Women are diagnosed as having PCOS if they are positive for at
 5 least two of the following symptoms: oligoovulation or anovulation, excess androgen activity or the presence of polycystic ovaries.

In addition to the short term consequence of infertility, the pathophysiology of PCOS also involves disturbances in energy metabolism, with symptoms similar to the metabolic syndrome (Lindholm, A., L. Andersson, M. Eliasson, M. Bixo, and I.
 10 Sundstrom-Poromaa, *Prevalence of symptoms associated with polycystic ovary syndrome*. Int J Gynaecol Obstet, 2008. **102**(1), 39-43). Thus, patients suffering from PCOS do not only suffer the well known fertility related morbidities, but also suffers the same health problems as other victims of the metabolic syndrome not having the PCOS, including increased risk for CVD etc (Wild, S., T. Pierpoint, P. McKeigue, and H.
 15 Jacobs, *Cardiovascular disease in women with polycystic ovary syndrome at long-term follow-up: a retrospective cohort study*. Clin Endocrinol (Oxf), 2000. **52**(5), 595-600). Like metabolic syndrome, PCOS has been associated to chronic systemic inflammation (Gonzalez, F., N.S. Rote, J. Minium, A.L. Weaver, and J.P. Kirwan, *Elevated circulating levels of macrophage migration inhibitory factor in polycystic ovary syndrome*.
 20 Cytokine. 2010. epub July 1).

Diabetes mellitus is a group of diseases resulting in elevated levels of plasma glucose. Diabetes is currently defined (WHO/ADA) as symptoms of diabetes plus:

random plasma glucose concentration above 11.1 mmol/L [200mg/dl], or

fasting plasma glucose above 7.0 mmol/L [126mg/dl], or

25 2-h plasma glucose concentration after 75 g anhydrous glucose in an oral glucose, tolerance test above 11.1 mmol/L [200mg/dl].

In the absence of symptoms, diabetes should not be diagnosed on a single glucose measurement but needs confirmation. There are two main types of diabetes, Type 1 (T1DM) and Type 2 (T2DM). T1DM is an autoimmune disease where
 30 pancreatic beta cells are destroyed, and the patients are thus dependent on exogenous insulin administration. T1DM is characterized by elevated glucose levels and low insulin levels, as the pancreas is unable to secrete insulin in response to the elevation in plasma glucose.

In diabetes, especially in T2DM, there is a relation between elevated markers for ongoing systemic inflammatory processes and disease development (Devaraj, S., U. Singh, and Jialal, *Human C-reactive protein and the metabolic syndrome*. Curr Opin Lipidol, 2009. **20**(3), 182-91), indicating that inflammatory processes are important in diabetes. Before T2DM has developed, the patients are going through a period of pre-diabetes. In this period insulin resistance has developed, but fasting plasma glucose is normal due to an increased secretion of insulin. Insulin resistance is diagnosed as elevated fasting insulin levels with normal fasting glucose levels, or as increased HOMA-IR, the product of fasting glucose and fasting insulin levels. Also insulin resistance in pre-diabetic individuals is associated to low-grade systemic inflammation.

Phosphorylcholine (PC) antibodies (anti-PC) are natural antibodies that belong to the innate immune system (Binder, C.J., P.X. Shaw, M.K. Chang, A. Boullier, K. Hartvigsen, S. Horkko, Y.I. Miller, D.A. Woelkers, M. Corr, and J.L. Witztum, *The role of natural antibodies in atherogenesis*. J Lipid Res, 2005. **46**(7), 1353-63.). Natural antibodies have scavenging functions and are a part of the first line defence against certain infections. Thus, these antibodies can recognize PC – containing epitopes of certain infectious agents such as some parasites and bacteria, e.g. streptococcus bacteria. They can also recognize PC (neo)epitopes formed in membranes during cell ageing and senescence, and during inflammation. In biological membranes, these immunogenic PC epitopes are generated by oxidative and/or enzymatic modification of the membrane phospholipid phosphatidylcholine. It is shown that membranes containing immunogenic PC induce inflammation in other cells, and that this inflammation can be blocked by anti-PC (Chang, M.K., C.J. Binder, Y.I. Miller, G. Subbanagounder, G.J. Silverman, J.A. Berliner, and J.L. Witztum, *Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory*. J Exp Med, 2004. **200**(11), 1359-70). Further, it is also shown that anti-PC can inhibit the uptake of oxidized LDL particles by macrophages, and it is suggested that this effect can prevent against atherosclerosis (Boullier, A., K.L. Gillotte, S. Horkko, S.R. Green, P. Friedman, E.A. Dennis, J.L. Witztum, D. Steinberg, and O. Quehenberger, *The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein*. J Biol Chem, 2000. **275**(13), 9163-9).

Low serum levels of anti-PC (WO 2005/100405) have been shown to be related to an increased risk of developing atherosclerosis. Active immunization with PC-conjugates and passive immunization with antibodies directed to PC-conjugates have

been suggested for the prevention and treatment of cardiovascular disease (WO 2005/100405).

However, there is currently no information in the art showing that anti-PC is of any importance in the pathophysiology of the metabolic syndrome, diabetes, insulin resistance or PCOS. Neither has anti-PC been suggested for the prevention or treatment of metabolic diseases.

SUMMARY OF THE INVENTION

The present invention is based on the surprising findings that low levels of antibodies reactive with a PC-conjugate are related to an increased risk of developing metabolic diseases. The present inventors have shown that the progression of metabolic diseases, such as insulin resistance, can be reduced by administration of a composition that increases the levels of anti-PC antibodies, and that risk of metabolic diseases, such as polycystic ovary syndrome, can be identified by low levels of anti-PC antibodies.

Accordingly, a first aspect of the present invention provides a composition comprising at least one phosphorylcholine (PC) conjugate, or an antibody preparation with reactivity to PC or a PC conjugate, for use in the immunization or prophylaxis against, or the prevention or treatment of, metabolic diseases in mammals.

To put it another way, the first aspect of the present invention provides for the use of a composition comprising at least one PC conjugate, or an antibody preparation with reactivity to PC or a PC conjugate, in the manufacture of a medicament for the immunization or prophylaxis against, or the prevention or treatment of, metabolic diseases in mammals.

Thus, according to the first aspect of the present invention, there is provided a method for the immunization or prophylaxis against, or the treatment of, metabolic diseases in a mammal, the method comprising the step of administering to the mammal a pharmaceutical composition comprising at least one PC conjugate, or an antibody preparation with reactivity to PC or a PC conjugate. The method may thus include administration of a therapeutically effective amount of a composition comprising at least one PC conjugate or an antibody preparation with reactivity to PC or a PC conjugate is administered to the mammal

According to the first aspect of present invention, any mammal may be treated, although in one embodiment the mammal may be a human.

According to the first aspect of present invention, any metabolic disease may be addressed. Exemplary metabolic diseases include a condition selected from the group
5 consisting of metabolic syndrome, insulin resistance, glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS).

According to the first aspect of present invention the composition comprising at least one PC conjugate may, for example, be a pharmaceutical composition comprising
10 at least one PC conjugate, and may optionally include an adjuvant. Any suitable adjuvant, for example aluminium hydroxide, may be used.

According to the first aspect of present invention the antibody preparation with reactivity to PC or a PC conjugate may, for example, comprise a polyclonal antibodies, or a monoclonal antibody, with reactivity to PC or a PC conjugate.

15 The first aspect of present invention may provide, for example, for the therapeutic treatment of a mammal suffering from metabolic disease, or for the prophylactic treatment of a mammal facing the risk of developing metabolic disease. The mammal may be identified as being of risk of developing metabolic disease by a method according to the second aspect of the present invention, as discussed further
20 below.

Accordingly, in one embodiment, the first aspect of the invention provides the use of at least one PC conjugate, or an antibody preparation, for example a monoclonal antibody, with reactivity to PC or a PC conjugate, in the manufacture of a medicament for immunization and prophylaxis, prevention or treatment of mammals,
25 including humans, against metabolic diseases, such as metabolic syndrome, insulin resistance (IRS), glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS). The medicament is intended to provide immunization having immunogenic or therapeutic properties against metabolic
30 diseases.

In another embodiment, the first aspect of the invention provides a method for immunization and treatment of a mammal, including a human, against metabolic diseases, such as metabolic syndrome, insulin resistance (IRS), glucose intolerance,

hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS), the method comprising the step of administering to the mammal a pharmaceutical composition comprising at least one PC conjugate, or an antibody preparation, for example a monoclonal antibody, with reactivity to PC or a PC conjugate. The pharmaceutical composition is intended to provide immunization having immunogenic or therapeutic properties against metabolic diseases.

In another embodiment, the first aspect of the invention provides the use of one or more of the PC conjugates as defined in relation to the preceding aspects of the invention, in the manufacture of a pharmaceutical composition, optionally in combination with an adjuvant, for immunotherapy or therapy for the prevention, prophylaxis and/or treatment of metabolic diseases.

In another embodiment, the first aspect of the invention provides a method of prophylactic or therapeutic treatment of a mammal, which may be a human being, suffering from metabolic disease or facing the risk of developing metabolic disease, whereby a therapeutically effective amount of at least one PC conjugate or an antibody preparation, for example a monoclonal antibody, with reactivity to PC or a PC conjugate is administered.

A second aspect of the present invention provides a method for diagnosing metabolic disease, or assessing a patient's risk of developing or progression of metabolic disease, the method comprising the steps of –

- (a) assessing the patient's level of antibodies with reactivity to PC or a PC conjugate; and
- (b) diagnosing metabolic disease or determining the patient's level of risk of developing or progression of metabolic disease based on the assessed levels of antibodies with reactivity to PC or a PC conjugate.

The method of the second aspect of the present invention may assess the level of all of the patient's antibodies with reactivity to PC or a PC conjugate, or may comprise the assessment of a particular isotype, such as the patient's level of IgM, IgG or IgA antibodies with reactivity to PC or a PC conjugate.

Typically, although not necessarily, the patient's level of antibodies with reactivity to PC or a PC conjugate are assessed by analysis of an *ex vivo* sample taken

from the patient. Thus, the sample may be a blood, plasma or serum sample that has been obtained from the patient.

The method of the second aspect of the present invention may be employed to diagnose metabolic disease, or assess a patient's risk of developing or progression of metabolic disease, in any mammalian patient, although in one embodiment the patient is human.

According to the findings of the inventors, lower levels of antibodies with reactivity to PC or a PC conjugate are indicative of the presence of metabolic disease and/or the risk of developing or progression of metabolic disease. Accordingly, in the method of the second aspect of the present invention, the patient's level of antibodies with reactivity to PC or a PC conjugate may correlate negatively with the patient's risk of developing or progression of the metabolic disease.

The method of the second aspect of the present invention may be employed to diagnose any metabolic disease, or assess a patient's risk of developing or progression of any metabolic disease. Exemplary metabolic diseases include a condition selected from the group consisting of metabolic syndrome, insulin resistance, glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS).

Accordingly, the second aspect of the invention provides a method of diagnosing the presence or absence of antibodies, for example IgM, IgG or IgA antibodies, related to increased or decreased risk of developing metabolic diseases, using PC or a PC conjugate.

The second aspect of the present invention also provides for the use of PC or a PC conjugate in a method for diagnosing metabolic disease and/or for assessing a patient's risk of developing or progression of metabolic disease in which the patient's levels of antibodies (for example, all antibodies, or a particular isotype, such as IgM, IgG or IgA antibodies) with reactivity to PC or the PC conjugate are assessed.

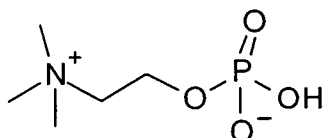
DETAILED DESCRIPTION OF THE INVENTION

The invention relates to pharmaceutical compositions comprising a PC conjugate, or an antibody preparation, for example a monoclonal antibody, with reactivity to PC or a PC conjugate, and the use of these compositions in the treatment,

prophylaxis or prevention of metabolic diseases, such as metabolic syndrome, insulin resistance (IRS), glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS). Furthermore, the invention also relates to the use
5 of PC conjugates or said antibody preparation, for example monoclonal antibody to produce a pharmaceutical composition optionally with an adjuvant.

Furthermore the invention relates to diagnosing the absence, presence and/or levels of antibodies, for example IgM, IgG or IgA antibodies, reactive with a PC-conjugate (such as PC-BSA) related to increased or decreased risk of displaying or
10 developing metabolic diseases.

By PC is meant phosphorylcholine according to the formula.



By a PC conjugate is meant a PC moiety linked to a carrier, optionally via a spacer. The PC moiety can be covalently or non-covalently linked to the carrier. For
15 example, the PC moiety may be linked to the carrier via the phosphate group.

Any suitable carrier can be used. For example, the carrier may be selected from the group consisting of a protein, a carbohydrate, a polymer, latex beads, or colloid metal.

The PC conjugate may for example be a protein-PC conjugate, such as a
20 human serum albumin (HSA)-PC conjugate, a transferrin-PC conjugate, a keyhole limpet hemocyanin (KLH)-PC conjugate or a bovine serum albumin (BSA)-PC conjugate. Examples of PC-conjugates and generation of anti-PC antibodies are, e.g., described in WO 2005/100405 and U.S. Patent 5,455,032, the contents of both of which are incorporated by reference.

25 Where the PC conjugate comprises PC linked to a carrier via a spacer, then any suitable spacer may be used. Non-limiting examples of spacers include coupling agents (typically, bi-functional compounds), such as a di-carboxylic acids like succinic and glutaric acid, the corresponding di-aldehydes, di-amines such as 1,6

diaminohexane, di-substituted phenols such as p-amino-phenol, p-diazo-phenol, p-phenylenediamine, p-benzoquinone, and the like.

Metabolic diseases that can be treated, prevented and/or diagnosed according to the first or second aspects of the present invention are exemplified, but not limited to, metabolic syndrome, insulin resistance (IRS), glucose intolerance, hyperglycemia, type I diabetes also referred to as insulin-dependent diabetes mellitus or IDDM, type II diabetes also referred to as noninsulin-dependent diabetes mellitus or NIDDM, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS).

Thus, an individual for treatment according to the method or use of the present invention may be an individual that is identified as suffering from, or being at risk of suffering from, a metabolic disease, such as, but not limited to, metabolic syndrome, insulin resistance (IRS), glucose intolerance, hyperglycemia, type I diabetes also referred to as insulin-dependent diabetes mellitus or IDDM, type II diabetes also referred to as noninsulin-dependent diabetes mellitus or NIDDM, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS).

In one embodiment, the individual for treatment is an individual that is not suffering from a disease or condition as discussed in WO 2005/100405 or WO 2010/003602, the contents of both of which are incorporated by reference.

Optionally, the individual to be treated may be an individual who is not suffering from, and/or has not been diagnosed as suffering from or being at risk of the progression or development of, conditions selected from one or more of Alzheimer's disease, atherosclerosis, atherosclerosis related disease, cardiovascular disease, or ischemic cardiovascular diseases. In another option, the individual to be treated may be an individual who is suffering from, and/or has been diagnosed as suffering from or being at risk of the progression or development of, conditions selected from one or more of Alzheimer's disease, atherosclerosis, atherosclerosis related disease, cardiovascular disease, or ischemic cardiovascular diseases, and who is receiving therapy or prophylaxis for that condition by administration of a therapeutic or prophylactic agent other than a composition comprising at least one phosphorylcholine (PC) conjugate, or an antibody preparation with reactivity to PC or a PC conjugate.

Preferably, in the use according to the first aspect of the invention, or the method according to the second aspect of the invention, the medicament is for

administration by injection. However, in practice it can be administered by any suitable means that allows the PC-conjugate to provoke an immune response in, or allows efficient delivery of the antibody preparation to, the subject to which it is administered.

A clinician can determine the most appropriate administrative regimen for an individual based on factors such as the individual's weight, age, gender, diagnosis or prognosis, and the half-life of the administered therapeutic molecule. However, in general it may be suitable to treat an individual with a single dose, or multiple doses, of the composition comprising at least one PC conjugate and/or an antibody preparation with reactivity to PC or a PC conjugate. Where multiple administrations are made, these may be made at a rate of, for example, once, twice, three times, four times or more often per day, week or month, and may be continued for a period of time necessary and effective to increase the levels of anti-PC antibodies in the individual and thereby obtain a therapeutically or prophylactically-beneficial effect in respect of metabolic disease. For example treatment may continue for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more days, weeks, months or years, or even for the rest of the life of the subject. In the case of the use of anti-PC antibodies, then administration would most typically be made weekly, or once or twice per month, and continue for as long as is clinically beneficial. In the case of the administration with a PC conjugate then, in one embodiment, the treatment may involve an initial immunisation, followed by a further administration as a booster (for example, within about one month of the initial immunisation), and optionally followed by yearly further administrations, continued for as long as is clinically beneficial.

The first aspect of the invention provides active (where the composition comprises at least one PC-conjugate) or passive (where the composition comprises the defined antibody) immunization having immunogenic or therapeutic properties against metabolic diseases.

In other words, the invention provides at least one PC-conjugate, or an antibody preparation (for example a monoclonal antibody) with reactivity to PC and/or a PC-conjugate, for use in the prophylaxis, prevention and/or treatment of metabolic diseases, and provides a method for immunization and treatment against metabolic diseases. The method may comprise the step of administering to a subject a pharmaceutical composition comprising at least one PC-conjugate, or an antibody preparation (for example a monoclonal antibody) with reactivity to PC and/or a PC-conjugate. The pharmaceutical composition is intended to provide active or passive

immunization having immunogenic or therapeutic properties against metabolic diseases.

Typically the prophylaxis, prevention and/or treatment of metabolic diseases according to the present invention is for the treatment of humans. The human subject
5 may, for example, be aged at least 10, 20, 30, 40, 50, 60, 65, 70, 75, 80, 85 or more years. The human subject may be one that has been diagnosed as being at increased risk of development or progression, of one or more metabolic diseases, for example by using a method of diagnosis based on the assessment of anti-PC levels according to the other aspects of the present invention. For example, in one embodiment, the
10 present invention provides a method for the prophylaxis, prevention and/or treatment of polycystic ovary syndrome (PCOS) in females belonging to older age groups, such as human females aged at least 40, 50, 60, 65, 70, 75, 80, 85 or more years and/or females that are pre-, peri-, and/or post-menopausal.

Active immunization:

15 One embodiment of the present invention is thus to use a PC-conjugate for the preparation of a pharmaceutical composition to be used in the treatment, prophylaxis and/or prevention of metabolic diseases, such as metabolic syndrome, insulin resistance (IRS), glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and
20 polycystic ovary syndrome (PCOS).

The conjugate can, for example, be PC linked to a pharmaceutically acceptable carrier such as a protein, carbohydrate, or polymer. The pharmaceutical composition is preferably given by injection, but can in practice be administered by any suitable means that allows the PC-conjugate to provoke an immune response in the subject to
25 which it is administered.

For the purposes of active immunisation of a patient, one or more PC-conjugate molecules are prepared in an immunogenic formulation, optionally containing suitable adjuvants and carriers and administered to the patient in known ways. Suitable adjuvants include Freund's complete or incomplete adjuvant, muramyl dipeptide, the
30 "Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminium hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic polyols or the Ribi adjuvant system (see, for example GB-A-2 189 141). "Pluronic" is a Registered Trade Mark.

The proposed method of active immunization will modulate (preferably, increase) the titre of anti-PC antibodies which in turn will have a positive effect on the development of metabolic diseases (that is, the development of metabolic disease will be reduced). Thus, active immunisation may be used to increase the titre of anti-PC antibodies to a level that, when assessed by the methods of diagnosis according to the present application, would not be said to be "low" or indicative of an increased risk of development, or progression, of metabolic diseases.

Thus, a method of active immunisation according to the present invention may be used to increase anti-PC levels, such as IgM anti-PC levels, in an individual to a level that is greater than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 or 65 U/ml when tested by the methods described below. Accordingly, the method of active immunisation according to the present invention may be used to increase anti-PC levels to a level that is above the mean and/or median average, or above a particular percentile value determined with reference to the wider population, such as above the 5th, 10th, 20th or 25th percentile, such as to a level wherein the odds ratio is below one, the p-value is < 0.05 and the upper limit of the odd ratio confidence interval is less than one, indicating a statistically significant level of low risk for a metabolic disease.

Passive immunization.

Another embodiment of the invention is the use an antibody preparation, for example a monoclonal antibody, recognizing PC or a PC-conjugate for the preparation of a pharmaceutical composition to be used in the treatment, prophylaxis and/or prevention of metabolic diseases, such as metabolic syndrome, insulin resistance (IRS), glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS).

Monoclonal antibodies can be produced using methods known in the art and/or as discussed further below. Other antibody preparations may be used, such as anti-PC enriched preparations obtained from Intravenous immunoglobulin preparations, recombinantly produced anti-PC antibodies and/or other artificially created anti-PC antibody derivatives, as discussed above.

Thus, passive immunisation may be used to increase the titre of anti-PC antibodies in an individual to a level that, when assessed by the methods of diagnosis according to the present application, would not be said to be "low" or indicative of an increased risk of development, or progression, of metabolic diseases. Thus, a method

of passive immunisation according to the present invention may be used to increase anti-PC levels, such as IgM anti-PC levels, in an individual to a level that is greater than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 or 65 U/ml when tested by the methods described above. Accordingly, the method of active immunisation according to the present invention may be used to increase anti-PC levels to a level that is above the mean and/or median average, or above a particular percentile value determined with reference to the wider population, such as above the 5th, 10th, 20th or 25th percentile, such as to a level wherein the odds ratio is below one, the p-value is < 0.05 and the upper limit of the odd ratio confidence interval is less than one, indicating a statistically significant level of low risk.

Monoclonal antibodies reactive against PC and /or PC-conjugate can be produced using any standard method known in the art. See for example Briles *et al.*, 1982. *J Exp Med* 156, 1177-1185 or Spira *et al.*, 1988. *J Immunology* 140, 2675-2680.

Other antibodies against a phosphorylcholine and/or its conjugate can be prepared using methods well known to those skilled in the art. For example, a subfraction with anti-PC activity of a human immunoglobulin preparation can be prepared, for example as described below, for example by affinity purification using a phosphorylcholine conjugate. Intravenous immunoglobulin preparations (e.g., IGIV; Baxter and others) are highly purified preparations of IgG commercially available and is used in the treatment of patients who have no, or very low levels of antibody production. Immunoglobulin preparations include those available from the following manufacturers: Baxter (US), e.g., Gammagard®, Isiven (Antimo Naples, Italy), Omrix (Tel-Hashomer, Israel), Miles (Biological Products Division, West Haven, CT), Sclavo (Lucca, Italy), Sandoz (Novartis, Basel, Switzerland), e.g., Sandoglobulin®, Biotest Diagnostic Corporation (Deville, NJ). Examples of immunoglobulin preparations are GammagardS/D®, GammarIV®, Gaimnar-PIV®, Gammimune N®, Iveegam®, Panglobulin®, Polygam S/D®, Sandoglobulin®, Venoglobulin®. Immunoglobulin preparations typically contain some IgM as well as IgG. Trace amounts of IgM are present in Gammagard®. Pentaglobin (Biotest) is an enriched IgM preparation which has been used for treatment of SARS. The subfraction with anti-PC activity may comprise both IgG and IgM, or may be selected to comprise mainly IgG (for example by starting with an IgG-rich preparation such as Gammagard® and/or by selecting for IgG); or mainly IgM (for example by starting with an IgM-rich preparation such as Pentaglobin and/or by selecting for IgM).

Additionally, the present invention contemplates the use of recombinantly produced anti-PC antibodies and/or other artificially created anti-PC antibody derivatives, such as CDR-grafted and/or humanised antibodies, scFv, dAb, Fab, or Fv or other molecules which comprise or consists of PC-binding fragments of an antibody.

5 An antibody preparation with specificity to a PC-conjugate binds to unconjugated PC and may also bind to PC present in PC-containing compounds in which PC is exposed, for example in lysophosphatidylcholine (see for example, Kim *et al.*, 2002 *J Exp Med.* 196, 655-65). Thus, an antibody preparation with specificity to a PC-conjugate may also bind to lysophosphatidylcholine.

10 The inventors have surprisingly demonstrated that, typically, low levels of antibodies with reactivity to PC and/or a PC-conjugate are indicative of an increased risk of developing metabolic diseases. Conversely, high levels of antibodies with reactivity to the PC and/or a PC-conjugate are indicative of a reduced risk of developing metabolic diseases.

15 A second aspect of the present invention provides a method for diagnosing metabolic disease, or assessing a patient's risk of developing or progression of metabolic disease, the method comprising the steps of –

- (a) assessing the patient's level of antibodies with reactivity to PC or a PC conjugate; and
- 20 (b) diagnosing metabolic disease or determining the patient's level of risk of developing or progression of metabolic disease based on the assessed levels of antibodies with reactivity to PC or a PC conjugate.

Typically, the method of the second aspect of the invention comprises exposing PC or the PC conjugate to a sample (for example, an *ex vivo* sample) from an
25 individual and detecting antibodies which have bound to PC or the PC conjugate.

Preferably, in the second aspect of the invention, the individual is a human.

Preferably, in the method of the second aspect of the invention, the sample is blood, serum or plasma. Serum may be preferred in one embodiment.

Optionally, in the second aspect of the invention, PC is linked to a carrier via a
30 spacer. In this embodiment, typically the carrier may, for example, be a protein, such as KLH (keyhole limpet hemocyanin), transferrin, human serum albumin (HSA) or

bovine serum albumin (BSA). In an alternative embodiment, the carrier may be latex beads.

Typically, according to the second aspect of the invention, antibodies which have bound to the PC conjugate are determined by an assay, preferably an immunoassay.

The patient's levels of antibodies, e.g., of all or a particular isotype such as IgM, IgG or IgA antibodies, with reactivity to PC or the PC conjugate may be assessed using an immunoassay. Examples of suitable immunoassays are described below and will in any case be apparent to those skilled in the art.

Typically, in the second aspect of the invention, low levels of antibodies with reactivity to PC or the PC conjugate are indicative of the presence of metabolic disease and/or an increased risk of developing metabolic disease. Conversely, high levels of antibodies with reactivity to PC or the PC conjugate are indicative of the absence of metabolic disease and/or a reduced risk of developing metabolic disease. Typically, although not necessarily, antibodies are determined in a sample of patient blood, plasma or serum.

In any given population, levels of antibodies with reactivity to PC or a PC conjugate are likely to vary. The level of antibodies with reactivity to PC or a PC conjugate determined for any given individual may be categorised as high or low by reference to the range observed in the wider population. For example, a level of such antibodies below a particular percentile value determined with reference to the wider population may be categorised as a low level. Suitably, a low level may correspond to a value below the 25th percentile, or below the 20th, 10th or 5th percentile. A high level may correspond to a value of above the 5th, 10th, 20th, or 25th percentile, for example.

Where an individual is characterised as possessing low levels of antibodies with reactivity to PC or a PC conjugate, this information may assist in the diagnosis or prognosis of the presence of, or the increased risk of development or progression of, metabolic disease. A clinician may take other factors into account in arriving at a diagnosis or prognosis.

It may be desirable to measure antibodies reactive with platelet activating factor (PAF; also known as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) or a PAF conjugate as well as measuring antibodies, e.g., IgM, IgG and/or IgA antibodies, with reactivity to the PC conjugate. It may further be desirable to measure antibodies

reactive with oxidised low density lipoprotein (oxLDL) or malondialdehyde modified LDL (MD-LDL) as well as measuring antibodies, e.g., IgM, IgG and/or IgA antibodies, with reactivity to the PC conjugate. It may alternatively or in addition be desirable to measure levels of lipoprotein associated phospholipase A₂ (LpPLA₂), homocystein, C-reactive protein (CRP), HSP70, high density lipoprotein (HDL), TNF, in particular TNF α , and/or HSP60 as well as measuring antibodies, e.g., IgM, IgG and/or IgA antibodies, with reactivity to the PC conjugate. Assaying for such factors may assist in the diagnosis or prognosis of increased risk of development or progression of metabolic disease.

10 Other factors presented by a patient may also be taken into account clinician in arriving at a diagnosis or prognosis.

For example, where the individual is assessed for the presence of, or the increased risk of development or progression of, metabolic syndrome then a clinician may take into account any one, two, three or more of the presence of obesity, insulin resistance, diabetes, hypertension and hyperlipidemia in the patient. Thus, for
15 example, the clinician may also take into account whether the patient presents at least one, two, three or more of the following features (i) blood pressure >130/85 mmHg or antihypertensive treatment, (ii) fasting plasma glucose > 6.1 mmol/l, (iii) serum triglycerides >1.7 mmol/l, (iv) waist circumference > 102 cm in men and >88 cm in
20 women, and (v) HDL-cholesterol < 1.0 mmol/l in men and <1.3 in women.

Where the individual is assessed for the presence of, or the increased risk of development or progression of, polycystic ovary syndrome (PCOS) the clinician may also take into account whether the female patient presents at least one, two or more of the following features: (i) oligoovulation or anovulation, (ii) excess androgen activity or
25 (iii) the presence of polycystic ovaries.

Where the individual is assessed for the presence of, or the increased risk of development or progression of, diabetes mellitus, the clinician may also take into account whether the individual (such as male or female) patient presents and one, two or more of the following features: (i) random plasma glucose concentration above 11.1 mmol/L [200mg/dl], or (ii) fasting plasma glucose above 7.0 mmol/L [126mg/dl], or (iii)
30 2-h plasma glucose concentration after 75 g anhydrous glucose in an oral glucose, tolerance test above 11.1 mmol/L [200mg/dl].

Where the individual is considered to have metabolic disease, treatments (including treatment in accordance with the first aspect of the present invention) and/or

life-style changes may be recommended. Where the individual is considered to have an increased risk of developing metabolic disease, prophylactic treatments (including prophylactic treatment in accordance with the first aspect of the present invention) and/or life-style changes may be recommended. Where the individual is diagnosed as
5 having a progressive metabolic disease, his or her clinician may recommend treatments and/or life-style changes tailored to the individual.

In the second aspect of the invention, levels of antibodies may be characterised by assaying for all antibodies with reactivity to PC or a PC conjugate, or for only antibodies of a particular isotype, such as IgM, IgG or IgA, or for a combination of two
10 or more antibody isotypes. Preferably, the level of IgM is determined.

Immunoassays can be competitive or noncompetitive. In a typical competitive immunoassay, the antibody in the sample competes with labeled antibody to bind with the PC conjugate. The amount of labeled antibody bound to the PC conjugate is then measured. There is an inverse relationship between concentration of antibody in the
15 sample and the quantity of labeled antibody detected. In noncompetitive immunoassays, antibody in the sample is bound to the PC conjugate, then a labeled detection reagent, typically an anti-immunoglobulin antibody, is bound to the antibody. The amount of labeled detection reagent bound to the antibody is then measured. Unlike the competitive method, the results of the noncompetitive method will be directly
20 proportional to the concentration of the antibody.

In a noncompetitive immunoassay or western blot, a labeled detection reagent, typically an anti-immunoglobulin antibody, is used to detect antibody bound to the PC conjugate. A suitable anti-immunoglobulin antibody must bind specifically to immunoglobulin of the species from which the sample is obtained. It may bind to all
25 immunoglobulin isotypes of that species, or only a subset of isotypes. For example, it may bind only to IgA, IgD, IgE, IgG or IgM, or combinations of two or more of these isotypes. The anti-immunoglobulin antibody may bind specifically only to certain subtypes of any given isotype. Subtypes of human IgA are IgA1 and IgA2. The anti-immunoglobulin antibody may bind to one or both of these subtypes. Subtypes of
30 human IgG are IgG1, IgG2, IgG3 and IgG4. The anti-immunoglobulin may bind to one or more of these human IgG subtypes. It will be appreciated that there are different isotypes and subtypes in different vertebrate species.

In radioimmunoassay, the antibody or detection reagent is labeled with a radioisotope, such as ¹³¹I or ¹²⁵I. In enzyme immunoassays, the antibody or detection

reagent is labeled with an enzyme. Suitable enzymes are capable of being detected with the use of a chromogenic substrate. A chromogenic substrate is a substance which, as a result of the reaction with the enzyme, gives rise to a coloured product which can thus be detected spectrophotometrically. Enzymes such as horse radish
5 peroxidase, alkaline phosphatase, beta-galactosidase, and pyrophosphatase from *E.coli* have been widely employed. Chemi-luminescent systems based on enzymes such as luciferase can also be used. Other labels include fluorescent labels such as fluorophores of the Alexa series.

Conjugation of the antibody or detection reagent with the vitamin biotin is
10 frequently used since this can readily be detected by its reaction with enzyme- or fluorophore-linked avidin or streptavidin to which it binds with great specificity and affinity.

In a typical noncompetitive enzyme immunoassay, the sample to be analyzed is placed in contact and incubated with the PC conjugate adsorbed on a solid substrate.
15 Any anti-PC conjugate antibodies that are possibly present in the sample are thus specifically bound by the PC conjugate adsorbed on the solid substrate, producing a PC conjugate /anti- PC conjugate antibody complex. The sample is then separated from the solid substrate so as to eliminate non-bound materials, for example, by washing. In the next step of the method, an indicator antibody capable of binding any
20 anti-PC conjugate antibodies that are present on the substrate in the form of a PC conjugate /anti-PC conjugate antibody complex is added to the solid substrate, thus producing a PC conjugate /anti-PC conjugate antibody/indicator antibody complex. The indicator antibody may, for example, be an anti-human IgG immunoglobulin raised in a non-human animal species. Finally, the presence of the PC conjugate / anti-PC
25 conjugate antibody/ indicator antibody complex on the solid substrate is detected, the presence of said complex on the solid substrate being indicative of the presence of anti- PC conjugate antibodies in the sample from the individual.

Typically, the solid substrate is a micro-titration plate, for example, of the type commonly used for performing ELISA immunological assays. The micro-titration plate
30 is preferably a polystyrene plate. Other suitable solid substrates are latex particles, beads and coated red blood cells. Conveniently, the PC conjugate is adsorbed to the solid substrate by incubating the PC conjugate in a buffer with the solid substrate. Suitable buffers include carbonate buffer or phosphate buffered saline. Alternatively, the PC conjugate may be covalently linked to the solid substrate. Typically, after
35 adsorption or covalent linkage of the PC conjugate to the solid substrate, the solid

substrate is incubated with a blocking agent to reduce non-specific binding of matter from the sample to the solid substrate. Suitable blocking agents include bovine serum albumin.

It is preferred that a quantitative estimate of antibody which can bind to PC or the PC conjugate is obtained by one or more of the above techniques. In typical non-competitive assays, a linear relationship between the measured variable, whether it be optical density or some other read-out, and antibody concentration, is assumed. For example, if sample A has double the optical density of sample B in the assay (background having been subtracted from both), it is assumed that the concentration of antibody is double in A compared to B. However, it is preferable to construct a standard curve of serial dilutions of a pool of positive serum samples. Preferably, such dilutions are assayed at the same time as the test samples. By doing this, any variation from the linear relationship may be taken into account in determining the quantity of antibody in the samples.

A particularly preferred immunoassay for assessing the levels of antibodies to PC and/or the PC-conjugate in a sample uses the commercially available CVDefine™ assay kit available from Athera Biotechnologies AB. The CVDefine™ assay has been discussed in numerous publications, such as Grönlund, H., Hallmans, G.; Jansson, J.H., Boman, K., Wikström, M., de Faire, U., Frostegård, J. *Low levels of IgM antibodies against phosphorylcholine predict development of acute myocardial infarction in a population-based cohort from northern Sweden*. Eur J Cardiovasc Prev Rehabil. 2009. **16**(3) 382-6; Frostegård, J., Tao, W., Georgiades, A., Råstam, L., Lindblad, U., and Lindeberg, S. *Atheroprotective natural anti-phosphorylcholine antibodies of IgM subclass are decreased in Swedish controls as compared to non-westernized individuals from New Guinea*. Nutr. Metab. (Lond). 2007. **4**(7) 1-10; Sjöberg, B., Su, J., Dahlbom, I., Grönlund, H., Wikström, M., Hedblad, B., Berglund, G., de Faire, U., and Frostegård, J. *Low levels of IgM antibodies against phosphorylcholine - A potential risk marker for ischemic stroke in men*. Atherosclerosis. 2009. **203**(2) 184-9; and Su, J., Georgiades, A., Wu, R., Thulin, T., de Faire, U., and Frostegård, J. *Antibodies of IgM subclass to phosphorylcholine and oxidized LDL are protective factors for atherosclerosis in patients with hypertension*. Atherosclerosis 2006. **188**(1), 160-66.

The CVDefine™ assay is an indirect non-competitive enzyme immunoassay for quantitative determination of anti-phosphorylcholine (anti-PC) IgM antibodies in human serum or plasma. The wells of a microplate are coated with PC antigen. PC-specific

IgM antibodies present in the patient sample bind to the antigen. In a second step an enzyme labelled second antibody (conjugate) binds to the antigen-antibody complex which leads to the formation of an enzyme labelled conjugate-antibody-antigen complex. The enzyme labelled antigen-antibody complex converts the added substrate to form a coloured solution. The rate of colour formation from the chromogen is a function of the amount of conjugate complexed with the bound antibody and thus is proportional to the initial concentration of the respective antibodies in the patient sample. The CVDefine™ kit contains the following reagents –

- Microplate Strips: 12 strips × 8 wells (for 96 determinations) coated with PC conjugated to bovine serum albumin (BSA), maintained in a foil pouch containing desiccant.
- Calibrators: vials of anti-PC IgM antibody calibrators at concentrations of 0-6.25-12.5-25-50-100 U/ml in a buffer containing BSA, 0.095% (w/v) sodium azide, detergent and human serum, 1.5 ml each, ready to use.
- Control High: a vial of buffer containing BSA, 0.095% (w/v) sodium azide, detergent and human serum, 1.5 ml, ready to use. The level of IgM anti-PC in this control should be high enough to provide an optical density of greater than 0.6 when assayed by the following procedure.
- Control Low: a vial of buffer containing BSA, 0.095% (w/v) sodium azide, detergent and human serum, 1.5 ml, ready to use. The level of IgM anti-PC in this control should be low enough to provide an optical density of less than 0.2 when assayed by the following procedure.
- Wash Buffer Concentrate: a vial of 20x PBS concentrate and detergent, 75 ml
- Sample Diluent: a vial of PBS containing BSA, with <0.1% (w/v) sodium azide and detergent (Tween 20), 100 ml (yellow coloured), ready to use.
- IgM HRP Conjugate: a vial of anti-human IgM HRP (horse-radish peroxidase) from goat, 20 ml, ready to use.
- Substrate TMB: a vial of TMB (3, 3', 5, 5' Tetramethylbenzidine), < 0.05 % (w/v) in 20 ml water, ready to use,
- Stop Solution: a vial of 0.5 M H₂SO₄, 20 ml (colourless), ready to use

The CVDefine™ assay procedure is as follows –

- (i) Dilute serum/plasma (1:101) using Sample Diluent.
- (ii) Remove microplate strips from pouch and put firmly into strip holder. Plates are coated with PC-BSA (10 µg/mL) 50 µL/well in phosphate-buffered saline (PBS). Coated plates were incubated overnight at 4°C. After washing with PBS, the plates were blocked with 2% BSA/PBS for 2 hours at room temperature and washed with PBS.
- (iii) Dispense 100 µl of calibrators, high and low controls and diluted patient samples into appropriate wells.
- (iv) Incubate for 30 minutes.
- (v) Aspirate fluid from wells and wash wells 3 times with Wash Buffer with soaking steps in between, as follows. Dispense 300 µl of 1x Wash Buffer into each well and incubate for 20 seconds; remove Wash Buffer from the wells by aspiration or by inverting the plate over a sink and vigorously shaking ; remove residual Wash Buffer by tapping the inverted plate on clean absorbent paper. Repeat procedure 2 further times.
- (vi) Dispense 100 µl of IgM HRP Conjugate into all wells.
- (vii) Incubate for 30 minutes.
- (viii) Aspirate fluid from wells and wash wells 3 times with Wash Buffer with soaking steps in between (see step (v) above).
- (ix) Dispense 100 µl of Substrate TMB into all wells.
- (x) Incubate for 10 minutes in the dark.
- (xi) Dispense 50 µl of Stop Solution into all wells.
- (xii) Read absorbance (OD) at 450 nm, max. 30 minutes after adding the Stop Solution. A reference wavelength of 620 nm is recommended.

The direct results of the CVDefine™ assay are initially expressed in arbitrary units. Calibrators and controls are adjusted and traceable to an in-house human reference serum preparation established at Athera Biotechnologies AB. The assay has a measuring range of 6.25 U/ml - 100 U/ml and a detection limit of 0.5 U/ml.

For quantitative evaluation of the results from this type of assay, an individual calibration must be performed for each run. Quantitative evaluation can be performed manually or by the use of data reduction software.

The manual procedure requires calculation of the mean absorbance (OD) value of each calibrator, which is plotted against the calibrator concentrations of 0, 6.25, 12.5, 25, 50, 100 U/ml on suitable graph paper. A smooth curve is then drawn considering all

calibrator points, and the concentrations of the samples can then be read from the calibration curve.

When using data reduction software, a suitable computer program is selected that uses a 4 parameter or Cubic Spline curve fitting algorithm. In case the implemented curve fitting algorithm is not able to automatically handle standards with 0 U/ml, assign a minimal value to Calibrator 1 (0 U/ml), which is derived one log scale below the Calibrator 2 (e.g. 0.625 U/ml for Calibrator 1). Samples which give absorbances above that of the highest Calibrator are out of range of this assay and should be stated as > 100 U/ml. Such samples should be diluted as appropriate and reassayed.

Quantile cut-offs can be based on the anti-PC distribution in control groups. The associations between serum levels of anti-PC and risk of developing a metabolic disease can be determined by conditional logistic regression models with calculation of odds ratios (ORs) and 95% confidence intervals (CI). Test samples and controls may be age and gender matched for by design of the study. To test for differences in means/medians between cases and controls, the t-test may be used for normally distributed variables and the Wilcoxon Rank sum test for non-normally distributed variables. The Chi-Square test (and Fisher's exact for small samples) may be used to test for differences in proportions. The non-parametric Spearman Rank Correlation Coefficient may be used to test for correlations. Linear trend in proportions may be assessed through the Cochran-Armitage trend test. Linear trend in ORs over quantiles may be assessed by the Score-test (a Cochran-Armitage trend test) of quantile included as a continuous variable in SAS PROC LOGISTIC. A two-tailed p-value < 0.05 may be considered as significant. SAS may be used for the statistical analyses (release 9.2, SAS Institutet Inc.Cary,NC).

An example of a method to determine the absence or presence and/or level of IgM antibodies with reactivity to a PC conjugate which is related to an increased or decreased risk of developing metabolic diseases is described. Other methods known in the art can also be used.

Similar methods may be used to determine the absence or presence and/or level of IgG or IgA antibodies with reactivity to a PC conjugate.

As discussed above, the level of antibodies with reactivity to PC and/or a PC-conjugate determined for any given individual may be categorised as high or low by reference to the range observed in the wider population or test cohort. It may be

appropriate to assess anti-PC levels blood samples taken from individuals in a cohort *before* the onset of metabolic disease (incident cases) compared to three unrelated age- and sex-matched controls at blood draw (+/- 1 year), and/or to assess anti-PC levels blood samples taken from individuals in a cohort *after* the onset of disease (prevalent cases) compared to three unrelated age- and sex-matched controls at blood draw (+/- 1 year). It may be possible to match the controls to more than one test case and so the effective number of controls may therefore be less than 3 x number of cases. The total number of test and controls individuals in a suitable cohort may be greater than 100, such as about 200, 300, 400, 500, 600, 700, 800, 900 or 1000.

Where a test case shows a level of anti-PC antibodies below the mean average, or below a particular percentile value determined with reference to the wider population or cohort, it may be categorised as a low level. Suitably, a low level may correspond to a value below the 25th percentile, or below the 20th, 10th or 5th percentile. A high level may for example, correspond to a value of above the 5th, 10th, 20th, or 25th percentile, or above the mean average level.

In practice, the skilled person will appreciate that any percentile value cut-off point can be used to indicate a low level of anti-PC that is associated with the presence of, or the increased risk of developing, a metabolic disease, so long as, when conditional logistic regression analysis is performed on the anti-PC levels generated from a test cohort:-

- the calculated odds ratio for all individuals within that percentile group is greater than 1 (indicating that a person having an anti-PC level within the levels associated with that percentile is more likely to develop a metabolic disease than a person having an anti-PC level above that percentile); and
- the p-value calculated from the anti-PC values for individuals within that percentile group is less than 0.05 and the 95% odds ratio confidence interval for that group provides a range in which the lower limit is above 1 (wherein such p-values and CI values indicate that the odds ratio value ascribed to individuals with anti-PC levels falling within that percentile group is statistically significant).

In practice, therefore, the skilled person can readily determine by statistical analysis of the data from a cohort, the highest percentile value for which anti-PC levels indicate a statistically significant risk of developing a metabolic disease, and can also calculate associated (and incrementally higher) hazard ratios for individuals with anti-PC levels falling within lower percentile values.

Additionally, or alternatively, the level of antibodies with reactivity to PC and/or a PC-conjugate determined for any given individual may be categorised as high or low by reference to the absolute level of anti-PC antibody within a sample taken from that individual, in view of the inventors' findings. Thus, when the level of anti-PC is determined quantitatively, for example by using the CVDefine™ kit assay, the inventors have found that mean levels of anti-PC IgM levels in a population are typically about 40-50 U/ml (corresponding to about 4-5 µg/ml), and median levels in female humans are around 68 U/ml (corresponding to about 6.8 µg/ml) in PCOS patients and around 84 U/ml (corresponding to about 8.4 µg/ml) in a control group. Values in a sample at or below any one or more of these levels, for example, less than about 84, 80, 75, 70, 68, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, or less U/ml (wherein 1 U/ml correspond to about 1 µg/ml) may be considered as being low. anti-PC IgM levels of, or below, about 25-20 U/ml (which corresponds to about 2.5-3 µg/ml) are typically representative of values below the about the 25th percentile, and values under about 17 U/ml (which corresponds to about 1.7 µg/ml) antibody are typically representative of values below about the 10th percentile. Thus, anti-PC levels, such as IgM anti-PC levels, in a sample at or below about 50, 40, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10 or less U/ml (wherein 1 U/ml is equal to about 100 ng of antibody per ml) may be particularly associated with an increased risk of developing a metabolic disease, such as PCOS, and/or as a marker of actually having an already developed metabolic disease.

An example of a method to determine the absence or presence and/or level of IgM antibodies with reactivity to a PC-conjugate which is related to an increased or decreased risk of developing metabolic diseases is described below. Other methods known in the art can also be used. Similar methods may be used to determine the absence or presence and/or level of IgG or IgA antibodies with reactivity to a PC-conjugate.

Where an individual is characterised as possessing low levels of antibodies with reactivity to PC and/or a PC-conjugate, this information will assist in the diagnosis, or prognosis of increased risk of development or progression, of a metabolic disease.

Accordingly, methods that allow for the determination of absence, presence and/or levels of antibodies with reactivity to PC and/or a PC-conjugate can be used as an early-warning mechanism (i.e. a predictive method) for the likelihood of developing metabolic diseases and/or as a marker of the presence of the disease.

As discussed above, an aspect of the invention is to provide a method of diagnosing the absence, presence and/or levels of antibodies, for example IgA, IgM or IgG antibodies, with reactivity towards PC and/or a PC-conjugate (that is, anti-PC antibodies) which factor is related to an increased or decreased risk of developing metabolic diseases, using a PC-conjugate and to the use of this information to determine whether an individual is at risk of developing metabolic diseases. A preferred method is an immunoassay. The method may be used in assessing an individual's risk of developing or progression of metabolic diseases and/or may be used to monitor the efficacy of the treatment methods of the invention by active or passive immunisation, insofar as they are directed at increasing the anti-PC titre in an individual in order to effect prophylaxis, prevention and/or treatment of a metabolic disease.

Typically the method of diagnosis will be performed on a sample, such as an *ex vivo* sample, taken from the test subject. The sample may, for example, be an *ex vivo* serum sample or an *ex vivo* plasma sample. Typically the test subject will be human. The human subject from which the test sample is taken may, for example, be aged at least 10, 20, 30, 40, 50, 60, 65, 70, 75, 80, 85 or more years. In the case of polycystic ovary syndrome (PCOS) in females belonging to older age groups, such as human females aged at least 40, 50, 60, 65, 70, 75, 80, 85 or more years, and/or females that are pre-, peri-, and/or post-menopausal, may be selected.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein. Similarly, any embodiment discussed with respect to one aspect of the invention may be used in the context of any other aspect of the invention.

Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value. Alternatively, it may be used to signify a value that is $\pm 20, 10, 5, 4, 3, 2, 1$ or less than 1% of the stated value.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

Other objects, features and advantages of the present invention will be apparent from the detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and
5 modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

The examples disclosed below are provided only for the purpose of illustrating the present invention and should not be considered as any limitation of the scope as outlined in the appended claims. Document referred to herein are hereby incorporated
10 by reference.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a graph illustrating the IgM anti-PC levels in patients with PCOS and in healthy controls.

Figure 2 shows a graph illustrating the plasma levels of anti-PC after PC-BSA
15 vaccination.

Figure 3 shows a graph illustrating the association between anti-PC levels and waist circumference.

EXAMPLES

20 Example 1 - low levels of anti-PC antibodies in PCOS.

Material and methods:

Patients

One-hundred eleven women with PCOS with a median age of 37 years (range 27 – 46 years) were recruited from the departments of Obstetrics and Gynecology at
25 Sunderby Hospital, Umea University Hospital and Uppsala University Hospital. The patients were recruited among women seeking care for oligomenorrhea and/or hirsutism or among patients participating in a long-term follow-up of middle-aged PCOS patients. PCOS was defined according to the Rotterdam criteria and two of the following three features had to be present for the PCOS diagnosis 1) oligomenorrhea
30 with eight or fewer menstruations in the previous 12 months or amenorrhea; 2) clinical

and/or biochemical signs of hyperandrogenism such as testosterone > 2.7 nmol/l, elevated DHEAS, free androgen index ≥ 5.0 , or hirsutism (> 7 on the Ferriman and Gallway scale); 3) polycystic ovaries on ultrasound examination (> 12 follicles 2 to 9 mm in diameter and/or increased ovarian volume (> 10 ml)). PCOS diagnosis also implied no evidence of thyroid disease, adrenocortical dysfunction or, hyperprolactinemia. All women with PCOS were premenopausal and were currently not using any hormonal compounds.

Included were also 79 healthy control subjects with a median age of 39 years (range 26 – 46 years), recruited after advertisement in local newspapers. All control subjects had regular menstrual cycles, normal androgens and no signs of polycystic ovaries on transvaginal ultrasound.

All subjects gave written informed consent and The Ethical Review Board at Uppsala University, Sweden approved the study.

Clinical measures

All PCOS patients and controls were examined with the following measures: Height (to the nearest cm) and weight (to the nearest 200 g) was measured in stockinged feet and light clothes using standardized equipment. Body mass index (BMI) was calculated as the weight in kilogram (kg) divided by the square of height in meters. Waist circumference (to the nearest 0.5 cm) was measured horizontally around the smallest circumference between the ribs and iliac crest. Hip circumference (to the nearest 0.5 cm) was measured around the maximal girth of the hips. Seated blood pressure was taken (average of three readings) after five minutes rest. Ovarian morphology was assessed in each subject, including the control subjects, by transvaginal ultrasound.

After overnight fasting, blood samples for analyses of glucose, insulin, triglycerides (TG), cholesterol, testosterone, sex hormone binding globulin (SHBG), and IgM anti-PC were drawn between 08.00 and 08.30. The blood samples were stored at - 70° C until analyzed. Free androgen index (FAI) was calculated as testosterone (nmol/L)/SHBG (nmol/L) x 100 and homeostasis model assessment of insulin resistance index (HOMA)-IR as fasting serum insulin (mU/L) x fasting serum glucose (mmol/L)/22.5.

Assays

Insulin, SHBG, and testosterone were analyzed on a Modular E170 (Roche Diagnostics, Mannheim, Germany). The total coefficients of variation of the instrument were 1.6% at 7.0 mU/L for insulin, 1.5% at 43 nmol/L for SHBG, and 6.8% at 3.9 nmol/L for testosterone. Triglycerides and glucose were analyzed on an Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA). The total imprecision (coefficient of variation, CV) of the instrument were 1.1% at 0.9 mmol/L for triglycerides, 1.0% at 4.4 mmol/L for glucose.

Detection of IgM anti-PC was performed with ELISA (CVDefine®, Athera Biotechnologies AB, Stockholm, Sweden) according the manufacturer's instruction. Serum samples were tested in dilution 1:101 and antibody levels were expressed as arbitrary units (U/ml) calculated from a six-point calibrator curve containing 0, 6.25, 12.5, 25, 50, and 100 U/ml of IgM anti-PC. The between CV of the assay was 5.2% and the within CV was 1.9%.

Statistical methods

All values are presented as mean $\pm$ SD or as median (10th - 90th percentiles). Comparisons between groups were made with independent t-test or Mann-Whitney U-test, depending on normal distribution of the variable. Subject were sub-grouped according to age quintiles and PCOS patients in the oldest age-quintile (43 years and older) were compared to control subjects of similar age as well as to younger PCOS patients and younger control subjects by Kruskal-Wallis test.

IgM anti-PC levels were also categorized according to the median level for all participants and the proportion of subjects with IgM anti-PC levels below the median level were compared between groups by Chi-Square test. Stepwise logistic regression analysis with below-median IgM anti-PC as outcome measure, and group (PCOS patients vs. control subjects), age, BMI, testosterone, and HOMA-IR as independent confounders was performed. Nagelkerke pseudo R^2 was used as a measure of the goodness of fit between the logistic regression models.

SPSS statistical package was used for all analyses (SPSS, Inc., Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.

Results

Subject characteristics

There was no difference in age or BMI between women with PCOS and control subjects (Table 1). Women with PCOS had significantly higher waist to hip ratio, triglycerides and testosterone serum concentration, whereas SHBG serum concentrations were significantly lower than in control subjects. Insulin resistance, expressed as HOMA-IR, did not differ between groups (Table 1).

Table 1. Subject characteristics of patients with polycystic ovaries syndrome (PCOS) and healthy controls.

	PCO patients	Control subjects
	mean $\pm$ SD	mean $\pm$ SD
	(n = 111)	(n = 79)
Age, year	36.7 $\pm$ 6.9	38.0 $\pm$ 6.8
BMI	28.2 $\pm$ 5.7	26.8 $\pm$ 5.4
Waist to hip ratio	0.85 $\pm$ 0.09*	0.82 $\pm$ 0.09
Systolic blood pressure (mm Hg)	122 $\pm$ 16	123 $\pm$ 13
Diastolic blood pressure (mm Hg)	78 $\pm$ 10	77 $\pm$ 10
Triglycerides, mmol/L	1.26 $\pm$ 0.81**	0.99 $\pm$ 0.44
Cholesterol, mmol/L	4.8 $\pm$ 0.9	4.9 $\pm$ 1.0
Glucose, mmol/L	4.9 $\pm$ 0.8	4.8 $\pm$ 0.7
Insulin, mU/L	10.3 $\pm$ 8.3	8.3 $\pm$ 6.7
HOMA-IR	2.3 $\pm$ 2.0	1.8 $\pm$ 1.7
Testosterone, nmol/L	1.4 $\pm$ 0.8 [#]	1.2 $\pm$ 0.7
SHBG, nmol/L	45.6 $\pm$ 27.1 ^{##}	56.4 $\pm$ 29.7
Free androgen index	4.9 $\pm$ 5.4**	2.8 $\pm$ 2.5

Age, body mass index (BMI), waist to hip ratio, systolic blood pressure, diastolic blood pressure, triglycerides, cholesterol, glucose, insulin, homeostasis model assessment of insulin resistance index (HOMA-IR), free androgen index.

*) $p < 0.05$ t-test **) $p < 0.01$ t-test, independent t-test, #) $p < 0.05$, ##) $p < 0.01$, Mann-Whitney U-test

IgM Anti-PC

The median IgM anti-PC level in PCOS patients (68 U/ml, 32 - 283 U/ml) was not significantly different compared to control subjects (84 U/ml, 33 - 316 U/ml). However, the proportion of PCOS patients with low IgM anti-PC levels, defined as number of individuals below the median level (70.8 U/ml), was significantly higher than among healthy controls (65.3% of subjects with low values IgM anti-PC levels had PCOS vs. 34. 7% of the control subjects, $p < 0.05$).

The median age of PCOS patients and control subjects were in same range but the age distribution between the two groups differed slightly. There was no correlation between IgM anti-PC levels and age either among PCOS patients or control subjects ($r = -0.02$ and $r = 0.15$, respectively) but PCOS patients in the oldest age quintile had significantly lower IgM anti-PC level than all other groups, (Figure 1).

Table 2. The association between polycystic ovary syndrome (PCOS) and low levels of IgM anti-phosphorylcholine antibodies (IgM anti-PC) after stepwise logistic regression displayed as odds ratios (OR) and 95% confidence intervals (CI).

Variable	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)
	Age	Age, testosterone	Age, testosterone, BMI	Age, testosterone, BMI, HOMA-IR
Nagelkerke	0.03	0.057	0.09	0.10
PCOS diagnosis	1.83* (1.01 - 3.31)	2.28* (1.2 - 4.33)	2.26* (1.18 - 4.31)	2.14* (1.09 - 4.21)
Age	1.02 (0.98 - 1.07)	1.02 (0.97 - 1.07)	1.02 (0.97 - 1.07)	1.03 (0.97 - 1.08)
Testosterone		0.79 (0.53 - 1.18)	0.72 (0.48 - 1.09)	0.69 (0.44 - 1.08)
BMI			1.06* (1.01 - 1.13)	1.07 (0.99 - 1.16)
HOMA-IR				1.04 (0.83 - 1.30)

The logistic regression was made using below-median IgM anti-PC as outcome measure. Model 1: The relation for low IgM anti-PC and PCOS, adjusted for age. Model 2-4: Adjustments of Model 1 by adding the explanatory variables body mass index (BMI), testosterone levels, and homeostasis model assessment of insulin resistance index (HOMA-IR) in a stepwise fashion. Low levels of IgM anti-PC are

consistently associated with PCOS diagnosis throughout all models. In model 3 PCOS diagnosis and BMI were independently associated with IgM anti-PC but the association with BMI was lost when insulin resistance was brought into model 4.

*) $p < 0.05$, **) $p < 0.01$.

5 The stepwise logistic regression analysis indicated that PCOS diagnosis was significantly and independently associated with low levels of IgM anti-PC even following the stepwise adjustments for age, BMI, testosterone levels and HOMA-IR (Table 2). In fact, the relation between PCOS and low IgM anti-PC levels became even stronger when these adjustments were made; the OR increased from 1.83 in the first model with
10 age and group (PCOS vs. control subjects) to an OR of 2.14 when all other confounders were included in the regression model.

Discussion

 The main finding of the present study was that, even though most participants had IgM anti-PC levels well above the 17 -30 U/ml which have previously been
15 reported as a cut off for the association with increased CVD risk (17-19), the proportion of PCOS patients with below-median levels of IgM anti-PC was increased compared to healthy controls. This difference between women with PCOS and controls persisted even though adjustments were made for known CVD risk factors such as age, BMI, testosterone levels and insulin resistance.

20 Furthermore we found that PCOS patients older than 43 years of age had lower median IgM anti-PC levels than both younger women with PCOS and healthy control subjects of similar and younger age. However, IgM anti-PC levels did not differ in the overall comparison between PCOS patients and control subjects. As levels of IgM anti-PC tend to decrease with increasing age (20) the finding of low IgM anti-PC levels
25 predominantly in older women with PCOS may indicate a more rapid age-related decrease of IgM anti-PC levels in women with PCOS, or possibly, decreased levels of IgM anti-PC does not become evident until women with PCOS reach premenopausal ages. Hence, the overall difference between women with PCOS and healthy controls maybe would have been more pronounced if primarily older women had been included
30 in the study.

 As low levels of IgM anti-PC have been reported to be associated with both inflammatory responses involved in atherosclerosis (19) and increased risk for CVD (17-19), low levels of IgM anti-PC might be a pathway through which part of the

increased risk for CVD reported among PCOS patients operate. The generally lower levels of IgM anti-PC among the oldest PCOS patients might also support further investigations into the use of IgM anti-PC as a marker for risk of developing CVD in this specific group of women.

5 Although direct comparisons of IgM anti-PC levels in-between studies may be limited, it was evident that our study population of relatively young and healthy women displayed higher median IgM anti-PC levels than previous studies have reported. The relatively high IgM anti-PC levels in our cohort are presumably attributed to the female study population, the relatively low age of our subjects and the fact that cardiovascular
10 events are rare in premenopausal women (21). IgM anti-PC levels decrease with increasing age (20) and are higher in women compared to men (16, 19). Also, the IgM anti-PC autoantibodies are suggested to have an atheroprotective function, by reducing development of atherosclerosis in mice and humans (14-16). Furthermore, in a non-westernized population with very low prevalence rates for CVD significantly higher anti-
15 PC IgM levels were found as compared to a Swedish age- and sex-matched population (20). In fact, because of the gender-related differences in IgM anti-PC levels, it has been hypothesized that higher levels of these antibodies could be one underlying factor that contributes to the lower risk of CVD in women as compared to men of similar age (22). From a gynecological perspective, the later presentation of cardiovascular
20 disease in women has generally been attributed to the protective effects of premenopausal estradiol levels (21). Although hormone replacement therapy currently is not recommended for postmenopausal women for the prevention of cardiovascular diseases (23), ongoing trials are investigating its use in younger, perimenopausal women (24). Further studies of how endogenous estradiol levels and estradiol
25 treatment affects IgM anti-PC levels are warranted.

In conclusion, the results of this study indicate that women with PCOS more frequently display below-median IgM anti-PC levels than controls and that this result persists even after the adjustment for other known risk factors for CVD.

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45 **Example 2**

Background

apoE^(-/-) mice fed an atherogenic diet (a so called Western diet) become severely hyperlipidemic, and atherosclerosis will develop rapidly. After some time on the diet, these mice will show features of the metabolic syndrome (hypertension, hyperlipidemia, insulin resistance and obesity) as a consequence of the lipid laden diet.

Thus, it is possible to investigate the effects of intervention on not only atherosclerosis in this experimental model, it is also possible to investigate effects features of the metabolic syndrome.

5 This prophetic example describes means to investigate the effects of PC antibodies on the development of diabetes, wherein apoE^(-/-) mice will be immunized with PC conjugated to BSA, as the PC molecule itself is too small to trigger an antibody reaction. Aluminium hydroxide (Alum, Aluminium hydroxide hydrate, batch 034K3685, Sigma, St Louis, MO) is used as adjuvance.

10 Male apoE^(-/-) mice (Taconics, Denmark) will be used in the current study. At age from 8 to 10 weeks weeks, they will be switched from a standard mouse chow diet to a Western type diet containing 21.2% and 0.15% (wt/wt) fat and cholesterol, respectively (Lantmännen R368). The mice will be divided into groups, receiving 200 µL subcutaneous injections containing 5 to 25 µg/mouse of the PC-BSA conjugate and one mg alum (PC group), 5 to 25 µg BSA and Alum (sham) or saline (n = 11 per group). For active immunisation, PC-BSA will be added to alum (5 mg/ml in NaCl), and 15 the mixture gently shaken for an hour before administration. 200 µL will be injected subcutaneously in the neck at each occasion. For the sham group, PC-BSA will be replaced by BSA, and in the controls, only NaCl will be administered. Starting when the mice were placed on the Western diet, they will receive injections every second week 20 until termination of the experiment after 16 weeks on the diet. Fasting blood samples (4 hours fasting) will be taken from the tail vein or v saphena before the experiment starts and before each immunisation.

Fasting insulin (F_{insulin}) and glucose will be measured every 2nd week throughout the study. Tail vein blood samples will be obtained after a 4 hour fasting period. Fasting 25 blood glucose (FPG) levels will be measured using an Accu-Chek Compact glucometer (Roche Diagnostics Corp., Indianapolis; Indiana, USA), and insulin will be determined by a ELISA kit specific for mouse insulin (Ultra Sensitive Mouse Insulin ELISA kit #90080, Crystal Chem Inc.).

Insulin sensitivity will be estimated as HOMA-IR, obtained as the product of FPG and F_{insulin} , or as elevated F_{insulin} . 30

The levels of antibodies against PC will be measured using the ELISA method as described above.

Results.

In line with our experience, the active immunisation is expected to result in markedly elevated levels of IgM antibodies against PC, while levels in the other groups will be low. After approximately two weeks, antibody levels will begin to rise, and reach maximal levels after 6-8 weeks in actively immunized mice. After this they remain high. IgG antibodies against PC will appear after 6 to 10 weeks in the actively immunized mice, and remain low in the other groups. Although the methods used to measure the IgM and IgG antibodies are not quantitative, the levels of IgG anti-PC will appear to be lower than levels of the IgM anti-PC. As the PC was linked to BSA, we will also measure if antibodies towards BSA are formed.

It is well known that apoE^(-/-) mice develop insulin resistance or prediabetes when administered an atherogenic diet (see above). Throughout the study period of 16 weeks FPG will remain constant in all study groups, i.e. the mice will not become diabetic. Insulin resistance can be measured as increased fasting insulin levels in serum, or as HOMA_{IR}. Both fasting insulin levels and HOMA_{IR} will increase significantly, and will show the development of peripheral insulin resistance. This process will start after approximately 8 weeks on the atherogenic diet, and insulin resistance will then increase steadily.

Compared to controls, mice actively immunized with PC-BSA will have better peripheral insulin sensitivity than the other groups, as shown by significantly lower increases in fasting insulin and HOMA_{IR}, without major differences in fasting glucose levels. Mice receiving BSA and adjuvant will not show any improvement in fasting insulin or glucose levels compared to saline treated animals.

Conclusions.

In accordance with the paradigm of the present invention, immunisation of mice with PC-BSA will be shown to result in markedly elevated levels of antibodies against PC, especially of the IgM isotype. A surprising aspect to the present invention is that the PC immunisation will also reduce the development of insulin resistance in the mice. To the best of our knowledge, there are no data previously available showing that anti-PC has any role in the pathophysiology of peripheral insulin resistance, type 2 diabetes or the metabolic syndrome in large. Thus, this finding will show that increasing the levels of such antibodies, either through active immunisation as in the present case, or through passive immunisation with polyclonal or monoclonal antibodies, is an attractive therapeutic option for patients suffering from insulin resistance, type 2 diabetes and/or

the metabolic syndrome or patients suffering other diseases which display these symptoms.

Example 3

Background

5 apoE^(-/-) mice fed an atherogenic diet (a so called Western diet containing 21.2% fat and 0.15 % cholesterol) becomes severely hyperlipidemic, and atherosclerosis develops rapidly. After some time on the diet, these mice will show features of the metabolic syndrome (hypertension, hyperlipidemia, insulin resistance and obesity) as a consequence of the lipid laden diet (de Roos, B., G. Rucklidge, M. Reid, *et al.*,
10 *Divergent mechanisms of cis9, trans11-and trans10, cis12-conjugated linoleic acid affecting insulin resistance and inflammation in apolipoprotein E knockout mice: a proteomics approach*. FASEB J, 2005. **19**(12): p. 1746-8; Hansmann, G., R.A. Wagner, S. Schellong, *et al.*, *Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor-gamma activation*. Circulation, 2007. **115**(10): p. 1275-84; Phillips, J.W., K.G. Barringhaus, J.M. Sanders, *et al.*,
15 *Rosiglitazone reduces the accelerated neointima formation after arterial injury in a mouse injury model of type 2 diabetes*. Circulation, 2003. **108**(16): p. 1994-9). Thus, it is possible to investigate the effects of intervention on not only atherosclerosis in this experimental model, it is also possible to investigate effects features of the metabolic
20 syndrome.

We have shown that administering a western type diet for 16 weeks to apoE^(-/-) mice increases fasting insulin levels from less than 1 ng/ml to 4.3 and 6.0 ng/ml in two separate studies, confirming the findings in the studies cited above.

To investigate then potential of PC as a vaccine to induce the formation of IgM
25 and IgG anti-PC, apoE^(-/-) mice were immunized with PC conjugated to BSA, as the PC molecule itself is too small to trigger an antibody reaction. Using this approach, we could also investigate if the vaccination protocol could prevent the rise of fasting insulin, i.e. to prevent development of insulin resistance.

Male apoE^(-/-) mice (Charles River) were used in the experiment. At age from 8
30 to 10 weeks weeks, they were switched from a standard mouse chow diet to a Western type diet containing 21.2% and 0.15% (wt/wt) fat and cholesterol, respectively (Lantmännen R368). The mice received 200 µL subcutaneous injections containing 25 µg/mouse of a PC-BSA conjugate and one mg alum as adjuvans (n = 11). PC-BSA was

added to alum (5 mg/ml in NaCl), the mixture was gently shaken for an hour before administration. 200 µL was injected subcutaneously in the neck at each occasion. Starting when the mice were placed on the Western diet, they received injections every second week until termination of the experiment after 16 weeks on the diet. Fasting blood samples (4 hours fasting) were taken from the tail vein or vena saphena before the experiment started and before each immunisation.

The levels of antibodies against PC were measured using the ELISA method as described above. Fasting insulin and glucose were measured before the mice were placed on the western diet, and after 16 weeks on the diet. Fasting blood glucose levels were measured using an Accu-Chek Compact glucometer (Roche Diagnostics Corp., Indianapolis; Indiana, USA), and insulin was determined by a ELISA kit specific for mouse insulin (Ultra Sensitive Mouse Insulin ELISA kit #90080, Crystal Chem Inc.).

Results

The immunisation resulted in markedly elevated levels of IgM and IgG antibodies against PC (see figure 2). After approximately two weeks, IgM antibody levels began to rise, and reached maximal levels after 6-8 weeks. After this they remain high. IgG antibodies against PC appeared after 6 to 10 weeks. Although the methods used to measure the IgM and IgG antibodies are not quantitative, the levels of IgG anti-PC appear to be lower than levels of the IgM anti-PC.

During the 16 week period of western diet and vaccination, fasting plasma glucose was within the normal range for apoE^{-/-} mice (8 to 10 mM). At the same time, fasting insulin increased from 0.9 ± 0.1 ng/ml to only 1.3 ± 0.1 ng/ml. Although this increase was statistically significant, the increase was modest and clearly indicate that insulin resistance did not develop to any marked extent in this experiment.

Conclusions

Immunisation of mice with PC-BSA results in markedly elevated levels of antibodies against PC. A surprising finding was that insulin resistance did not develop in these mice. To the best of our knowledge, there are no previous data available showing that anti-PC has any role in the pathophysiology of peripheral insulin resistance, type 2 diabetes or the metabolic syndrome. Thus, this finding shows that increasing the levels of such antibodies, either through active immunisation as in the present case, or through passive immunisation with polyclonal or monoclonal antibodies, is an attractive therapeutic option for patients suffering from insulin

resistance, type 2 diabetes and/or the metabolic syndrome or patients suffering other diseases which display these symptoms.

Example 3.

5 Association between waist circumference and low anti-PC levels

Obesity is one of the components of the metabolic syndrome, and increased waist circumference is one of the symptoms of the metabolic syndrome.

The present study evaluated if anti-PC was related to cardiovascular (CV) risk factors and the metabolic syndrome in a general population. In the Prospective
10 Investigation of the Vasculature in Uppsala Seniors (PIVUS) study, 1016 subjects aged 70 were investigated regarding circulating anti-PC levels as well as classical and non-classical CV risk factors.

Anti-PC levels were not related to classical CV risk factors, but in subjects in the lowest decedtile of anti-PC levels (<17.2 U/ml) an increased prevalence of the
15 metabolic syndrome (NCEP-criteria) was found ($p=0.03$). This was mainly due to a marked increase in waist circumference in subjects with low anti-PC levels ($p=0.0003$).

Figure 3 show that individuals in the decedtile of subjects with the lowest IgM anti-PC also had a significantly higher waist circumference. We have previously noted that anti-PC levels roughly corresponding to the lowest decedtile is also associated to
20 an increased risk for premature CVD (Sjoberg, B.G., J. Su, I. Dahlbom, et al., *Low levels of IgM antibodies against phosphorylcholine-A potential risk marker for ischemic stroke in men. Atherosclerosis*, 2008. **203**(2): p. 528-32). However, no previous association between anti-PC levels and the metabolic syndrome has been observed.

The present findings indicate that increasing anti-PC levels in subjects with the
25 metabolic syndrome and/or obesity is beneficial.

CLAIMS

1. A composition comprising at least one phosphorylcholine (PC) conjugate, or an antibody preparation with reactivity to PC or a PC conjugate, for use in the immunization or prophylaxis against, or the prevention or treatment of, metabolic diseases in mammals.
5
2. Use of a composition comprising at least one PC conjugate, or an antibody preparation with reactivity to PC or a PC conjugate, in the manufacture of a medicament for the immunization or prophylaxis against, or the prevention or treatment of, metabolic diseases in mammals.
- 10 3. A method for the immunization or prophylaxis against, or the treatment of, metabolic diseases in a mammal, the method comprising the step of administering to the mammal a pharmaceutical composition comprising at least one PC conjugate, or an antibody preparation with reactivity to PC or a PC conjugate.
- 15 4. The composition of claim 1, the use of claim 2, or the method of claim 3, wherein the mammal is a human.
5. The composition of claim 1 or 4, the use of claim 2 or 4, or the method of claim 3 or 4, wherein metabolic disease is a condition selected from the group consisting of metabolic syndrome, insulin resistance, glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia,
20 hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS).
6. The composition of claim 1, 4 or 5, the use of claim 2, 4 or 5, or the method of claim 3, 4 or 5, wherein the composition comprising at least one PC conjugate is a pharmaceutical composition comprising at least one PC conjugate,
25 optionally in combination with an adjuvant.
7. The composition of claim 1, 4 or 5, the use of claim 2, 4 or 5, or the method of claim 3, 4 or 5, wherein the antibody preparation with reactivity to PC or a PC conjugate comprises a monoclonal antibody with reactivity to PC or a PC
30 conjugate.

8. The composition of any of claims 1 or 4 to 7, the use of any of claims 2 or 4 to 7, or the method of any of claims 3 to 7, for the therapeutic treatment of a mammal suffering from metabolic disease, or for the prophylactic treatment of a mammal facing the risk of developing metabolic disease.
- 5 9. The method of any of claims 3 to 8, wherein a therapeutically effective amount of a composition comprising at least one PC conjugate or an antibody preparation with reactivity to PC or a PC conjugate is administered to the mammal.
- 10 10. A method for diagnosing metabolic disease, or assessing a patient's risk of developing or progression of metabolic disease, the method comprising the steps of –
- (a) assessing the patient's level of antibodies with reactivity to PC or a PC conjugate; and
- 15 (b) diagnosing metabolic disease or determining the patient's level of risk of developing or progression of metabolic disease based on the assessed levels of antibodies with reactivity to PC or a PC conjugate.
11. The method of claim 10 comprising the step of assessing the patient's level of IgM, IgG or IgA antibodies with reactivity to PC or a PC conjugate.
- 20 12. The method of claim 10 or 11, wherein the patient's level of antibodies with reactivity to PC or a PC conjugate are assessed by analysis of an *ex vivo* sample taken from the patient.
13. The method of any of claims 10 to 12, wherein the patient is human.
- 25 14. The method of any of claims 10 to 13, wherein the patient's level of antibodies with reactivity to PC or a PC conjugate correlates negatively with the patient's risk of developing or progression of the metabolic disease.
- 30 15. The method of any of claims 1- to 14 wherein metabolic disease is a condition selected from the group consisting of metabolic syndrome, insulin resistance, glucose intolerance, hyperglycemia, type I diabetes, type II diabetes, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and polycystic ovary syndrome (PCOS).

16. Use of PC or a PC conjugate in a method of diagnosing metabolic disease and/or for assessing a patient's risk of developing or progression of metabolic disease as defined by any of claims 10 to 15.

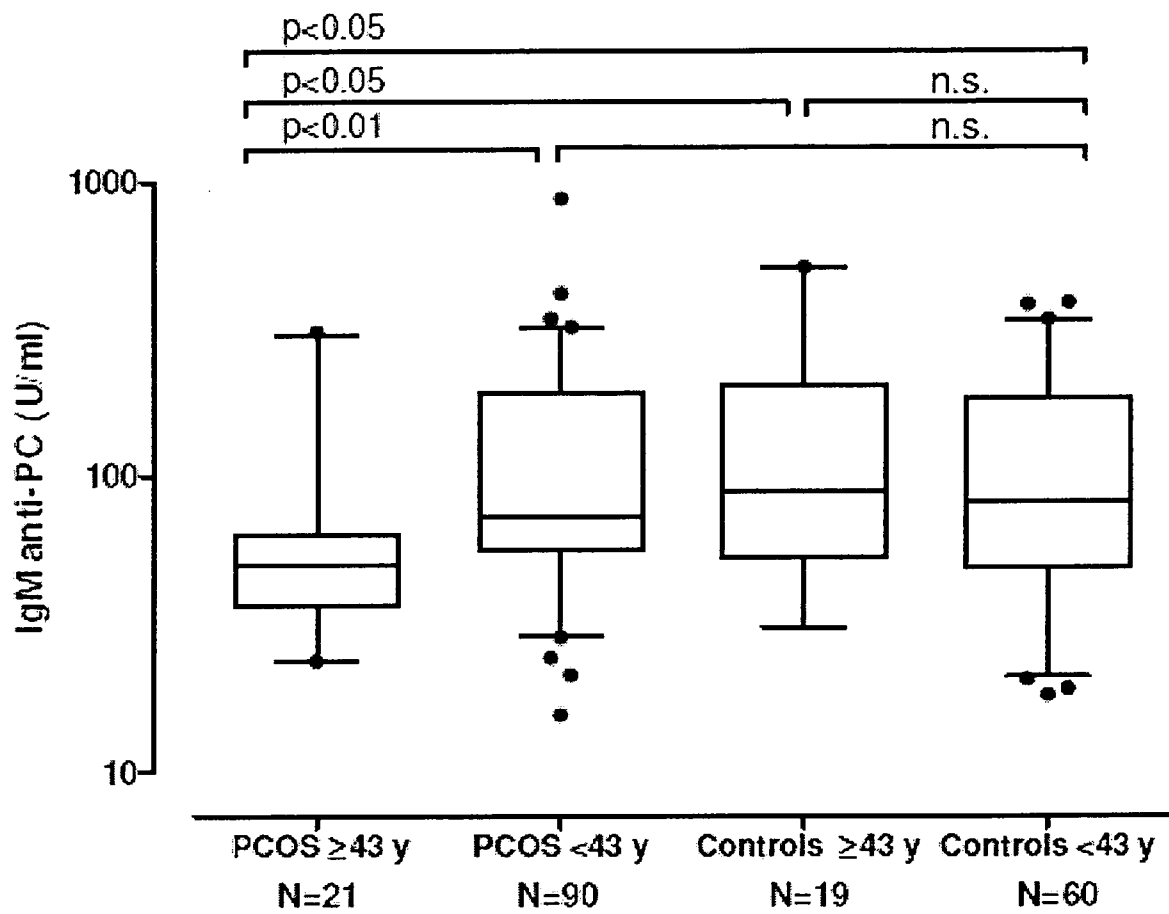
Figure 1

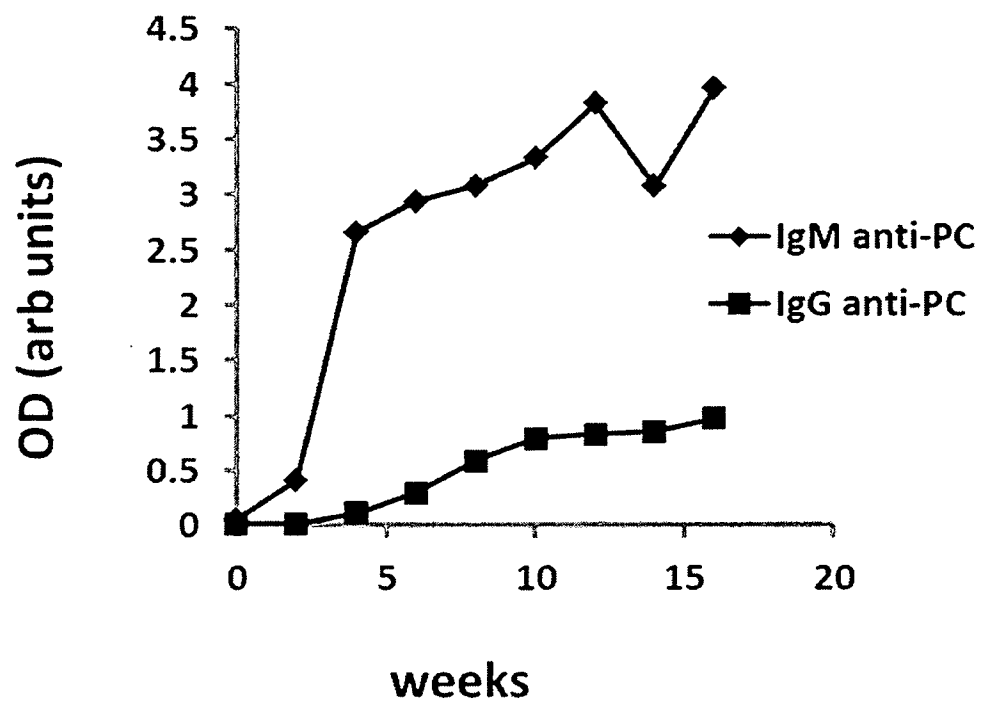
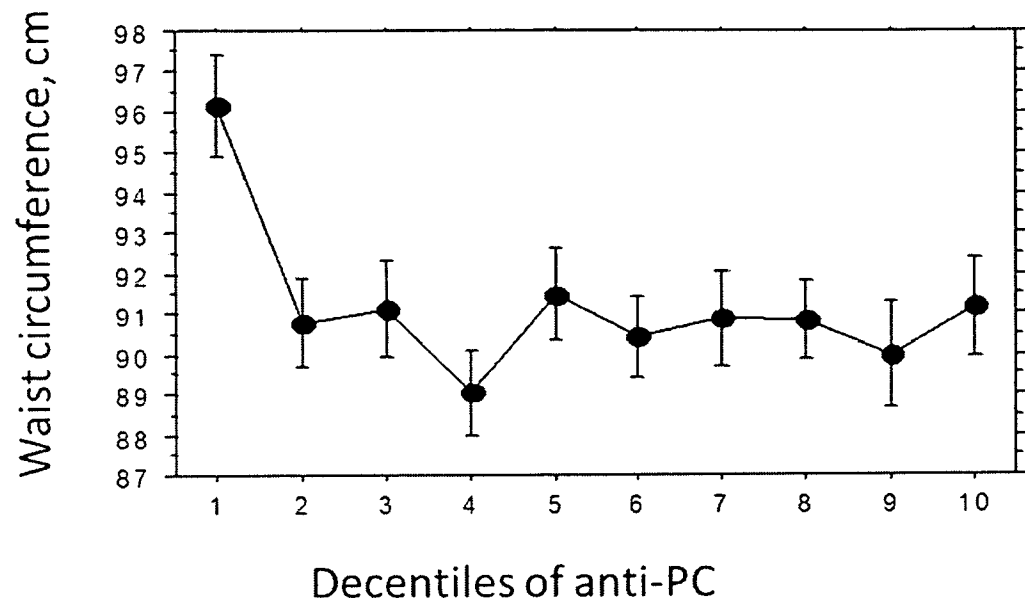
Figure 2

Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/003604

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K39/00 A61K39/395 A61K47/48 A61P3/08 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) A61K		
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 practical, search terms used) EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, SCISEARCH, 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 2005/100405 A2 (ATHERA BIOTECHNOLOGIES AB [SE]; DE FAIRE ULF [SE]; FROSTEGAARD JOHAN []) 27 October 2005 (2005-10-27) pages 4,5 pages 16,17,25 claims 1-3,10,16	1-4, 6-14,16
Y	----- FISKESUND ROLAND ET AL: "Low levels of antibodies against phosphorylcholine predict development of stroke in a population-based study from northern Sweden.", STROKE; A JOURNAL OF CEREBRAL CIRCULATION APR 2010 LNKD- PUBMED:20150554, vol. 41, no. 4, April 2010 (2010-04), pages 607-612, XP002664275, ISSN: 1524-4628 the whole document ----- -/-	1-3,10, 16
☒ Further documents are listed in the continuation of Box C. ☒ 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 document 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	
1 December 2011	13/12/2011	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Domingues, Helena	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/003604

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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International application No

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