ADIPONECTIN ANTIBODIES AND METHODS TO MEASURE ADIPONECTIN

Field of the Invention

The invention pertains to methods for measuring different forms of human adiponectin that are present in human plasma/serum, and more specifically methods are based on an ELISA assay that utilizes different monoclonal antibodies directed against adiponectin, in combination with different polyclonal antibodies directed against different domains of human adiponectin. The invention also provides unique isoforms of adiponectin and antibodies thereto, including polyclonal and monoclonal antibodies.

Background of the Invention

Adiponectin is an adipocyte specific protein, that has recently been shown to possess a variety of activities including anti-inflammatory, antiatherogenic, preventive for metabolic syndrome, and insulin sensitizing activities. Adiponectin is encoded by a single gene, giving rise to a polypeptide chain of about 30 kilodaltons. In plasma/serum, adiponectin exists in various molecular forms that include monomers, trimer, hexamers, and various combinations thereof. No information is currently known in the art about which specific molecular forms of adiponectin possess which types of activities. While current ELISA methods exist to measure adiponectin levels in human plasma/serum, it is still not possible to accurately and rapidly measure the different adiponectin forms, nor are there useful adiponectin measures that predict the relationship of the levels of specific adiponectin forms to a specific physiologic state or disease. For example, current adiponectin measures include ELISA methods which measure total adiponectin, and do not provide a means of readily predicting the physiologic state based on the levels of adiponectin. Other measures such as velocity sedimentation or gel filtration, which can discriminate between high and low molecular weight adiponectin forms include complicated methods of fractionation on the basis of size, and are not readily amenable to high throughput screening procedures.

Summary

The present disclosure relates to the measurement of different adiponectin forms, specifically the ability to measure these forms in human plasma/serum. Additionally, the

disclosure also provides the ability to correlate the levels of these different forms to a specific physiologic state, and preferably using the correlating levels of said different forms to diagnose individuals at risk of developing disease, including but not limited to diabetes, specifically Type II diabetes. Other potential disease indications where adiponectin isoforms may provide significant value include, but are not limited to metabolic syndrome, cardiovascular disease, inflammatory diseases, NASH (Nonalcoholic steatohepatitis), dyslipidemia, and other diseases.

The disclosure also provides unique isoforms of adiponectin and antibodies thereto, including polyclonal and monoclonal antibodies.

In a first aspect, there is provided a monoclonal antibody directed to a N-terminus of adiponectin, wherein the antibody comprises a light chain variable region set forth in Seq. Id. No. 4 and a heavy chain variable region set forth in Seq. Id. No. 5.

In a second aspect, there is provided a monoclonal antibody directed to a N-terminus of adiponectin, wherein the antibody comprises a light chain variable region set forth in Seq. Id. No. 6 and a heavy chain variable region set forth in Seq. Id. No. 7.

In a third aspect, there is provided a polynucleotide sequence comprising a sequence encoding the light chain variable region selected from Seq. Id. No. 4 or Seq. Id. No. 6.

In a fourth aspect, there is provided a polynucleotide sequence comprising a sequence encoding the heavy chain variable region selected from Seq. Id. No. 5 or 7.

The disclosure also demonstrates that different adiponectin isoforms do exist, and that they can be readily measured using ELISA methods of the present disclosure.

In a fifth aspect, there is provided a method for the diagnosis of pre-disposition to diabetes of a human subject, comprising at least one adiponectin monoclonal antibody selected from the group consisting of an antibody earlier described and mAb A12820 and at least one adiponectin polyclonal antibody selected from the group consisting of globular, N-terminus and C-terminus antibody in an assay to screen for adiponectin in a patient sample, wherein assay values corresponding to Receiver Operating Characteristic curve (ROC) plots with AUC of 0.65 or higher indicate a pre-disposition to diabetes.

In a sixth aspect, there is provided an antibody according to those earlier described or the mAb A12820 in combination with at least one adiponectin polyclonal antibody selected from the group consisting of globular, N-terminus and C-terminus antibody for the diagnosis of pre-disposition to diabetes of a human subject.

As such, the utility of these assays, and the identified novel adiponectin antibodies and isoforms utilized therein, can be used to monitor disease progression, response to treatment, prediction of treatment responses, as well as the diagnosis of early forms of disease, more particularly diabetes and cardiovascular disease.

Description of the Drawings

Figure 1: Amino Acid Sequence of Human Adiponectin (Seq. Id. No. 19). The amino acid sequence of human adiponectin is shown, indicating the signal peptide cleavage site, and the start of the mature protein. The site of the potential N-linked N-glycosylation is shown in the figure.

Figure 2: Schematic representation of different adiponectin molecular forms. Adiponectin has been shown to exist in multiple forms, ranging from a monomer, trimer, hexamer, as well as higher ordered aggregates. Any of the aggregate forms can have different conformations. The functional role of these different molecular forms of adiponectin remains to be elucidated. It is not known whether there are additional post-translational modifications on adiponectin that modify either the molecular state or biological role of adiponectin.

Figure 3: Schematic representation of the various adiponectin peptide sequences (N-terminal, C-terminal and globular peptides) that were used (epitopes) to develop monoclonal and polyclonal antibodies. The specific sites that the commercial monoclonal antibodies are directed to are not known at present.

Figure 4: Schematic representation of the adiponectin assay format. Briefly, monoclonal antibodies directed to adiponectin, are used to coat multiwell plates at a

concentration of 0.1 – 10 µg/well. After a coating period of 2 hours – 2 days, the wells are rinsed with buffer, and the biologic sample containing adiponectin is added to the plate, and incubated with antibody coated plate for several hours. The wells are washed with buffer, and a rabbit polyclonal antibody directed to different epitopes of adiponectin is added to the wells, and incubated for an hour. The wells are washed with buffer, and a developing antibody, donkey antirabbit IgG conjugated to HRP is added for an hour. The wells are washed several times, and a TMB substrate solution is added for a fixed time. The reaction is stopped with the addition of phosphoric acid, and the optical density at 450 nm is determined. The amount of adiponectin in the sample is determined by comparing the OD (optical density) of the sample, to the OD of a known amount of adiponectin which is used to generate a standard curve.

Figure 5: Schematic representation of a selective adiponectin assay. Specific combinations of antibodies are used to detect specific adiponectin forms within the mixture of adiponectin, which is generally found in biologic samples such as plasma. Only adiponectin isoforms recognized by two antibodies, one of which is a monoclonal antibody, and a second which is a polyclonal antibody, will be recognized and quantitated in these assays. There may be examples where certain combinations of antibodies are able to recognize multiple forms of adiponectin. One example of such an assay could be the commercial adiponectin assay from R&D systems, or some of the assays which we have developed, which recognize multiple forms of adiponectin (based on levels of adiponectin measured, as well as the different isoforms that are recognized from gradient fractionated plasma).

Figure 6: Schematic representation of the work flow in measuring adiponectin levels in plasma samples.

Figures 7: Standard curve for ELISA assays using MAB3604 and globular and N-terminal polyclonal antibodies. Examples are provided of an ELISA standard curve using the reference adiponectin 1-244 standard (C-term His-tag) 0.0195 to 10ng/well for the globular domain adiponectin pAb (Figure 7a), and for the N-terminal domain adiponectin pAb (Figure 7b) with the mAb MAB3604 at a 1:5000 dilution.

Figures 8: Standard curve for ELISA assays using mAb A12820 and three anti-adiponectin polyclonal antibodies. Examples are provided of an ELISA standard curve using the reference standard adiponectin 1-244 (C-term His tag) 0.0195 to 10 ng/well for the adiponectin globular domain pAb (Figure 8a), and for the N-terminal domain adiponectin pAb (Figure 8b). Figure 8c is an example of a standard curve using the reference standard adiponectin 16-247 (N-term FLAG tag) 0.0195 to 10 ng/well for the C-terminal domain adiponectin pAb. The mAb used in each example is A12820 at 0.5µg/ml.

Figures 9: Standard curve for ELISA assays using mAb MAB10651 and three anti-adiponectin polyclonal antibodies. Examples are provided of an ELISA standard curve using the reference standard adiponectin1-244 (C-term His tag) 0.0195 to 10 ng/well for the adiponectin globular domain pAb (Figure 9a), and for the N-terminal domain pAb (Figure 9b). Figure 9c is an example of a standard curve using the reference standard adiponectin 16-247 (N-term FLAG tag) for the C-terminal domain adiponectin pAb. The mAb is MAB10651 at 0.25 µg/ml.

Figures 10: Standard curve for ELISA assays using mAb Adiponectin M1 and three anti-adiponectin polyclonal antibodies. Examples are provided of an ELISA standard curve using the reference standard adiponectin1-244 (C-term His tag) 0.0195 to 10 ng/well for the adiponectin globular domain pAb (Figure 10a), and for the N-terminal domain pAb (Figure 10b). Figure 10c is an example of a standard curve using the reference standard adiponectin 16-247 (N-term FLAG tag) for the C-terminal domain adiponectin pAb. The mAb is Adiponectin-M1 at 1µg/ml.

Figures 11: Standard curve for ELISA assays using mAb Adiponectin M24 and three anti-adiponectin polyclonal antibodies. Examples are provided of an ELISA standard curve using the reference standard adiponectin1-244 (C-term His tag) 0.0195 to 10 ng/well for the adiponectin globular domain pAb (Figure 11a), and for the N-terminal domain pAb (Figure 11b). Figure 11c is an example of a standard curve using the reference standard adiponectin 16-247 (N-term FLAG tag) for the C-terminal domain adiponectin pAb. The mAb is Adiponectin-M24 at 1µg/ml.

Figure 12: Standard curve for the ELISA assays using the adiponectin kit from R & D Systems. An example is provided of an ELISA standard curve using the commercially available adiponectin ELISA kit from R&D Systems.

Figure 13: Fractionation of normal human plasma sample by velocity sedimentation, and measurement of adiponectin isoforms using the ELISA with MAB 3604. Human plasma was fractionated by velocity sedimentation, and fractions were collected. Fraction 1 is the top of the gradient, and fraction 20 is the bottom of the gradient. The adiponectin levels were measured using the MAB3604 in combination with the globular domain and the N-terminal domain polyclonal antibodies. The monoclonal antibody MAB3604 was used at a 1:5000 dilution as the capture antibody. 5µl of each fraction was assayed to determine the levels of adiponectin.

Figure 14: Fractionation of normal human plasma sample by velocity sedimentation, and measurement of adiponectin isoforms using the ELISA with mAb

A12820. Human plasma was fractionated by velocity sedimentation, and fractions were collected. Fraction 1 is the top of the gradient, and fraction 20 is the bottom of the gradient. The adiponectin levels were measured using the mAb A12820 in combination with the globular domain, the N-terminal domain and C-terminal domain polyclonal antibodies. The monoclonal antibody mAb A12820 was used at 0.5 µg/ml as the capture antibody. 5µl of each fraction was assayed to determine the levels of adiponectin.

Figure 15: Fractionation of normal human plasma sample by velocity sedimentation, and measurement of adiponectin isoforms using the ELISA with MAB 10651. Human plasma was fractionated by velocity sedimentation, and fractions were collected. Fraction 1 is the top of the gradient, and fraction 20 is the bottom of the gradient. The adiponectin levels were measured using the MAB 10651 in combination with the globular domain, the N-terminal domain and C-terminal domain polyclonal antibodies. The monoclonal antibody MAB 10651 was used at 0.25 µg/ml as the capture antibody. 5µl of each fraction was assayed to determine the levels of adiponectin.

Figure 16: Fractionation of normal human plasma sample by velocity sedimentation, and measurement of adiponectin isoforms using the ELISA with mAb adiponectin M1. Human plasma was fractionated by velocity sedimentation, and fractions were collected. Fraction 1 is the top of the gradient, and fraction 20 is the bottom of the gradient. The adiponectin levels were measured using the mAb adiponectin M1 in combination with the globular domain, the N-terminal domain and C-terminal domain polyclonal antibodies. The mAb adiponectin M1 was used at 1.0 µg/ml as the capture antibody. 5ul of each fraction was assayed to determine the levels of adiponectin.

Figure 17: Fractionation of normal human plasma sample by velocity sedimentation, and measurement of adiponectin isoforms using the ELISA with mAb adiponectin M24. Human plasma was fractionated by velocity sedimentation, and fractions were collected. Fraction 1 is the top of the gradient, and fraction 20 is the bottom of the gradient. The adiponectin levels were measured using the mAb adiponectin M24 in combination with the globular domain, the N-terminal domain and C-terminal domain polyclonal antibodies. The monoclonal antibody mAb adiponectin M24 was used at 1.0 µg/ml as the capture antibody. 5µl of each fraction was assayed to determine the levels of adiponectin.

Figure 18: Fractionation of normal human plasma sample by velocity sedimentation, and measurement of adiponectin isoforms using the R & D adiponectin ELISA. Human plasma was fractionated by velocity sedimentation, and fractions were collected. Fraction 1 is the top of the gradient, and fraction 20 is the bottom of the

gradient. The adiponectin levels were measured using the R & D adiponectin ELISA. The assay was used as described by the manufacturer. 5µl of each fraction was assayed to determine the levels of adiponectin.

Figures 19: Analysis of human plasma samples for adiponectin levels using MAB 3604 with globular (Figure 19a) and N-terminal (Figure 19b) adiponectin polyclonal antibodies. Human fasting plasma samples collected from normal, diabetic, impaired glucose tolerant (IGT), impaired fasting glucose (IFG) and both IGT & IFG individuals was assayed for levels of adiponectin. The antibody pairs for assaying were MAB 3604 in combination with globular adiponectin or N-terminal adiponectin polyclonal antibodies. The adiponectin levels (µg/ml) are plotted against the levels of fasting blood glucose (mg/dL). Individuals with normal fasting glucose levels - blue diamond, IGT - pink square, IFG - yellow triangles, IGT + IFG - light blue cross, and diabetic - blue asterisk. No significant correlation is apparent.

Figures 20: Analysis of human plasma samples for adiponectin levels using mAb A12820 with globular (Figure 20a), N-terminal (Figure 20b) and C-terminal (Figure 20c) adiponectin polyclonal antibodies. Human fasting plasma samples collected from normal, diabetic, impaired glucose tolerant (IGT), impaired fasting glucose (IFG) and both IGT & IFG individuals was assayed for levels of adiponectin. The antibody pairs for assaying were mAb A12820 in combination with globular adiponectin, N-terminal or C-terminal adiponectin polyclonal antibodies. The adiponectin levels (µg/ml) are plotted against the levels of fasting blood glucose (mg/dL). Individuals with normal fasting glucose levels - blue diamond, IGT - pink square, IFG -yellow triangles, IGT + IFG - light blue cross, and diabetic - blue asterisk. A significant correlation is apparent, especially with the N and C-terminal antibodies.

Figures 21: Analysis of human plasma samples for adiponectin levels using MAB 10651 with globular (Figure 21a) , N-terminal (Figure 21b) and C-terminal (Figure 21c) adiponectin polyclonal antibodies. Human fasting plasma samples collected from normal, diabetic, impaired glucose tolerant (IGT), impaired fasting glucose (IFG) and both IGT & IFG individuals was assayed for levels of adiponectin. The antibody pairs for assaying were MAB 10651 in combination with globular adiponectin, N-terminal or C-terminal adiponectin polyclonal antibodies. The adiponectin levels (µg/ml) are plotted against the levels of fasting blood glucose (mg/dL). Individuals with normal fasting glucose levels - blue diamond, IGT - pink square, IFG -yellow triangles, IGT + IFG - light blue cross, and diabetic - blue asterisk. No significant correlation is apparent.

Figures 22: Analysis of human plasma samples for adiponectin levels using mAb adiponectin M1 with globular (Figure 22a), N-terminal (Figure 22b) and C-terminal

(Figure 22c) adiponectin polyclonal antibodies. Human fasting plasma samples collected from normal, diabetic, impaired glucose tolerant (IGT), impaired fasting glucose (IFG) and both IGT & IFG individuals was assayed for levels of adiponectin. The antibody pairs for assaying were mAb adiponectin M1 in combination with globular adiponectin, N-terminal or C-terminal adiponectin polyclonal antibodies. The adiponectin levels (μg/ml) are plotted against the levels of fasting blood glucose (mg/dL). Individuals with normal fasting glucose levels - blue diamond, IGT - pink square, IFG - yellow triangles, IGT + IFG - light blue cross, and diabetic - blue asterisk. A significant correlation is apparent, especially with the globular and N-terminal antibodies.

Figures 23: Analysis of human plasma samples for adiponectin levels using mAb adiponectin M24 with globular (Figure 23a), N-terminal (Figure 23b) and C-terminal (Figure 23c) adiponectin polyclonal antibodies. Human fasting plasma samples collected from normal, diabetic, impaired glucose tolerant (IGT), impaired fasting glucose (IFG) and both IGT & IFG individuals was assayed for levels of adiponectin. The antibody pairs for assaying were mAb adiponectin M24 in combination with globular adiponectin, N-terminal or C-terminal adiponectin polyclonal antibodies. The adiponectin levels (μg/ml) are plotted against the levels of fasting blood glucose (mg/dL). Individuals with normal fasting glucose levels - blue diamond, IGT - pink square, IFG - yellow triangles, IGT + IFG - light blue cross, and diabetic - blue asterisk. A significant correlation is apparent, especially with the globular and N-terminal antibodies.

Figure 24: Analysis of human plasma samples for adiponectin levels using the R & D adiponectin ELISA. Human fasting plasma samples collected from normal, diabetic, impaired glucose tolerant (IGT), impaired fasting glucose (IFG) and both IGT & IFG individuals was assayed for levels of adiponectin. The R & D adiponectin ELISA assay was used to determine the levels of adiponectin. The adiponectin levels (μg/ml) are plotted against the levels of fasting blood glucose (mg/dL). Individuals with normal fasting glucose levels - blue diamond, IGT - pink square, IFG - yellow triangles, IGT + IFG - light blue cross, and diabetic - blue asterisk. No significant correlation is apparent.

Figures 25: Correlation of M1 Globular Form and Fasting Blood Glucose. Sample Characteristics: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126. Figure 25a: The level of adiponectin is in μg/ml, and is the M1 globular form. The odds ratio for the globular form (OR=0.84, 95% c.i. 0.74-0.95) was significantly less than 1, indicating that low levels of the M1 globular form are associated with Type II diabetes risk. Figure 25b: The ROC curve shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. FP stands for false positives. The area under the curve for the M1 globular form was 0.731.

Figures 26: Correlation of M1 N-terminus Form and Fasting Blood Glucose. Sample Characteristics: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126. Figure 26a: The level of adiponectin is in μg/ml, and is the M1 N-terminus form. The odds ratio for the globular form (OR=0.53, 95% c.i. 0.32-0.88) was significantly less than 1, indicating that low levels of the M1 N-terminal form are associated with Type II diabetes risk. Figure 26b: The ROC curve shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the M1 N-terminus form was 0.705.

Figures 27: Correlation of M24 Globular Form and Fasting Blood Glucose. Sample Characteristics: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126. Figure 27a: The level of adiponectin is in μg/ml, and is the M24 globular form. The odds ratio for the globular form (OR=0.47, 95% c.i. 0.30-0.72) was significantly less than 1, indicating that low levels of the M24 globular form are associated with Type II diabetes risk. Figure 27b: The ROC curve shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the M24 globular form was 0.742.

Figures 28: Correlation of M24 C-terminus Form and Fasting Blood Glucose. Sample Characteristics: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126. Figure 28a: The level of adiponectin is in μg/ml, and is the M24 C-terminus form. The odds ratio for the C-terminus form (OR=0.87, 95% c.i. 0.80-0.92) was significantly less than 1, indicating that low levels of the M24 C-terminus form are associated with Type II diabetes risk. Figure 28b: The ROC curve shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the M24 C-terminus form was 0.740.

Figures 29: Correlation of A12820 Globular Form and Fasting Blood Glucose. Sample Characteristics: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126. Figure 29a: The level of adiponectin is in μg/ml, and is the A12820 globular form. The odds ratio for the A12820 globular form (OR=0.87, 95% c.i. 0.80-0.92) was significantly less than 1, indicating that low levels of the A12820 globular form are associated with Type II diabetes risk. Figure 29b: The ROC curve shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the A12820 globular form was 0.714.

Figures 30: Correlation of A12820 N-terminus Form and Fasting Blood Glucose. Sample Characteristics: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126. Figure 30a: The level of adiponectin is in μg/ml, and is the A12820 N-terminus form. The odds ratio for the A12820 N-terminus form (OR=0.97, 95% c.i. 0.95-0.99) was significantly less than 1, indicating that low levels of the A12820 N-terminus form are associated with Type II diabetes risk. Figure 30b: The ROC curve shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the A12820 N-terminus form was 0.673.

Figures 31: Correlation of A12820 C-terminus Form and Fasting Blood Glucose. Sample Characteristics: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126. Figure 31a: The level of adiponectin is in μg/ml, and is the A12820 C-terminus form. The odds ratio for the A12820 C-terminus form (OR=0.97, 95% c.i. 0.93-0.99) was significantly less than 1, indicating that low levels of the A12820 C-terminus form are associated with Type II diabetes risk. Figure 31b: The ROC curve shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the A12820 C-terminus form was 0.663.

Figure 32: Nucleic acid sequence (Seq. Id. No. 16 and 17) and amino acid sequence (Seq. Id. No. 4 and 5) of the M1 light and heavy chain variable regions. The corresponding regions of the Adiponectin M1 hybridoma cDNA were cloned and sequenced. The amino acid sequence was deduced from the cDNA sequence. The variable region amino acids encode the specific binding region of the antibody, and confer the high degree of specificity of IgG binding to the adiponectin N-terminus amino acids.

Figure 33: Nucleic acid sequence (Seq. Id. No. 18) and amino acid sequence (Seq. Id. No. 6 and 7) of the M24 light and heavy chain variable regions. The corresponding regions of the Adiponectin M1 hybridoma cDNA were cloned and sequenced. The amino acid sequence was deduced from the cDNA sequence. The variable region amino acids encode the specific binding region of the antibody, and confer the high degree of specificity of IgG binding to the adiponectin N-terminus amino acids.

Figure 34: Amino Acid Sequence Comparisons and CDR Assignments of IgG Heavy Chain Variable Regions for Adiponectin Hybridoma Cell Lines M1 and M24.

Figure 35: Amino Acid Sequence Comparisons and CDR Assignments of IgG Light Chain Variable Regions for Adiponectin Hybridoma Cell Lines M1 and M24.

Definitions

All references cited herein are hereby incorporated by reference in their entirety.

The terms "protein" or "polypeptide" are intended to be used interchangeably. They refer to a chain of at least two (2) or more amino acids which are linked together via a peptide or amide bond, and is independent of post-translational modifications (e.g., glycosylation, acylation, phosphorylation, etc). Antibodies are specifically intended to be within the scope of this definition. The polypeptides of this invention may comprise more than one subunit, where each subunit is encoded by a separate DNA sequence. The protein may also encompass more than one polypeptide unit, comprising a dimer, trimer, tetramer, or various higher ordered structures. The different proteins may also be made up of additional proteins or components, for which we do not know the specific composition or identity.

"Isoform", refers to the different molecular forms proteins such as adiponectin, maybe found in human or animal, plasma, serum or other bodily fluids. The isoforms may also be produced by various recombinant methods, and be found secreted in the media from cells expressing adiponectin. For adiponectin, the isoform maybe comprised of multiple adiponectin subunits, and maybe modified by different post-translational modifications. The isoform may also consist of other proteins or components which together form a complex.

Adiponectin is an adipocyte specific protein that has recently been shown to possess a variety of activities including anti-inflammatory, antiatherogenic, preventive for metabolic syndrome, and insulin sensitizing activities. Adiponectin is encoded by a single gene, and consists of 244 amino acids (Seq. Id. No. 19), giving rise to a polypeptide chain of about 30 kilodaltons. The mature human adiponectin protein encompasses amino acids 19 – 244. A globular domain has been reported to encompass amino acids 107 – 244. In plasma/serum, adiponectin exists in various molecular forms that include monomers, trimers, hexamers, and various combinations thereof. No information is known in the art about which molecular forms of adiponectin possess which types of activities. While methods exist to measure adiponectin levels in human plasma/serum, it is still not possible to accurately measure the different adiponectin forms, nor to predict the relationship of the levels of specific adiponectin forms to a specific physiologic state or disease.

"Recombinant" adiponectin refers to proteins that are synthesized in cells that do not normally produce adiponectin. This technique is commonly used in the art for the preparation of large amounts of proteins for various types of studies. The "recombinant"

adiponectin can be produced in E.coli, yeast, or various mammalian cell lines such as 293 or CHO cells. The recombinant adiponectin isoforms produced in these cell types can be similar to, or can differ significantly from the adiponectin isoforms present in human plasma samples.

"N-terminus" or "N-terminal" refers to the amino acid residues which are closest to the N-terminal of the protein. These residues will begin with amino acid residue 19 of the protein, and can contain as many as 40 additional amino acids. We utilized amino acid residues 19 – 39 of adiponectin as the N-terminal sequence, to which antibodies were developed.

"C-terminus" or "C-terminal" refers to the amino acid residues which are closest to the C-terminal of the protein. These residues will end with amino acid residue 244 of the protein, and can contain as many as 40 additional amino acids before the end of the protein. We utilized residues 223 – 244 of adiponectin as the C-terminal sequence, to which antibodies were developed.

The phrase "substantially identical" with respect to an antibody polypeptide sequence shall be construed as an antibody exhibiting at least 70%, preferably 80%, more preferably 90% and most preferably 95% sequence identity to the reference polypeptide sequence. The term with respect to a nucleic acid sequence shall be construed as a sequence of nucleotides exhibiting at least about 85%, preferably 90% more preferably 95% and most preferably 97% sequence identity to the reference nucleic acid sequence. For polypeptides, the length of the comparison sequences will generally be at least 25 amino acids. For nucleic acids, the length will generally be at least 75 nucleotides.

The term "identity" or "homology" shall be construed to mean the percentage of amino acid residues in the candidate sequence that are identical with the residue of a corresponding sequence to which it is compared, after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent identity for the entire sequence, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art. Sequence identity may be measured using sequence analysis software (e.g., Sequence Analysis Software Package, Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Ave., Madison, Wis. 53705). This software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

The term "Insulin Sensitivity" as used herein refers to the measure of the body's ability to utilize glucose. This measure is dependent on the ability of insulin to stimulate glucose uptake. There are a variety of measures that can be used for this purpose, including fasting glucose, fasting insulin, oral glucose tolerance test, glucose clamp, or insulin clamp. All of these measures can be routinely used for this type of analysis.

The term "antigen" as used herein refers to the substance that stimulates an immune response, and that can be used to generate antibodies that specifically react with the antigen. The antibodies can be polyclonal or monoclonal in origin. Polyclonal antibodies are a group of antibody molecules that are produced from a large number of B cells, and are directed to a specific antigen. In polyclonal antibodies, there is a mixture of antibody molecules that recognize different epitopes or sequences of a protein or peptide. A monoclonal antibody is a single unique antibody molecule which is produced from a single B cell, and is directed specifically to an antigen, such as a protein or peptide.

The term "ELISA Assay" as used herein refers to enzyme linked immunosorbent assay. This assay is routinely used to measure levels of various entities, including proteins, peptides, and small molecules. It is not common to be able to identify different molecular forms of proteins, via traditional ELISA assays, other than post-translational modifications, such as phosphorylation, acylations, glcnacylation among others. Traditional ELISA methods do not have the ability to measure different molecular entities made up of the same subunit.

Detailed Description of the Invention

In a first object the present invention relates to a monoclonal antibody directed to the N – terminus of adiponectin.

In a preferred embodiment, an adiponectin peptide comprising Seq. Id. No. 3 was used to raise the antibody.

In a further preferred embodiment, the antibody comprises at least two CDR sequences selected from the group consisting of amino acids 21 – 36, amino acids 52 – 58 and amino acids 91 – 99 of Seq. Id. No. 4 or Seq. Id. No. 6 and of amino acids 25 – 33, amino acids 48 – 64 and amino acids 97 – 104 of Seq. Id. No. 5 or Seq. Id. No. 7.

In a further preferred embodiment, the antibody comprises at least three CDR sequences, more preferably at least four, even more preferably at least five, most preferably six CDR sequences selected from the group consisting of amino acids 21 – 36, amino acids 52 – 58 and amino acids 91 – 99 of Seq. Id. No. 4 or Seq. Id. No. 6 and of

amino acids 25 -33, amino acids 48 – 64 and amino acids 97 – 104 of Seq. Id. No. 5 or Seq. Id. No. 7.

In another preferred embodiment, the antibody comprises a light chain variable region selected from Seq. Id. No. 4 or Seq. Id. No. 6 and a heavy chain variable region selected from Seq. Id. No. 5 or 7.

In a further preferred embodiment, the antibody comprises the light chain variable region set forth in Seq. Id. No. 4 and the heavy chain variable region set forth in Seq. Id. No. 5.

In another preferred embodiment, the antibody comprises the light chain variable region set forth in Seq. Id. No. 6 and the heavy chain variable region set forth in Seq. Id. No. 7.

In a second object, the present invention relates to a polynucleotide sequence comprising a sequence encoding the light chain variable region selected from Seq. Id. No. 4 or Seq. Id. No. 6. and to a polynucleotide sequence comprising a sequence encoding the heavy chain variable region selected from Seq. Id. No. 5 or 7.

In a preferred embodiment, the polynucleotide sequence is selected from the group consisting of Seq. Id. No. 16, Seq. Id. No. 17 and Seq. Id. No. 18.

In a third object, the present invention relates to a method for the diagnosis of pre-disposition to diabetes of a human subject, comprising at least one adiponectin monoclonal antibody and at least one adiponectin polyclonal antibody in an assay to screen for adiponectin in a patient sample, wherein assay values corresponding to Receiver Operating Characteristic curve (ROC) plots with AUC of 0.65 or higher indicate a pre-disposition to diabetes.

In a preferred embodiment of the method of the present invention, the at least one monoclonal antibody is selected from an antibody of the present invention and/or mAb A12820.

In a further preferred embodiment of the method of the present invention, the at least one monoclonal antibody is M1 and the at least one polyclonal antibody is selected from the group consisting of globular and N-terminus antibody.

In a further preferred embodiment of the method of the present invention, the at least one monoclonal antibody is mAb adiponectin M24 and the at least one polyclonal antibody is selected from the group consisting of globular and C-terminus antibody.

In a further preferred embodiment of the method of the present invention, the at least one monoclonal antibody is mAb A12820 and the at least one polyclonal antibody is selected from the group consisting of globular, N-terminus and C-terminus antibody.

In a further preferred embodiment, the assay is an ELISA.

In a further preferred embodiment of the method of the present invention, the AUC value is 0.70 or higher.

In a further preferred embodiment of the method of the present invention, the patient sample is blood plasma or blood serum.

In a fourth object, the present invention relates to a use of an antibody of the present invention and/or the mAb A12820 in combination with at least one adiponectin polyclonal antibody for the diagnosis of pre-disposition to diabetes of a human subject.

In preferred embodiment, the polyclonal antibody is selected from the group consisting of globular, N-terminus and C-terminus adiponectin antibody.

In a further object, the present invention provides a polyclonal antibody directed to a N-terminus of adiponectin, wherein an adiponectin peptide comprising Seq. Id. No. 1 was used to raise the antibody.

In yet another object, the present invention relates to a polyclonal antibody directed to a C-terminus of adiponectin, wherein an adiponectin peptide comprising Seq. Id. No. 2 was used to raise the antibody.

In another object, the present invention relates to a polyclonal antibody directed to a globular domain of adiponectin, wherein an adiponectin peptide comprising amino acids 107 – 244 of Seq. Id. No. 19 was used to raise the antibody.

The invention pertains to methods for measuring different forms of human adiponectin that are present in human plasma/serum, and more specifically methods are based on an ELISA assay that utilizes different monoclonal antibodies directed against adiponectin, in combination with different polyclonal antibodies directed against different domains of human adiponectin.

When different combinations of monoclonal and polyclonal antibodies are combined to configure an ELISA assay, the different ELISA formats can measure different molecular forms of adiponectin. Moreover, some of these different molecular forms of adiponectin correlate with different physiologic states. The levels of the different adiponectin molecular forms can be correlated with insulin sensitivity or fasting blood

glucose, or with other physiologic states such as cardiovascular disease, steatosis, inflammation as well as other diseases.

The invention also provides unique isoforms of adiponectin and antibodies thereto, including polyclonal and monoclonal antibodies.

The methods of the invention are simple, reliable and reproducible, and are able to discriminate different adiponectin isoforms, including some forms (M1 globular, M1 N-terminus, M24 globular, M24 C-terminus, A12820 globular, A12820 N-terminus, A12820 C-terminus) which show a significant degree of correlation to the diabetic phenotype. Moreover, the invention demonstrates that prior methods which simply measure low or high molecular weight adiponectin isoforms is not sufficient to provide discriminatory power to identify individuals who have a form of type II diabetes. The assays of the invention can also be used to further define the association of adiponectin isoforms to other diseases such as cardiovascular disease, metabolic syndrome, inflammation, dyslipidemia, NASH, and others. The methods of the invention can also be used to monitor disease progression, response to treatment, prediction of treatment responses, as well as the diagnosis of early forms of disease.

As such, the utility of these assays, and the identified novel adiponectin antibodies and isoforms, can be used to monitor disease progression, response to treatment, prediction of treatment responses, as well as the diagnosis of early forms of disease.

Antibody Development & Characterization

Polyclonal antibodies were developed to the globular domain, N-terminal domain, and C-terminal domain of human adiponectin. These polyclonal antibodies can be used in Western blots and in the development of ELISAs for different forms of adiponectin that are found in body fluids, such as plasma, serum, etc. The polyclonal antibodies were raised in rabbits, and the procedure was performed by Pocono Rabbit Farm & Laboratory Inc, Canadensis, PA., following standard protocols. The standard protocol was used for antigen injection, boosting, and bleeding. The antibodies were purified from serum by affinity chromatography with Protein G-Agarose. The antigen for the globular-domain was the recombinant adiponectin protein, encoding amino acid residues 107 – 244. The protein was produced in E.coli, and purified by standard purification techniques. The N-terminal peptide antigen has the N-terminal sequence ETTTQGPGVLLPLPKGASTGC (Seq. Id. No. 1). The C-terminal peptide antigen has the sequence CYADNVNDSTFTGFLLYHDTN (Seq. Id. No. 2).

The novel monoclonal antibodies to human adiponectin N-terminus (M1 Seq. Id. No 4 and Seq. Id. No. 5, light and heavy chain variable regions respectively and M24 Seq.

Id. No. 6 and Seq. Id. No. 7, light and heavy chain variable regions respectively) were produced using the N-terminal sequence ETTQGPGVLLPLPKGAC (Seq. Id. No. 3). These antibodies were produced in mice by Antibody Solutions, Mountain View, CA 94043. Antibody Solutions used their standard protocol (techniques which are well known in the art) for immunization (PolyExpress proprietary protocol for antibody response to antigen), production of hybridoma libraries (final boost of immune cells, fusion with myeloma cells, and cryopreservation), and monoclonal cultures (preparation of monoclonal cultures using flow cytometry cell sorting).

The protocols used are standard protocols for the generations, propagation, and characterization of hybridoma cells, according to the general procedural method below. (See also, Oi. V. T.. and L. A. Herzenberg. 1979. In *Selected Methods in Cellular* Immunology. B. B. Mishell & *S* . M. Shiigi, eds. Freeman, San Francisco. p. 351, Reik, L. M., S. L. Maines. D. E. Ryan, W. Levin, S. Bandiera. and P. E. Thomas. 1987. A simple. non-chromatographic purification procedure for monoclonal antibodies. *J. Immunol. Methods* 100:123, said references hereby incorporated by reference). The primary screening of the hybridoma supernatants, was to detect reactivity of IgG in the hybridoma sups, with the N-terminal peptide coated onto 96 well plates. The secondary assay involved the capture of human adiponectin from plasma, followed by reactivity with polyclonal antibodies directed against human adiponectin, using an ELISA format. Monoclonal antibodies that were capable of capturing human adiponectin were deemed useful, and subsequently further characterized by ELISA analysis, and by western blot analysis.

ELISA Assay Development

The ELISA assays for measuring levels of human adiponectin rely on the ability to capture different adiponectin isoforms with specific monoclonal antibodies directed to adiponectin, and then to further identify the captured adiponectin isoforms with the use of different polyclonal antibodies that recognize different adiponectin epitopes. The configuration of these assays thus provides the ability to specifically recognize different adiponectin isoforms that are dependent on the ability of antibody pairs (a monoclonal antibody together with a polyclonal antibody) to specifically interact with unique adiponectin isoforms. We have used 5 different monoclonal antibodies (MAB3604 (Chemicon International), A12820 (611645) (BD Biosciences), MAB10651 (R&D Systems, Inc), N-ADIP-M1 (Roche), and N-ADIP-M24 (Roche) in this analysis, coupled with three different polyclonal antibodies (N-terminal, C-terminal and globular). Thus we have the potential to develop 15 different assays, and to potentially identify 15 different adiponectin isoforms (M1 globular, M1 N-terminus, M1 C-terminus, M24 globular, M24 C-terminus, M24 N-terminus, A12820 globular, A12820 N-terminus,

A12820 C-terminus, MAB10651 globular, MAB10651 N-terminus, MAB10651 C-terminus, MAB3604 globular, MAB 3604 N terminus). The combination of MAB 3604 C-terminus did not yield a signal. By using additional monoclonal and polyclonal antibodies, it is possible to identify even greater numbers of potential adiponectin isoforms.

Procedure for generation of rabbit polyclonal antibodies

Purified N-terminal or C-terminal adiponectin peptides were coupled to KLH, at a ratio of 1 mg of peptide to 1 mg of KLH via cysteine residues (conjugated peptide). 100 – 200 µg of conjugated peptide in complete Freund's adjuvant (CFA) was injected intradermally into rabbits. 14 days later, 50 – 100 µg of conjugated peptide was injected intradermally in incomplete Freund's adjuvant (IFA). 14 days later, 50 – 100ug of conjugated peptide was injected intradermally in incomplete Freund's adjuvant (IFA). The rabbits were bled 14 days later to obtain serum for analytical purposes. Thereafter, the rabbits were immunized subcutaneously every 30 days with 25 – 50 µg of conjugated peptide, then bled 14 days following immunization. The serum was collected, and then the IgG was purified as described below, and used in assay development. (reference for pAb production: Lui, F. T., Sinnecker, M., Hamaoka, J., and Katz, D. (1979) Biochemistry 18,690-697. This is one of many protocols, which are standardly employed fro those skilled in the art). The antigens used were N-terminal adiponectin peptide, C-terminal adiponectin peptide, and recombinant globular adiponectin, spanning amino acids 107 – 244, produced in E. coli.

The above procedure resulted in the production of three different polyclonal antibodies, which we refer to as N-terminal, C-terminal, and globular antibodies, as discussed above in the "Antibody characterization and development" section. Each of these polyclonal antibodies was used together with different monoclonal antibodies, to generate a unique ELISA assay which recognized different adiponectin isoforms.

Procedure for generation of monoclonal antibody

Immunization protocol and *mA b production.*

Female BALB/c mice were immunized i.p. with 50 µg of conjugated N-terminal peptide in CFA and then boosted i.p. 5 wk later with 50 µg of conjugated N-terminal peptide in CFA. After 6 wk, 50 ug of conjugated N-terminal peptide in PBS was injected i.p. followed by an identical boost 48 h later. Two days after this last boost, the spleen was removed, teased into a cell suspension, and fused with the HAT-sensitive murine myeloma cell Sp2/0 as described previously (1 9). There are many similar procedures that are commonly used by those skilled in the art. Hybridomas were selected in HAT media,

HB 101 media (Irvine Scientific, Irvine, CA), 7% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1X HAT, and 10% Hybridoma Cloning Factor (HCF; IGEN, Rockville, MD) (Kenney et al, Biotechnology, 13: 787, 1995). Aliquots of supernatant (50 ul) from wells exhibiting growth after 10 to 14 days were screened for anti-adiponectin N-terminal antibodies by ELISA. N-terminal peptide was coated on the bottoms of 96 well plates, and screened for reactivity with the hybridoma supernatants. Hybridomas which reacted with the N-terminal peptide were further screened by ELISA to determine whether they could interact with native adiponectin present in human plasma. Hybridomas of interest were cloned by limiting dilution in HAT media (Kenney et al, Biotechnology, 13: 787, 1995).

Ascitic fluid was produced by injecting cloned hybridomas into pristane-primed BALB/c mice (1-2 x l0E6 cells/mouse, i.p.). mAb were purified from heat-inactivated ascitic fluid by a combination of caprylic acid and ammonium sulfate precipitations as previously described. mAb were also produced by large scale culture, followed by protein-G chromatography to purify the IgG.

Optionally and preferably, a further enhancement to these protocols involves reversing the order of antibodies that are used in the assay. For example, the polyclonal antibodies can be used to capture different adiponectin isoforms, and then to further identify the captured adiponectin isoforms with the use of different monoclonal antibodies that recognize different adiponectin epitopes.

Quantification of the globular-, N-terminal-, and C-terminal domains of human adiponectin in plasma/serum and in fractions of plasma are measured in an ELISA format. Immunoplates are coated with one of the following monoclonal antibodies: MAB3604 (Chemicon International) at 1:5000 dilution, A12820(611645) (BD Biosciences) at 0.5ug/ml, MAB10651 (R&D Systems, Inc) at 0.25 µg /ml, N-ADIP-M1 (Roche) at 1ug/ml, and N-ADIP-M24 (Roche) at 1ug/ml. The plates with 100 µg of the appropriate monoclonal antibody are covered with plate sealers and incubated at 4°C for 16-20 hours. The plates are then washed 3 times with PBS – tween20 0.05% which is used for all subsequent washes. The plates were blocked with 300 µl of BSA 1% + Tween20 0.05% in PBS buffer for 1 hour and washed 3 times. The appropriate reference standards (0.0195 to 10ng/well) are added to each plate in a final volume of 50 µl which is used for all subsequent incubations. For the globular and N-terminal domain of adiponectin detection, adiponectin (1-244) from R&D Systems (cat#1065, with his-tag on the C-terminus) is used. For the C-terminal adiponectin detection, adiponectin (16-247) from Alexis Biochemicals (cat#ALX-522-063, with an N-terminal FLAG tag) is used. Plasma/serum or plasma fraction is diluted in PBS with BSA 0.1% + Tween20 0.05% and added to each plate. The plates are incubated at 21-23°C for 1 hour on an orbital shaker,

and then washed 4 times. The appropriate detection antibody is added to each plate in PBS with BSA 0.1% + Tween20 0.05 and incubated at 21-23°C for 1 hour on an orbital shaker followed by washing 4 times. The developing antibody, donkey anti-rabbit conjugated to horseradish-peroxidase, is added for 1 hour at 21-23°C on an orbital shaker followed by 4 washes. The bound antibody conjugated to horseradish-peroxidase is quantified in the presence of 3,3',5,5'-tetramethylbenzidine (TMB). 50µl of the TMB solution is added /well, the plates covered with plate sealers and incubated at 21-23°C while shaking on a miniorbital plate shaker. The reaction is terminated at the appropriate time with the addition of 50µl/well phosphoric acid 0.5M. The OD is read at 450nm and the amount of adiponectin determined by interpolation from the appropriate standard curve. The reaction is terminated at a time which gives an OD reading of less than 2. This is between 2 and 10 minutes depending on the capture antibody and the detection antibody.

Screening Fractionated Plasma Samples

Plasma fractions from three normal donors were fractionated using a 5–20% sucrose gradients in 10 mM HEPES, pH 8, 125 mM NaCl were poured stepwise in 2-ml thin walled ultracentrifuge tubes (Becton Dickinson) and allowed to equilibrate overnight at 4 °C. 600ul of total plasma was diluted 1:10, applied and centrifuged on the gradient. Following layering of the sample on top (diluted 1:10 with 10 mM HEPES, pH 8, 125 mM NaCl), gradients were spun at 55,000 rpm for 4 h at 4 °C in a TLS55 rotor in a Beckman TL-100 tabletop ultracentrifuge (Pajvani et al 2004). 150µl sample fractions were removed from the top of the tube, and labeled 1 – 20, with fraction 1 being the top of the gradient, while fraction 20 was on the bottom of the gradient. The gradient fractions were sequentially retrieved and analyzed by western or ELISA analysis as described below. Different antibodies were used for the western analysis, and were either monoclonal or polyclonal antibodies. For the ELISA analysis, each fraction was analyzed for the presence of adiponectin isoforms, by using an ELISA with different combinations of monoclonal antibody as a capture reagent, and different polyclonal antibodies as a developing reagent. The configuration can also be reversed, such that the polyclonal antibody is used as the capture antibody, and the monoclonal antibody is used as the developing antibody. A commercial human adiponectin ELISA (R&D Systems, Minneapolis, MN) was used for comparative purposes.

Screening Patient Plasma Collections

Collection of Human Plasma Samples

Both male and female diabetics and non-diabetics were recruited. Subjects were recruited following an overnight fast, and approximately 50 ml of blood was collected. The identity of the subjects was kept confidential (see informed consent form). The subjects include diabetics and age matched controls who are not diabetic.

Exclusion Criteria: Both case and control subjects can not have type 1 diabetes or secondary forms of diabetes (e.g. Cushing's disease, acromegaly). Subjects undergoing steroid treatment or having inflammatory diseases (e.g. dermatomyositis) will be excluded. Subjects with a life expectancy less than one year (investigator judgment) will also be excluded. In addition to these criteria, control subjects can not have been previously diagnosed with type 2 diabetes or gestational diabetes, have a family history of type 2 diabetes, or have impaired fasting glucose or impaired glucose tolerance (as described above).

It is expected that the adiponectin isoforms and antibodies hereby divulged and utilized within the ELISA methods of the present invention will lead to a better understanding of the causes of certain diseases, including Type 2 Diabetes, and that this understanding should lead to better treatment and preventive measures.

Key material and parameters

For indiviudals who are known diabetics, a total of about 50 ml of blood was collected following an overnight fast.

If the individual has not previously been diagnosed with type 2 diabetes, or if they are a diet-treated diabetic, a total of of about 50 ml of blood was collected following an overnight fast. Some individuals, were required to take a 75 gm oral glucose tolerance test. This test requires an overnight fast (at least 10 hours) and blood draws at 0, 30, and 120 minutes relative to administration of oral glucose. At most, a total of 60mL of blood was drawn.

Procedure for blood collection and plasma preparation

Venous blood is withdrawn from the patients according to standard procedures. All steps were carried out consecutively as fast as possible to ensure maximum sample quality and to prevent proteolysis.

Per patient, ~ 50 ml of blood is collected (4 x 10 ml vacutainer). Every vacutainer is inverted twice to mix the blood with the anti-coagulant and immediately put on ice (not frozen).

After having finished the blood withdrawal, all vacutainers are immediately centrifuged for 15 min at 1500 x g at 4°C.

The cooled (4°C or on ice) vacutainers are opened and the supernatant is transferred to a 10 ml Falcon tube (on ice) using a 1 ml disposable tip pipette. Leave approx. 2 mm of supernatant to prevent carryover of the sediment.

Centrifuge the Falcon tubes for 30 min at 3000 x g at 4°C. The centrifuge brake must be switched off.

The plasma supernatants in the Falcon Tubes were recovered with a pipette leaving all the sediment behind. All plasma samples were transferred to one 50 ml Falcon tube and gently mixed to ensure sample homogeneity.

The samples were shock-frozen in liquid nitrogen and stored in a -80 °C freezer. Shipment was on dry ice.

Screening

The human plasma samples were thawed and aliquoted into 96 wells in preparation for screening.

The assay procedure as described in the section Assay Development and as briefly outlined below was used in the in the standard curves for the assays Figures 7 - 12, and in the assays for the plasma fractions, Figures 13 – 18. The assay format was also used in determining the levels of adiponectin in the human plasma sample collections for correlation to disease state, Figure 19 - 31.

1) Appropriate capture antibody added to an immunoplate in a volume of 100 µl PBS for 16-20 hours at 4°C.
2) Aspirate and wash the plate 3 times
3) Plate blocked with BSA 1% + Tween20 0.05% + PBS in a volume of 300 µl for 1 hour at 21-23°C in a volume of 300ul.
4) Aspirate and wash the plate 3 times
5) Appropriate reference standards added to each plate with dilutions in BSA 0.1% + Tween20 0.05% + PBS in a final volume of 50 µl.
6) Plasma/serum added to each plate with dilutions in BSA 0.1% + Tween20 0.05% + PBS in a final volume of 50 µl.
7) Plate placed on an orbital shaker and incubated at 21-23°C for 1 hour.
8) Aspirate and wash the plate 4 times

9) Appropriate detection antibody added to each plate in BSA 0.1% + Tween20 0.05% + PBS in a final volume of 50µl and placed on an orbital shaker and incubated at 21-23°C for 1 hour

10) Aspirate and wash the plate 4 times

11) Developing antibody, donkey anti-rabbit-HRP, added to each plate in BSA 0.1% + Tween20 0.05% + PBS in a final volume of 50 µl and placed on an orbital shaker and incubated at 21-23°C for 1 hour

12) Aspirate and wash the plate 4 times

13) TMB substrate solution added to the plate in a volume of 50 µl and placed on an orbital shaker and incubated at 21-23°C until the appropriate development time

14) The reaction is terminated by adding phosphoric acid 0.5M in a volume of 50µl

15) The OD is read at 450nm and the amount of adiponectin determined by interpolation from the standard curve.

Quantification of the different human adiponectin isoforms in plasma/serum and in fractions of plasma are measured in an ELISA format. Immunoplates are coated with one of the following monoclonal antibodies: MAB3604 (Chemicon International) at 1:5000 dilution, A12820(611645) (BD Biosciences) at 0.5 µg /ml, MAB10651 (R&D Systems, Inc) at 0.25 µg /ml, N-ADIP-M1 (Roche) at 1µg/ml, and N-ADIP-M24 (Roche) at 1µg/ml. The plates with 100 µl of the appropriate monoclonal antibody are covered with plate sealers and incubated at 4°C for 16-20 hours. The plates are then washed 3 times with PBS – tween20 0.05% which is used for all subsequent washes. The plates were blocked with 300 µl of BSA 1% + Tween20 0.05% in PBS buffer for 1 hour and washed 3 times. The appropriate reference standards (0.0195 to 10ng/well) are added to each plate in a final volume of 50 µl which is used for all subsequent incubations. For the globular and N-terminal domain of adiponectin detection, adiponectin (1-244) from R&D Systems (cat#1065, with his-tag on the C-terminus) is used. For the C-terminal adiponectin detection, adiponectin (16-247) from Alexis Biochemicals (cat#ALX-522-063, with an N-terminal FLAG tag) is used. Plasma/serum or plasma fraction is diluted in PBS with BSA 0.1% + Tween20 0.05% and added to each plate. The plates are incubated at 21-23°C for 1 hour on an orbital shaker, and then washed 4 times. The appropriate detection antibody is added to each plate in PBS with BSA 0.1% + Tween20 0.05 and incubated at 21-23°C for 1 hour on an orbital shaker followed by washing 4 times. The developing antibody, donkey anti-rabbit conjugated to horseradish-peroxidase, is added for 1 hour at 21-23°C on an orbital shaker followed by 4 washes. The bound antibody conjugated to horseradish-peroxidase is quantified in the presence of 3,3',5,5'-tetramethylbenzidine (TMB). 50ul of the TMB solution is added /well, the plates covered with plate sealers and incubated at 21-23°C while shaking on a miniorbital plate shaker. The reaction is terminated at the appropriate time with the addition of 50 µl/well

phosphoric acid 0.5M. The OD is read at 450nm and the amount of adiponectin determined by interpolation from the appropriate standard curve. The reaction is terminated at a time which gives an OD reading of less than 2. This is between 2 and 10 minutes depending on the capture antibody and the detection antibody, and their concentrations.

Sequencing of Variable Heavy and Light Chain cDNAs for Adiponectin Hybrodomas M1 and M24

Materials and Methods:

Hybridoma cells were grown and total RNA extracted by standard procedures. See, e.g., "Characterization of a murine monoclonal antibody that mimics heparin-induced thrombocytopenia antibodies". G.M. Arepally, S. Kamei, K.S. Park, K. Kamei, Z.Q. Li, W. Liu, D.L. Siegel, W. Kisiel, D.B. Cines and M. Poncz BLOOD (March 1, 2000) Volume 95 Number 5 pp. 1533-1540 "Efficient amplification and direct sequencing of mouse variable regios from any immunoglobulin family" Thierry Chardes, Sylvie Villard, Gaelle Ferrieres, Marine Piechaczyk, Marine Cerutti, Gerard Devauchelle and Bernard Pau FEBS Letters 452 (1999) pp. 386-394

First strand cDNA was synthesized by standard procedures, using either oligo dT or the reverse PCR oligos as primers. Variable heavy and light chain region cDNAs were PCR amplified using the following conditions:

94C / 5 minutes

94C / 30 seconds

55C / 30 seconds

72C / 90 seconds

30 cycles

72C / 10 minutes

The following PCR oligos were used:

From Ref 1:

Heavy chain Framework 1 (forward primer) (Seq. Id. No. 8)

GAG GTG AAG CTG GTG GAG (AT)C(AT) GG

Heavy chain constant (reverse primer) (Seq. Id. No. 9)

GGG GCC AGT GGA TAG AC

Light chain Framework 1 (forward primer) (Seq. Id. No. 10)

CCA GTT CCG AGC TCC AGA TGA CCC AGA CTC CA

Light chain constant (reverse primer) (Seq. Id. No. 11)

GTT GGT GCA GCA TCA GC

From Ref 2:

Heavy chain Framework 1 (forward primer) (Seq. Id. No.12)

CAG GT(GC) (AC)A(GA) CTG (GC)(A T)G (GC)AG (TA)C(AT) GG

Heavy chain constant (reverse primer) (Seq. Id. No.13)

CGA CAA GTC GAC TAG CCC TTG ACC AGG CAT CC

Light chain Framework 1 (forward primer) (Seq. Id. No.14)

GAC ATT (CG)AG CT(GC) ACC CAG TCT CCA

Light chain constant (reverse primer) (Seq. Id. No.15)

CGA CTA GTC GAC TGG TGG GAA GAT GGA TAC AG

Products were analyzed by agarose gel electrophoresis. Light chain amplicons were gel isolated and sequenced uncloned. Heavy chain amplicons were subcloned, and the sequences determined from cloned plasmids.

Results

Assay development and standardization

Different assays for human adiponectin were developed. The assay format used was an ELISA assay, which utilizes a specific monoclonal antibody as a capture antibody, a solution containing the adiponectin, such as a suitable standard, or human plasma or serum, followed by the addition of a polyclonal antibody (Figure 4 & 5). Each combination of monoclonal and polyclonal antibody represents a different assay format. The specific combination of monoclonal antibody, coupled with a polyclonal antibody, provides for a specific adiponectin assay (Figure 5). In some instances, the specific

combination will not be able to measure adiponectin at all (such as the combination of MAB3604 C-terminus), while with others different levels of adiponectin are measured.

To facilitate development of the ELISAs, we developed novel monoclonal antibodies that are specific for the N-terminus of adiponectin. These mAbs reacted specifically with N-terminal adiponectin peptide, and also specifically reacted with adiponectin as determined by ELISA, western blot analysis, and immunofluorescence. These mAbs were further used in combination with polyclonal antibodies to measure levels of adiponectin in human plasma using ELISA assays.

The basic format of the assay involved immobilizing a specific anti-adiponectin monoclonal antibody on a 96 well plate (Figure 4 & 5). The different anti-adiponectin monoclonal antibodies used in the studies included A12820, MAB3604, MAB10651, M1 and M24. The second component of the ELISA assay was a polyclonal antibody that was directed to adiponectin. There were three different polyclonal antibodies that were used. These included N-term, C-term and globular polyclonal antibodies. These three polyclonal antibodies recognize different adiponectin domains. A schematic diagram of the different steps in the Adiponectin ELISA assays is presented in Figure 6. It is possible to configure additional assays, using different monoclonal antibodies or polyclonal antibodies that are directed to different domains of adiponectin. Such combinations of antibodies, might provide additional assay formats that have discriminatory properties for various adiponectin isoforms that are correlated with disease states.

The different assay formats were standardized using different recombinant adiponectin proteins, which were obtained from commercial sources (Figures 7 – 12). Thus the absolute levels of adiponectin that are reported are based on the quantitation relative to the standards which were used for each assay.

Not all assays are able to detect unique adiponectin isoforms. So for each mAb in combination with each of the three polyclonal antibodies, all of the ELISA assays were not able to measure adiponectin, be it a standard adiponectin or adiponectin in human plasma. In some cases only two of the polyclonal antibodies in combination with a monoclonal antibody are useful to measure adiponectin levels. For other monoclonal antibodies, all three combinations of polyclonal antibodies are able to detect adiponectin. We have also observed that the levels of adiponectin isoform detected by the antibody combination, can vary significantly between the assays. For some, adiponectin levels of about 0.05 µg /ml (M24 – N-terminus pAb), while for others, levels of about 15 µg /ml can be detected (M24 – C-terminus pAb), and others have levels as high as about 60 µg /ml (A12820 – globular pAb).

Characterization of Adiponectin Isoforms recognized by different assays

Once the different assays were established, we noticed that different assays were able to recognize different levels of adiponectin. We then sought to determine the nature of the differences between the assays. Since adiponectin has been reported to exist in several different molecular forms we sought to determine whether the assays could recognize different adiponectin molecular forms (reviewed in Berg et al, Trends Endocrinol Metab. 2002 Mar;13(2):84-9.; Trujillo and Scherer, J Intern Med. 2005 Feb;257(2):167-75). Velocity gradient sedimentation provides a simple means of separating molecules on the basis of size. The separation of adiponectin via velocity sedimentation has been reported (Pajvani UB et al (2003). J. Biol. Chem. 278:9073-9085). There are additional ways of separating molecules on the basis of size, such as gel filtration chromatography which could also be used for this purpose.

Human plasma samples were separated by velocity sedimentation as outlined, and the fractions were collected. To determine the distribution of adiponectin, aliquots of the fractions were measured by ELISA, using the different assay formats. The amount of adiponectin in each fraction was determined by the different assay formats, and then plotted relative to fraction number from the velocity gradient fractionation. The data are presented in figures 13 – 18. The observed patterns indicate that adiponectin levels vary from assay to assay. MAB3604 in combination with globular or N-terminus pAbs, primarily recognizes only smaller molecular weight adiponectin isoforms, present in fractions 3 – 7 (Figure 13). On the other hand, MAB10651 in combination with all three pAbs, primarily recognizes a larger molecular weight adiponectin isoform, present in fractions 10 – 16 (Figure 15). The amount of adiponectin determined by the assays varies, with MAB10651 in combination with the globular pAb measuring the highest levels, while the MAB10651 in combination with N-terminus pAb, measures the smallest amount of adiponectin. Thus, these three different assays can detect primarily a larger adiponectin isoform, however the levels of adiponectin protein detected varies, suggesting some differences in the adiponectin molecules recognized by the different combinations of antibodies.

When fractionated human plasma samples were assayed for adiponectin using mAb A12820 in combination with the three different polyclonal antibodies a very different pattern was observed (Figure 14). Adiponectin levels were found in a number of different fractions, with a peak in fraction 5 corresponding to smaller molecular weight adiponectin. There was also a broad peak of adiponectin protein, from fraction 7 – 17, having a peak around fraction 13. A similar pattern was observed for all three assays, but the levels of adiponectin detected by each assay were different (Figure 14). Thus, these three different assays can detect different adiponectin isoforms, and the levels of

adiponectin protein detected varies by the assay, suggesting some differences in the adiponectin molecules recognized by the different combinations of antibodies.

When fractionated human plasma samples were assayed for adiponectin using mAb adiponectin M1 in combination with the three different polyclonal antibodies a very different pattern was observed (Figure 16). mAb Adiponectin M1 in combination with C-terminus pAb yielded the highest levels of adiponectin. The adiponectin recognized by this form was widely distributed, in fractions 3 – 20. Detectable levels of adiponectin were also observed for mAbs adiponectin M1 in combination with globular and N-terminus pAbs. The levels were much lower, and appeared to be distributed over a number of different fractions.

When fractionated human plasma samples were assayed for adiponectin using mAb adiponectin M24 in combination with the three different polyclonal antibodies a very different pattern was observed (Figure 17). mAb Adiponectin M24 in combination with C-terminus pAb yielded the highest levels of adiponectin. The adiponectin recognized by this form was widely distributed, in fractions 3 – 20. Barely detectable levels of adiponectin were also observed for mAbs adiponectin M24 in combination with globular and N-terminus pAbs. The levels were much lower, and appeared to be distributed over a number of different fractions.

Fractionated human plasma samples were assayed for adiponectin using the adiponectin R&D assay (Figure 18). Adiponectin was found to be distributed in fractions 3 – 20, with two peaks, one at fraction 5, and the second more abundant peak in fraction 13. The pattern observed was different from the other adiponectin assays.

The velocity sedimentation results clearly demonstrate that different combinations of monoclonal and polyclonal antibodies, recognize different molecular weight forms of adiponectin. Not only are the sizes of the adiponectin, as determined by velocity sedimentation different, but the levels of adiponectin detected are also very different. Therefore, the different antibody combinations represent different assay formats that specifically recognize different adiponectin molecular forms (isoforms). The availability of these assay formats provides the opportunity to assess whether the adiponectin isoforms correlate with different physiologic or disease states.

Screening of Normal and Diabetic Plasma Samples

The levels of each of the adiponectin isoforms was determined for a population of individuals consisting of normals, diabetics, impaired glucose tolerant, and impaired fasting glucose. Fasting plasma samples were collected and stored at -80°C prior to use. Sample aliquots were then tested to determine the levels of the various adiponectin

isoforms. The levels of adiponectin determined by the different ELISA assay formats was plotted against the fasting blood glucose levels, and is presented in figures 19 – 24. The distribution of the adiponectin levels relative to fasting blood glucose levels varies between the different assays. The statistical analysis is presented below (summarized in Table 1). Some of the adiponectin assays do not show any discrimination between adiponectin levels and fasting blood glucose. These assays include the commercial adiponectin R&D assay (Figure 24), the MAb 3604 assays (Figure 19), and the MAB 10651 assays (Figure 21) (summarized in Table 1). In contrast, the adiponectin assays using adiponectin mAb M1 (Figure 22), adiponectin mAb M24 (Figure 23), and mAb 12820 (Figure 20) demonstrate significant correlations of the adiponectin levels to fasting glucose values (summarized in Table 1).

Statistical Analysis of results

The ability of the various adiponectin forms to distinguish controls from Type II diabetics was examined with logistic regression models using the logistic procedure of SAS 9.1 (SAS Institute, Carey, NC). Separate models were run for each of the adiponectin forms. In each model group status (i.e. control or diabetic) served as the outcome variable and predictor variables included one of the adiponectin forms. Age, body mass index (BMI) and insulin served as covariates in each model. Sensitivity and specificity for the adiponectin forms were calculated using the freq and logistic procedures of SAS 9.1 Receiver operator characteristic (ROC) plots were calculated to show the variability of sensitivity and specificity for various cutoff points of adiponectin concentration for the three polyclonal forms. The area under the curve (AUC) is provided as a summary statistic for each ROC curve. The AUC can be interpreted as the probability that true case (i.e. Type II diabetic) will be correctly identified from a normal patient. For example, an AUC of 1.0 would represent a perfect test with no mis-classification whereas assays with AUC's of less than 0.5 would do no better than random assignment. Assays with ROC plots of the AUC of 0.65 and higher, with ROC plots of the AUC of 0.70 and higher being more preferred, demonstrate significant correlation and predictive value as an indicator for the predisposition to the disease state investigated, such as for example diabetes (Type II). The results for all of the different assays are summarized in Table 1.

Table 1: Logistic regression and correlation results for the various adiponectin forms based on 126 Type II diabetics and 124 normal controls. Separate regression models were run for each form adjusting for age, body mass index (BMI) and insulin levels. Pearson correlation coefficients are presented for each form indicating the strength of its relationship to fasting blood glucose.

	Regression Model			Correlation of Fasting Blood Glucose and Adiponectin Form	
Adiponectin Form	Wald Chi-Square	p-value	ROC AUC	r2	p-value
M1 Globular form	7.26	0.0100	0.73	-0.27	0.0001
M1 N-terminus form	6.01	0.0100	0.70	-0.26	0.0001
M1 C-terminus form	3.23	0.0700	0.66	-0.25	0.0001
M24 Globular form	11.97	0.0005	0.74	-0.25	0.0001
M24 N-terminus form	22.38	0.0001	0.76	-0.43	0.0001
M24 C-terminus form	15.99	0.0001	0.74	-0.40	0.0001
A12820 Globular form	11.91	0.0006	0.71	-0.24	0.0001
A12820 N-terminus form	5.18	0.0228	0.67	-0.25	0.0001
A12820 C-terminus form	4.91	0.0267	0.66	-0.23	0.0001
MAB10651 Globular form	0.49	0.4851	0.58	-0.10	0.3100
MAB10651 N-terminus form	1.77	0.1838	0.64	-0.22	0.0007
MAB10651 C-terminus form	0.40	0.5272	0.56	-0.14	0.0030
MAB3604 Globular form	0.69	0.4055	0.47	-0.05	0.3900
MAB3604 N-terminus form	1.59	0.2203	0.48	-0.02	0.7960
R&D form	4.14	0.0419	0.51	-0.05	0.3400

Differences between the M1 and M24 hybridomas are indicated

Prior to analysis the data were screened for normality and the presence of outliers. The data conformed to assumptions of normality therefore no transformations were applied. The Grubb's test was used to test for the presence of outliers and data points determined to be significant outliers at $p<0.05$ were removed prior to the analysis.

The population sample characteristics are the following: N=126 Type II diabetics (T2D) and N=124 normals were examined. The diagnostic criteria used for T2D in this sample was fasting blood glucose (FBG) > 126.

The logistic regression results for the M1 globular form shows that the globular form was a significant predictor of group status (Wald Chi-Square = 7.26; $p<0.01$). The odds ratio for the globular form (OR=0.84, 95% c.i. 0.74-0.95) was significantly less than 1, indicating that low levels of the M1 globular form are associated with Type II diabetes risk (Figure 25). The ROC curve in Figure 25 shows the sensitivity and specificity for globular concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the M1 globular form was 0.731.

The logistic regression results for the M1 N-terminus form shows that the N-terminus form was a significant predictor of group status (Wald Chi-Square = 6.01;

p<0.01). The odds ratio for the globular form (OR=0.53, 95% c.i. 0.32-0.88) was significantly less than 1, indicating that low levels of the M1 N-terminus form are associated with Type II diabetes risk (Figure 26). The ROC curve in Figure 26 shows the sensitivity and specificity for N-terminus concentration cutoff values of 0.8 and 1.0 μg/ml. The area under the curve for the N-terminus form was 0.705.

The logistic regression results for the M24 globular form shows that the globular form was a significant predictor of group status (Wald Chi-Square = 11.9; p<0.0005). The odds ratio for the globular form (OR=0.47, 95% c.i. 0.30-0.72) was significantly less than 1, indicating that low levels of the M24 globular form are associated with Type II diabetes risk (Figure 27). The ROC curve in Figure 27 shows the sensitivity and specificity for globular concentration cutoff values of 1.2 and 1.5 μg/ml. The area under the curve for the M1 globular form was 0.742.

The logistic regression results for the M24 C-terminus form shows that the C-terminus form was a significant predictor of group status (Wald Chi-Square = 15.9; p<0.0001). The odds ratio for the C-terminus form (OR=0.87, 95% c.i. 0.80-0.92) was significantly less than 1, indicating that low levels of the M24 C-terminus form are associated with Type II diabetes risk (Figure 28). The ROC curve in Figure 28 shows the sensitivity and specificity for C-terminus concentration cutoff values of 10.0 and 15.0 μg/ml. The area under the curve for the M24 C-terminus form was 0.740.

The logistic regression results for the A12820 globular form shows that the A12820 globular form was a significant predictor of group status (Wald Chi-Square = 15.9; p<0.0001). The odds ratio for the A12820 globular form (OR=0.87, 95% c.i. 0.80-0.92) was significantly less than 1, indicating that low levels are associated with Type II diabetes risk (Figure 29). The ROC curve in Figure 29 shows the sensitivity and specificity for A12820 Globular Form concentration cutoff values of 3.0, 4.0, and 5.0 μg/ml. The area under the curve for the M1 globular form was 0.714.

The logistic regression results for the A12820 N-terminus form shows that the A12820 N-terminus form was a significant predictor of group status (Wald Chi-Square = 5.18; p=0.02). The odds ratio for the A12820 N-terminus form (OR=0.97, 95% c.i. 0.95-0.99) was significantly less than 1, indicating that low levels are associated with Type II diabetes risk. (Figures 30). The ROC curve in Figure 30 shows the sensitivity and specificity for A12820 N-terminus Form concentration cutoff values of 11.0 and 16.0 μg/ml. The area under the curve for the M1 N-terminus form was 0.673.

The logistic regression results for the A12820 C-terminus form. The A12820 C-terminus form was a significant predictor of group status (Wald Chi-Square = 4.91;

p=0.03). The odds ratio for the A12820 C-terminus form (OR=0.97, 95% c.i. 0.93-0.99) was significantly less than 1, indicating that low levels are associated with Type II diabetes risk (Figure 31). The ROC curve in Figure 31 shows the sensitivity and specificity for A12820 C-terminus Form concentration cutoff values of 12.0 and 15.0 µg/ml. The area under the curve for the M1 globular form was 0.663.

The statistical analysis of the remaining assays is summarized in Table 1. These do not appear to show significance from the analysis which was performed.

Sequence determination of the CDRs of the hybridoma cell lines M1 & M24.

The sequence of the CDRs of mAb adiponectin M1 and M24 IgG cDNA was determined and is shown in Figures 32 and 33. The sequence of the two IgGs is very similar, yet different. Both of these IgG proteins bind to the N-terminal adiponectin region, encompassing amino acids 19 – 34, ETTQGPGVLLPLPKGAC (SEQ ID NO. 3).

Discussion

ELISA Assays that recognize Adiponectin

ELISA assays recognizing adiponectin were developed. Different monoclonal antibodies were used as capture antibodies, in combination with three different polyclonal antibodies. In most cases, the antibody combinations were able to recognize adiponectin, while some combinations were not able to detect adiponectin (MAB 3604 C-terminus). All of the assays could be standardized by using recombinant forms of adiponectin. The standardization provided a basis for determining the levels of adiponectin that are found in biological samples such as plasma. The ELISA assays could then be used to measure adiponectin levels, to determine the size distribution of the adiponectin molecular forms, and to determine whether the concentration of specific adiponectin isoforms correlated to a specific physiologic status. It is important to note that the assays of the invention do not require any sample pretreatment, or denaturation, of samples prior to measurement. As such, the assays measure native forms of adiponectin.

Different Adiponectin Molecular Forms

To demonstrate that different adiponectin molecular forms are being recognized by the various ELISA assays, human plasma was fractionated by velocity sedimentation, and the fractions were assayed for adiponectin levels using the different ELISA assays. Figures 13- 18, clearly demonstrate that the distribution of adiponectin levels is significantly different depending on the combination of polyclonal and monoclonal antibodies that

are used. For some of the assays, only a low molecular weight adiponectin form is recognized (Figure 13, fractions 4, 5 6). For other assays, only a higher molecular weight form of adiponectin is recognized (Figure 15, fractions 11 – 14). Finally, some of the assays recognize both higher and lower molecular weight forms of adiponectin (Figures 14, 16, 17). Not only do the assays recognize different sizes of adiponectin as demonstrated by the velcosity sedimentation profiles (Figures 13 – 18), but some of the assays (Figures 14, 15, 16, 17) recognize different levels of adiponectin, again suggesting that there are unique differences in the types of adiponectin molecules which are being recognized by the different assays. The fact that the assays recognize different adiponectin molecular weight forms, provides a powerful simple tool to assess the differences in adiponectin between individuals, and to determine whether any of the adiponectin forms correlates to disease status.

Levels of Adiponectin Forms vary between Individuals

When different assays were used to determine the levels of adiponectin in different individuals, there were significant differences in the levels of adiponectin that were observed. For some of the assays, the levels of adiponectin differ by 10 – 50 fold. These results suggest, that some adiponectin assays can measure most of the adiponectin isoforms that are present in human plasma or serum, while other assays measure a very specific adiponectin isoform(s) For example, mAb 12820 in combination with the globular domain pAb, detects from 30 – 120 µg/ml of adiponectin. In contrast, mAb adiponectin M24 in combination with N-terminus polyclonal antibody detects only 0.02 – 0.1 µg /ml. The difference represents about a 1000 fold difference in the levels of adiponectin which are detected in human plasma. These differences are apparent in Figures 13 – 18, where the adiponectin levels are determined in fractionated human plasma. These differences are also observed in figures 19 – 24. Some of these differences are significant in terms of correlation to phenotype (summarized in Table 1) while others are not. The ability to characterize plasma samples from a wide variety of disease states will provide the correlations that are significant.

Certain Adiponectin Forms correlate to glucose levels

All of the different adiponectin assays were used to screen human plasma sample collections to look for correlations to glucose. We observed, that certain adiponectin isoforms show moderate strength correlations to glucose. The assays which show significant correlations to glucose are the mAb adiponectin M1 globular and N-terminus (Figure 22, 25, 26), the mAb adiponectin M24 globular and C-terminus (Figure 23, 27, 28) the mAb A12820 globular, N-terminus and C-terminus (Figure 20, 29, 30, 31), and MAB1051 N-terminus and C-terminus (summarized in Table 1). None of the other

assays, including the R&D commercial adiponectin assay, demonstrate any significant association to glucose.

Certain Adiponectin forms are predictive of the diabetic state

Low adiponectin levels of the mAb adiponectin M1 globular and N-terminus (Figure 25, 26), the mAb adiponectin M24 globular and C-terminus (Figure 27, 28), and mAb 12820 globular (Figure 29), are significant predictors of diabetes (summarized in Table1). This observation suggests that these assays will be of interest to ascertain which normal individuals will become diabetic, which individuals have the best response to a specific diabetes treatment, and which individuals will have the best prognosis for their diabetes. The ROC plots show that the specific forms have good levels of specificity and sensitivity for utility in the clinic. Specifically, levels of sensitivity/specificity of 60% or greater can be useful in the clinic. The ability to use these assays for further predictive value depends on collecting longitudinal samples, to determine the correlation to disease progression and to treatment outcomes.

The above examples illustrating the monoclonal and polyclonal antibodies of the invention, the Elisa methods of the invention and the predicative/diagnostic methods of adiponectin forms/levels to certain disease states such as diabetes, are not meant to limit the scope of the invention. These examples are included for purposes of illustration and the present invention is limited only by the scope of the claims herein.

Claims

1. A monoclonal antibody directed to a N-terminus of adiponectin, wherein the antibody comprises a light chain variable region set forth in Seq. Id. No. 4 and a heavy chain variable region set forth in Seq. Id. No. 5.

2. A monoclonal antibody directed to a N-terminus of adiponectin, wherein the antibody comprises a light chain variable region set forth in Seq. Id. No. 6 and a heavy chain variable region set forth in Seq. Id. No. 7.

3. A polynucleotide sequence comprising a sequence encoding the light chain variable region selected from Seq. Id. No. 4 or Seq. Id. No. 6.

4. A polynucleotide sequence comprising a sequence encoding the heavy chain variable region selected from Seq. Id. No. 5 or 7.

5. The polynucleotide sequence of claims 3 or 4, wherein said sequence is selected from the group consisting of Seq. Id. No. 16, Seq. Id. No. 17 and Seq. Id. No. 18.

6. A method for the diagnosis of pre-disposition to diabetes of a human subject, comprising at least one adiponectin monoclonal antibody selected from the group consisting of an antibody of claim 1 and claim 2 and mAb A12820 and at least one adiponectin polyclonal antibody selected from the group consisting of globular, N-terminus and C-terminus antibody in an assay to screen for adiponectin in a patient sample,

wherein assay values corresponding to Receiver Operating Characteristic curve (ROC) plots with AUC of 0.65 or higher indicate a pre-disposition to diabetes.

7. The method of claim 6, wherein the at least one monoclonal antibody is the antibody of claim 1 and the at least one polyclonal antibody is selected from the group consisting of globular and N-terminus antibody.

8. The method of claim 6, wherein the at least one monoclonal antibody is the antibody of claim 2 and the at least one polyclonal antibody is selected from the group consisting of globular and C-terminus antibody.

9. The method of claim 6, wherein the at least one monoclonal antibody is mAb A12820 and the at least one polyclonal antibody is selected from the group consisting of globular, N-terminus and C-terminus antibody.

10. The method of claims 6-9, wherein the assay is an ELISA.

11. The method of claims 6-10, wherein the AUC value is 0.70 or higher.

12. An antibody according to claims 1 – 2 or the mAb A12820 in combination with at least one adiponectin polyclonal antibody selected from the group consisting of globular, N-terminus and C-terminus antibody for the diagnosis of pre-disposition to diabetes of a human subject.