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(57) Abstract: The present invention relates generally to a new cluster of molecules that affects oxidative stress, inflammation, and/or insulin signaling in white blood cells, particularly monocytes, and to identifying the optimal method or system to modulate the activity of said molecules. Thereby reducing the risk of diseases associated with activated monocytes such as obesity and obesity-related metabolic syndrome disorder phenotype characterized by dyslipidemia, hypertension, glucose intolerance, insulin resistance and diabetes, lipid homeostasis disorders and/or cardiovascular diseases. More in particular these molecules are microRNAs (miRNAs or miRs) that can be present in the cell, in cell-derived vesicles that are secreted in blood, and can be detected in plasma or serum. In addition, we present a method, for instance a diagnostics method or system, for instance a diagnostic, which provides information on how to modulate the molecules to treat or prevent obesity, to separate responders from non-responders, and to treat or prevent the obesity-related metabolic syndrome disorders.

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IRAK-RELATED INTERVENTIONS AND DIAGNOSIS

BACKGROUND OF THE INVENTION

A. Field of the Invention

The present invention relates generally to a new cluster of molecules that affects oxidative stress, inflammation, and/or insulin signaling in white blood cells, particularly monocytes, and to identifying the optimal method or system to modulate the activity of said molecules. Thereby reducing the risk of diseases associated with activated monocytes such as obesity and obesity-related metabolic syndrome disorder phenotype characterized by dyslipidemia, hypertension, glucose intolerance, insulin resistance and diabetes, lipid homeostasis disorders and/or cardiovascular diseases. More in particular these molecules are microRNAs (miRNAs or miRs) that can be present in the cell, in cell-derived vesicles that are secreted in blood, and can be detected in plasma or serum. In addition, we present a method, for instance a diagnostics method or system, for instance a diagnostic, which provides information on how to modulate the molecules to treat or prevent obesity, to separate responders from non-responders, and to treat or prevent the obesity-related metabolic syndrome disorders.

Several documents are cited throughout the text of this specification. Each of the documents herein (including any manufacturer's specifications, instructions etc.) are hereby incorporated by reference. However, there is no admission that any document cited is indeed prior art of the present invention.

B. Description of the Related Art

Despite considerable advances in the comprehension of the pathogenesis of atherosclerosis, cardiovascular diseases remain the leading cause of mortality and morbidity [1]. This can be explained by the increasing prevalence of obesity and type-2 diabetes mellitus (T2DM) [2,3]. The epidemic of obesity is a global health issue across all age groups, especially in industrialized countries (American Obesity Association, 2006). According to WHO's estimate there are more than 300 million obese people (BMI>30) world-wide. Today, for example

almost 65% of adult Americans (about 127 million) are categorized as being overweight or obese. There is also evidence that obesity is increasing problem among children, for example in the USA, the percentage of overweight children (aged 5-14 years) has doubled in the last 30 years, from 15% to 32%. The degree of health impairment of obesity is determined by three factors: 1) the amount of fat 2) the distribution of fat and 3) the presence of other risk factors. It is the second leading cause of preventable death in the Western society and an increasing cause on modernizing societies. Obesity affects all major bodily systems -heart, lung, muscle and bones-and is considered as a major risk factor for several chronic disease conditions, including coronary heart disease, type 2 diabetes mellitus, hypertension, stroke, and cancers of the breast, endometrium, prostate and colon [4].

A large number of medical conditions have been associated with obesity. Health consequences are categorized as being the result of either increased fat mass (osteoarthritis, obstructive sleep apnea, social stigma) or increased number of fat cells (diabetes, cancer, cardiovascular disease, non-alcoholic fatty liver disease) [5]. Mortality is increased in obesity, with a BMI of over 32 being associated with a doubled risk of death [6]. There are alterations in the body's response to insulin (insulin resistance), a pro-inflammatory state and an increased tendency to thrombosis (pro-thrombotic state) [5]. Disease associations may be dependent or independent of the distribution of adipose tissue. Central obesity (male-type or waist-predominant obesity, characterized by a high waist-hip ratio), is an important risk factor for the metabolic syndrome, the clustering of a number of diseases and risk factors that heavily predispose for cardiovascular disease. These are diabetes mellitus type 2, high blood pressure, high blood cholesterol, and triglyceride levels (combined hyperlipidemia) [7,8]. Apart from the metabolic syndrome, obesity is also correlated with a variety of other complications. For some of these complaints, it has not been clearly established to what extent they are caused directly by obesity itself, or have some other cause (such as limited exercise) that causes obesity as well. Cardiovascular: congestive heart failure, enlarged heart and its associated arrhythmias and dizziness, varicose veins, and pulmonary embolism. Endocrine: polycystic ovarian syndrome (PCOS), menstrual disorders, and infertility [9] Gastrointestinal:

gastroesophageal reflux disease (GERD), fatty liver disease, cholelithiasis (gallstones), hernia, and colorectal cancer. Renal and genitourinary: erectile dysfunction [10], urinary incontinence, chronic renal failure [11], hypogonadism (male), breast cancer (female), uterine cancer (female), stillbirth. Integument (skin and appendages): stretch marks, acanthosis nigricans, lymphedema, cellulitis, carbuncles, intertrigo. Musculoskeletal: hyperuricemia (which predisposes to gout), immobility, osteoarthritis, low back pain. Neurologic: stroke, meralgia paresthetica, headache, carpal tunnel syndrome, dementia [12], idiopathic intracranial hypertension. Respiratory: obstructive sleep apnea, obesity hypoventilation syndrome, asthma. Psychological: Depression, low self esteem, body dimorphic disorder, social stigmatization.

The economic cost attributable to obesity is substantial and is close to $100 billion/yr (Wolf & Colditz 1998). Obesity accounts for 2-6% of total health care costs in several developed countries; some estimates put the figure as high as 7%. The true costs are undoubtedly much greater as not all obesity-related conditions are included in the calculations.

One of the emerging cardiovascular risk factors is subclinical chronic low-grade inflammation [13]. Population studies showed a strong correlation between pro-inflammatory biomarkers (such as high sensitive C-reactive protein (hc-CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α)) and perturbations in glucose homeostasis, obesity, and atherosclerosis[14]. Another emerging risk factor is oxidized LDL (ox-LDL) that activates circulating monocytes, thereby increasing their ability to infiltrate the vascular wall [15]. This increased infiltration is a key event in atherogenesis. The metabolic syndrome clusters several cardiovascular risk factors including obesity, dyslipidemia, hypertension, and insulin resistance (IR). Increased inflammation [16,17] and oxidative stress [18-21] were found to be associated with the metabolic syndrome. It is a primary risk factor for diabetes and cardiovascular diseases. Recent data suggest that increased oxidative stress in adipose tissue is an early instigator of the metabolic syndrome and that the redox state in adipose tissue is a potentially useful therapeutic target for the obesity-associated metabolic syndrome [22]. Oxidative damage of adipose tissues is associated with

impaired adipocyte maturation, production of pro-inflammatory adipocytokines by dysfunctional adipocytes, and increased infiltration of activated macrophages into the adipose tissues of obese persons where they produce inflammatory chemokines [23]. This enhanced infiltration is causatively related to the loss of insulin signaling.

There is thus a clear need in the art to have accurate molecules for a proper treatment of obesity associated disorders such oxidative stress and inflammation, metabolic syndrome, insulin resistance and type 2 diabetes and related cardiovascular disorders for persons in need thereto, an to separate responders from non-responders to such a treatment.

A number of miRNAs of the present invention have previously been identified. For example, WO2010133970 discloses that **miR-103** is upregulated in liver cells of obese mice and that inhibition of miR-103 leads to an improvement of several obesitas/insulin resistance parameters. Surprisingly, as also described in more detail herein below, the inventors of the current application have found that the opposite is true in monocytes.

Iliopoulos et al. (2010) disclose that **miR-181b-1** is pro-inflammatory in endothelial or cancer cells. They also show that miR-181b-1 directly inhibits expression of CYLD, which in its turn is known to inhibit NF-κB activity [24]. Surprisingly, as also described in more detail herein below, the inventors of the current application have found that miR-181b is anti-inflammatory in monocytes.

WO2010129919 focuses on the influence of **let-7** (including let-7a - let-7i) on asthma and lung inflammation. They show that let-7a, and likely the other let-7 miRNAs, directly targets IL-13 expression. Furthermore, *in vivo* experiments show that inhibition of miR-155 (a let-7 family member) reduces inflammation in lungs. Surprisingly, as also described in more detail herein below, the inventors of the current application have found that let-7c and let-7g are anti-inflammatory in monocytes.

Lee et al. (2011) show that **miR-130** potently suppresses PPARγ in adipocytes [25]. Since PPARγ is known to inhibit inflammation in

monocytes [26], one would thus expect miR-130 to stimulate inflammation in monocytes. However, as described in more detail herein below, the inventors of the current application have found that miR-130 is associated with decreased inflammation in monocytes.

The above examples clearly show that the same miRNA can have very different, even opposite, effects in different tissues and diseases [27].

Present invention provides such solution to these problems in the art.

SUMMARY OF THE INVENTION

In accordance with the purpose of the invention, as embodied and broadly described herein, the invention is broadly drawn to molecules for treatment of the condition and for testing and predicting the efficacy of his or her treatment.

The present invention solves the problems of the related art by providing a combination of molecules for the treatment of an activated monocyte, which is characterized by an increased inflammatory state and/or an increased oxidative stress state and/or a deregulated insulin signaling. Treatment of said monocyte is particularly useful in the treatment of related diseases such as metabolic syndrome disorder, an inflammatory disorder, an oxidative stress disorder, an impaired glucose tolerance, an insulin resistance condition, the progression of an adipocyte tissue disorder, such as an impaired adipose tissue accumulation or adipocyte function; metabolic syndrome, and related cardiovascular diseases. In addition, the diagnostic tools for testing and predicting the efficacy of the optimal combination of molecules are provided.

The biomarkers of present invention in white blood cells (WBCs), or leukocytes (also spelled "leucocytes") for instance T lymphocytes, monocytes and neutrophils and most preferably the monocyte type of white blood cell can be analysed using high speed microfluidic single cell impedance cytometry as for instance been described by David Holmes and Hywel Morgan [28].

In particular this invention identifies miRNAs which regulate monocyte activation and inflammation, oxidative stress and/or insulin signaling in tissues infiltrated by these monocytes (e.g. adipose tissues, vascular tissues). Said miRNAs are relevant for treatment of an oxidative stress state and/or inflammatory state and/or insulin signaling deregulation in monocytes, which leads to the prevention and/or treatment of obesity and obesity-related metabolic syndrome disorders, and cardiovascular diseases.

Adiponectin is an antidiabetic adipokine, which enhances insulin action and inhibits the oxidative stress state and inflammatory state in monocytes, thereby inhibiting causes of among others metabolic syndrome and cardiovascular diseases. However, resistance to adiponectin has been shown in obesity and following chronic high fat feeding and adiponectin treatment may even contribute to lipid accumulation observed in these conditions. The present invention identifies miRNAs that regulate the inhibitory effects of adiponectin or adiponectin mimetics on oxidative stress and inflammation in monocytes. The present invention thus provides means to differentiate reponders from non-responders to adiponectin treatment. Furthermore, the present invention provides a medicinal solution for adiponectin resistance. In particular, the modulators of the miRNAs of the present invention can be used as a conjunctive therapy to a treatment with adiponectin or adiponectin mimetics.

In addition, miR-146b-5p, IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα are known to play an important role in inflammation, oxidative stress and/or insulin signaling in monocytes and are therefore a known target for the treatment of associated diseases such as obesity and insulin resistance. Therefore, the modulators of the miRNAs of the present invention can be used in a combination therapy with agents that modulate one or more targets known to play an important role in inflammation, oxidative stress and/or insulin signaling in monocytes, such as for example selected from miR-146b-5p, IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα. In a particular embodiment, the modulators of the miRNAs of the present invention can be used in a combination therapy with an IRAK3 modulator; more in particular with a modulator that increases the expression and/or activity of IRAK3. Said combination therapy can optionally further include agents that

modulate one or more targets known to play an important role in inflammation, oxidative stress and/or insulin signaling in monocytes, such as for example selected from miR-146b-5p, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα.

This method of IRAK3 activation is also particularly suitable for treating adiponectin resistance disorders or adiponectin deficiency pathological processes such as endoplasmatic reticulum stress–induced adiponectin downregulation -induced adiponectin –resistance and to decrease increased risk of cancer due to induced adiponectin downregulation and leptin upregulation for instance by obesity.

This method of IRAK3 activation is also particularly suitable for use in a conjunctive therapy with adiponectin or adiponectin mimetics to prevent stress-induced damage in the heart; to enhance adiponectin treatment of hyperproliferation or to enhance adiponectin inhibited angiogenesis in for instance an anti-cancer or anti-tumor therapy.

Furthermore, the present invention provides a means, for instance diagnostic tool or a diagnostic method to identify persons who have WBCs with an increased inflammatory state, an increased oxidative stress state and/or deregulated insulin signaling, in particular activated monocytes. The miRNAs of the present invention can be quantified or qualified in a sample obtained from said person. In a particular embodiment, said sample is a blood-derived sample. The miRNAs of present invention can for example be quantified or qualified on isolated WBCs, or leukocytes (also spelled "leucocytes") for instance T lymphocytes, monocytes and neutrophils and most preferably the monocyte type of white blood cell. A lab on chip microfluidic set-up to remove red blood cells from the sample and isolate WBCs uses electrodes to measure each blood cell's electrical properties and identify said cells as blood flows through the device's channels, suitable for distinguishing and counting the different types of cell, providing information used in the diagnosis, and in monitoring the treatment, of numerous diseases as for instance been described by David Holmes and Hywel Morgan [28] whereby the blood cells are identified as they flow through a microfluidic device. Alternative methods are power-free microfluidics using capillary forces to pull the blood or other samples. The currently routine blood analysis uses flow

cytometry. In another particular embodiment, the miRNAs of the present invention can be quantified or qualified on isolated microvesicles, particularly on monocyte-derived microvesicles.

The above means also allows to identifying persons in which treatment with adiponectin or adiponectin mimetics can decrease the risk of monocyte activation in inflammation and/or oxidative stress-related and/or insulin resistance-related diseases such as obesity, type 2 diabetes, and the metabolic syndrome, atherosclerosis and/or cardiovascular diseases. The quantification or qualification of the miRNAs of the present invention in WBCs, particularly monocytes, can be further complemented with the quantification or qualification of other markers, such as on or more targets known to play an important role in inflammation, oxidative stress and/or insulin signaling in monocytes, such as for example selected from miR-146b-5p, IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα.

Such method is particularly useful to identify the responder patients to a treatment of adiponectin or adiponectin mimetics. In particular in patients suffering from obesity, type 2 diabetes, and the metabolic syndrome, and atherosclerosis or cardiovascular diseases.

The present invention provides a method to identify miRNA to which mimics will improve and/or restore the anti-inflammatory and/or antioxidative and/or the insulin sensitizing response in blood monocytes and infiltrated tissues (e.g. adipose and vascular tissues), in particular in persons with obesity and obesity-related disorders as disclosed above. In addition, the present invention provides a method to identify miRNA to which mimics will improve and/or restore the anti-inflammatory and/or antioxidative and/or the insulin sensitizing actions of adiponectin.

Illustrative embodiments of the invention

DEFINITIONS

Myeloid refers to the nonlymphocytic groups of white blood cells, including the granulocytes, monocytes and platelets.

Dyslipidemia (From dys- + lipid (fat) + -emia (in the blood) = essentially, disordered lipids in the blood) is a disorder of lipoprotein metabolism. Dyslipidemias may be manifested by elevation of the triglyceride concentrations, and a decrease in the "good" high-density lipoprotein (HDL) cholesterol concentration in the blood. Dyslipidemia comes under consideration in many situations including diabetes, a common cause of lipidemia. For adults with diabetes, it has been recommended that the levels HDL-cholesterol, and triglyceride be measured every year. Optimal HDL-cholesterol levels are equal to or greater than 40 mg/dL (1.02 mmol/L), and desirable triglyceride levels are less than 150 mg/dL (1.7 mmol/L).

Insulinemia concerns an abnormally large concentration of insulin in the blood.

Glycemia concerns the presence of glucose in the blood. It is a medical term meaning that the blood glucose is elevated, typically above 100 mg/dl.

Hypercholesterolemia is manifested by elevation of the total cholesterol due to elevation of the "bad" low-density lipoprotein (LDL) cholesterol in the blood. Optimal LDL-cholesterol levels for adults with diabetes are less than 100 mg/dL (2.60 mmol/L).

Triglycerides are the major form of fat. A triglyceride consists of three molecules of fatty acid combined with a molecule of the alcohol glycerol. Triglycerides serve as the backbone of many types of lipids (fats). Triglycerides come from the food we eat as well as from being produced by the body. Triglyceride levels are influenced by recent fat and alcohol intake, and should be measured after fasting for at least 12 hours. A period of abstinence from alcohol is advised before testing for triglycerides. Markedly high triglyceride levels (greater than 500mg/dl) can cause inflammation of the pancreas (pancreatitis). Therefore, these high levels should be treated aggressively with low fat diets and medications, if needed. The word "triglyceride" reflects the fact that a triglyceride consists of three ("tri-") molecules of fatty

acid combined with a molecule of the alcohol glycerol ("-glyceride") that serves as the backbone in many types of lipids (fats).

HDL-cholesterol concerns lipoproteins, which are combinations of lipids (fats) and proteins, are the form in which lipids are transported in the blood. The high-density lipoproteins transport cholesterol from the tissues of the body to the liver so it can be gotten rid of (in the bile). HDL-cholesterol is therefore considered the "good" cholesterol. The higher the HDL-cholesterol level, the lower the risk of coronary artery disease. Even small increases in HDL-cholesterol reduce the frequency of heart attacks. For each 1 mg/dl increase in HDL-cholesterol there is a 2 to 4% reduction in the risk of coronary heart disease. Although there are no formal guidelines, proposed treatment goals for patients with low HDL-cholesterol are to increase HDL-cholesterol to above 35 mg/dl in men and 45 mg/dl in women with a family history of coronary heart disease; and to increase HDL-cholesterol to approach 45 mg/dl in men and 55 mg/dl in women with known coronary heart disease. The first step in increasing HDL-cholesterol levels is life style modification. Regular aerobic exercise, loss of excess weight (fat), and cessation of cigarette smoking cigarettes will increase HDL-cholesterol levels. Moderate alcohol consumption (such as one drink a day) also raises HDL-cholesterol. When life style modifications are insufficient, medications are used. Medications that are effective in increasing HDL-cholesterol include nicotinic acid (niacin), gemfibrozil (Lopid), estrogen, and to a lesser extent, the statin drugs.

Hypertension or High blood pressure is defined as a repeatedly elevated blood pressure exceeding 140 over 90 mmHg -- a systolic pressure above 140 with a diastolic pressure above 90. Chronic hypertension is a "silent" condition. Stealthy as a cat, it can cause blood vessel changes in the back of the eye (retina), abnormal thickening of the heart muscle, kidney failure, and brain damage. For diagnosis, there is no substitute for measurement of blood pressure. Not having your blood pressure checked (or checking it yourself) is an invitation to hypertension. No specific cause for hypertension is found in 95% of cases. Hypertension is treated with regular aerobic exercise, weight reduction (if overweight), salt restriction, and medications.

Diabetes, type 2 is one of the two major types of diabetes, the type in which the beta cells of the pancreas produce insulin but the body is unable to use it effectively because the cells of the body are resistant to the action of insulin. Although this type of diabetes may not carry the same risk of death from ketoacidosis, it otherwise involves many of the same risks of complications as type 1 diabetes (in which there is a lack of insulin). The aim of treatment is to normalize the blood glucose in an attempt to prevent or minimize complications. People with type 2 diabetes may experience marked hyperglycemia, but most do not require insulin injections. In fact, 80% of all people with type 2 diabetes can be treated with diet, exercise, and, if needed be, oral hypoglycemic agents (drugs taken by mouth to lower the blood sugar). Type 2 diabetes requires good dietary control including the restriction of calories, lowered consumption of simple carbohydrates and fat with increased consumption of complex carbohydrates and fiber. Regular aerobic exercise is also an important method for treating both type 2 diabetes since it decreases insulin resistance and helps burn excessive glucose. Regular exercise also may help lower blood lipids and reduce some effects of stress, both important factors in treating diabetes and preventing complications. Type 2 diabetes is also known as insulin-resistant diabetes, non-insulin dependent diabetes, and adult-onset diabetes.

Systolic: The blood pressure when the heart is contracting. It is specifically the maximum arterial pressure during contraction of the left ventricle of the heart. The time at which ventricular contraction occurs is called systole. In a blood pressure reading, the systolic pressure is typically the first number recorded. For example, with a blood pressure of 120/80 ("120 over 80"), the systolic pressure is 120. By "120" is meant 120 mm Hg (millimeters of mercury). A systolic murmur is a heart murmur heard during systole, the time the heart contracts, between the normal first and second heart sounds. "Systolic" comes from the Greek systole, meaning "a drawing together or a contraction." The term has been in use since the 16th century to denote the contraction of the heart muscle.

Osteoarthritis is a type of arthritis caused by inflammation, breakdown, and eventual loss of cartilage in the joints. It is also known as degenerative arthritis.

An ischemic stroke is death of an area of brain tissue (cerebral infarction) resulting from an inadequate supply of blood and oxygen to the brain due to blockage of an artery. Ischemic stroke usually results when an artery to the brain is blocked, often by a blood clot or a fatty deposit due to atherosclerosis. Symptoms occur suddenly and may include muscle weakness, paralysis, lost or abnormal sensation on one side of the body, difficulty speaking, confusion, problems with vision, dizziness, and loss of balance and coordination. Diagnosis is usually based on symptoms and results of a physical examination, imaging tests, and blood tests. Treatment may include drugs to break up blood clots or to make blood less likely to clot and surgery, followed by rehabilitation. About one third of people recover all or most of normal function after an ischemic stroke. Ischemic stroke occurs when local blood flow is suddenly limited by vessel occlusion. The rate of neuronal death varies with blood flow. If blood flow falls to less than 15 mL/100 g/min, energy failure and subsequent cell death occur within minutes. Even suboptimal flow for longer periods may cause the cells to die by an apoptotic mechanism over days to weeks. Rapid restoration of blood flow is essential to save brain tissue. The mechanism of stroke involving the PCA territory is variable. It is commonly due to embolization from the heart, the aortic arch, the vertebral artery, or the basilar artery. Other mechanisms include intrinsic atherosclerotic disease and vasospasm. Migrainous strokes tend to involve PCAs preferentially. Less commonly, the anterior circulation is to blame (e.g., internal carotid stenosis), when a fetal PCA is present. Rare causes of stroke may be considered when usual culprits such as coagulation abnormalities, vasculitis, sympathomimetic drugs, and metabolic disorders are not present.

Insulin resistance is the diminished ability of cells to respond to the action of insulin in transporting glucose (sugar) from the bloodstream into muscle and other tissues. Insulin resistance typically develops with obesity and heralds the onset of type 2 diabetes. It is as if insulin is "knocking" on the door of muscle. The muscle hears the knock, opens up, and lets glucose in. But with insulin resistance, the muscle cannot hear the knocking of the insulin (the muscle is "resistant"). The pancreas makes more insulin, which increases insulin levels in the blood and causes a louder "knock." Eventually, the pancreas produces

far more insulin than normal and the muscles continue to be resistant to the knock. As long as one can produce enough insulin to overcome this resistance, blood glucose levels remain normal. Once the pancreas is no longer able to keep up, blood glucose starts to rise, initially after meals, eventually even in the fasting state. Insulin resistance is an early feature and finding in the pathogenesis of type 2 diabetes associated with obesity is the development is insulin resistance, defined as impaired insulin-mediated glucose clearance in insulin-sensitive tissues (skeletal muscle, liver and adipose tissue). Insulin resistance is the condition in which normal amounts of insulin are inadequate to produce a normal insulin response from fat, muscle and liver cells. Insulin resistance in fat cells reduces the effects of insulin and results in elevated hydrolysis of stored triglycerides in the absence of measures which either increase insulin sensitivity or which provide additional insulin. Increased mobilization of stored lipids in these cells elevates free fatty acids in the blood plasma. Insulin resistance in muscle cells reduces glucose uptake (and so local storage of glucose as (glycogen), whereas insulin resistance in liver cells reduces storage of glycogen, making it unavailable for release into the blood when blood insulin levels fall (normally only when blood glucose levels are at low storage: Both lead to elevated blood glucose levels. High plasma levels of insulin and glucose due to insulin resistance often lead to metabolic syndrome and type 2 diabetes, including its complications. In 2000, there were approximately 171 million people, worldwide, with diabetes. The numbers of diabetes patients will expectedly more than double over the next 25 years, to reach a total of 366 million by 2030 (WHO/IDF, 2004). The two main contributors to the worldwide increase in prevalence of diabetes are population ageing and urbanization, especially in developing countries, with the consequent increase in the prevalence of obesity (WHO/IDF, 2004).

Cardiovascular diseases refer to the class of diseases that involve the heart or blood vessels (arteries and veins). While the term technically refers to any disease that affects the cardiovascular system, it is usually used to refer to those related to atherosclerosis (arterial disease). The circulatory system (or cardiovascular system) is an organ system that moves nutrients, gases, and wastes to and from

cells, helps fight diseases and helps stabilize body temperature and pH to maintain homeostasis. While humans, as well as other vertebrates, have a closed circulatory system (meaning that the blood never leaves the network of arteries, veins and capillaries), some invertebrate groups have open circulatory system. The present diagnostic invention is particularly suitable for a cardiovascular disease of the group consisting of hypertension, coronary heart disease, heart failure, congestive heart failure, atherosclerosis, arteriosclerosis, stroke, cerebrovascular disease, myocardial infarction and peripheral vascular disease.

The following terms are similar, yet distinct, in both spelling and meaning, and can be easily confused: arteriosclerosis, arteriolosclerosis and atherosclerosis.

Arteriosclerosis also called hardening of the arteries chronic disease is characterized by abnormal thickening and hardening of the walls of arteries, with a resulting loss of elasticity. The major form of arteriosclerosis is atherosclerosis, in which plaques of consisting of macrophages, fatty deposits in foam cells, or atheromas, form on the inner walls of the arteries. These fatty acids are largely due to the uptake of oxidized LDL by macrophages. Arteriosclerosis is a general term describing any hardening (and loss of elasticity) of medium or large arteries (in Greek, "Arterio" meaning artery and "sclerosis" meaning hardening); arteriolosclerosis is arteriosclerosis mainly affecting the arterioles (small arteries); atherosclerosis is a hardening of an artery specifically due to an atheromatous plaque. Therefore, atherosclerosis is a form of arteriosclerosis. Arteriosclerosis ("hardening of the artery") results from a deposition of tough, rigid collagen inside the vessel wall and around the atheroma. This increases the stiffness, decreases the elasticity of the artery wall.

Arteriolosclerosis (hardening of small arteries, the arterioles) is the result of collagen deposition, but also muscle wall thickening and deposition of protein ("hyaline").Calcification, sometimes even ossification (formation of complete bone tissue) occurs within the deepest and oldest layers of the sclerosed vessel wall.

Atherosclerosis causes two main problems. First, the atheromatous plaques, though long compensated for by artery enlargement, eventually lead to plaque ruptures and stenosis (narrowing) of the artery and, therefore, an insufficient blood supply to the organ it feeds. If the compensating artery enlargement is excessive, a net aneurysm occurs. Atherosclerosis chronic disease is caused by the deposition of fats, cholesterol, calcium, and other substances in the innermost layer (endothelium) of the large and medium-sized arteries. Atherosclerosis is a disease affecting the arterial blood vessel. It is commonly referred to as a "hardening" or "furring" of the arteries. It is caused by the formation of multiple plaques within the arteries.

These complications are chronic, slowly progressing and cumulative. Most commonly, soft plaque suddenly ruptures (see vulnerable plaque), causing the formation of a thrombus that will rapidly slow or stop blood flow, e.g. 5 minutes, leading to death of the tissues fed by the artery. This catastrophic event is called an infarction. One of the most common recognized scenarios is called coronary thrombosis of a coronary artery causing myocardial infarction (a heart attack). Another common scenario in very advanced disease is claudication from insufficient blood supply to the legs, typically due to a combination of both stenosis and aneurismal segments narrowed with clots. Since atherosclerosis is a body wide process, similar events also occur in the arteries to the brain, intestines, kidneys, legs, etc.

Pathologically, the atheromatous plaque is divided into three distinct components: the nodular accumulation of a soft, flaky, yellowish material at the centre of large plaques composed of macrophages nearest the lumen of the artery; sometimes with underlying areas of cholesterol crystals; and possibly also calcification at the outer base of older/more advanced lesions.

Thrombogenicity refers to the tendency of a material in contact with the blood to produce a thrombus, or clot. It not only refers to fixed thrombi but also to emboli, thrombi which have become detached and travel through the bloodstream. Thrombogenicity can also encompass events such as the activation of immune pathways and the complement system. All materials are considered to be thrombogenic with the exception of the endothelial cells which line the vasculature. Certain medical implants appear non-thrombogenic due to high flow

rates of blood past the implant, but in reality, all are thrombogenic to a degree. A thrombogenic implant will eventually be covered by a fibrous capsule, the thickness of this capsule can be considered one measure of thrombogenicity, and if extreme can lead to the failure of the implant.

Low-density lipoprotein (LDL) belongs to the lipoprotein particle family. Its size is approx. 22 nm and its mass is about 3 million Daltons; but, since LDL particles contain a changing number of fatty acids, they actually have a mass and size distribution. Each native LDL particle contains a single apolipoprotein B-100 molecule (Apo B-100, a protein with 4536 amino acid residues) that circles the fatty acids, keeping them soluble in the aqueous environment. In addition, LDL has a highly-hydrophobic core consisting of polyunsaturated fatty acid known as linoleate and about 1500 esterified cholesterol molecules. This core is surrounded by a shell of phospholipids and unesterified cholesterol as well as a single copy of B-100 large protein (514 kD). Cholesterol is an animal sterol that is normally synthesized by the liver. The main types, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) carry cholesterol from and to the liver, respectively. LDL-cholesterol concerns thus the cholesterol in low-density lipoproteins. Cholesterol is required in the membrane of mammalian cells for normal cellular function, and is either synthesized in the endoplasmic reticulum, or derived from the diet, in which case it is delivered by the bloodstream in low-density lipoproteins. These are taken into the cell by LDL receptor-mediated endocytosis in clathrin-coated pits, and then hydrolyzed in lysosomes. Oxidized LDL-cholesterol concerns a LDL-cholesterol that has been bombarded by free radicals; it is thought to cause atherosclerosis; the `bad' cholesterol; a high level in the blood is thought to be related to various pathogenic conditions

Metabolic syndrome is a combination of medical disorders that increase the risk of developing cardiovascular disease and type 2 diabetes. It affects a large number of people, and prevalence increases with age. Some studies estimate the prevalence in the USA to be up to 25% of the population. Metabolic syndrome is also known as metabolic syndrome X, syndrome X, insulin resistance syndrome, Reaven's

syndrome or CHAOS. Metabolic syndrome components were defined as detailed in the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in adults (ATPIII) report: 1) waist circumference ≥102 cm in men and ≥ 88 cm in women; 2) fasting triglycerides ≥ 150 mg/dl (1.70 mmol/l); 3) HDL-cholesterol <40 mg/dl (1.03 mmol/l) in men and < 50 mg/dl (1.29 mmol/l) in women; 4) blood pressure ≥ 130/85 mmHg or on anti-hypertensive medication; 5) fasting-glucose ≥ 100 mg/dl (5.55 mmol/l) or on anti-diabetic medication.

Activated monocytes are monocytes that are associated with increased inflammation, often due to activation of the toll-like receptor (TLR)-2 (and/or -4), an increase in interleukin-1 receptor-associated kinase (IRAK)-1 and 4, and a decrease in the interleukin-1 receptor-associated kinase (IRAK)-3 (sometimes called IRAKM) and an increase in NFκB activity [29,30], and/or an increased production of reactive oxygen species (ROS) and oxidative stress, often due to loss of antioxidant enzymes like superoxide dismutase (SODs) [21,31], and/or a loss of insulin signaling and insulin resistance, for example by loss of expression of the insulin receptor substrate (IRS)-1 and -2 [32]. Activation of monocytes renders them more prone to infiltration of in tissues (e.g. adipose, vascular, pancreas, liver) often due to increased expression of the monocyte chemotactic protein 1 (MCP1 or otherwise called chemokine CC motif ligand or CCL2) [33]. Once infiltrated, these activated monocytes are more prone to give rise to inflammatory M1 macrophages instead of anti-inflammatory M2 macrophages [34-37]. In addition, they lost their capacity to activate their anti-inflammatory (e.g. increase in IRAK-3) and antioxidative (e.g. increase in antioxidant SODs and decreased ROS) mechanisms, and thus their capacity to switch their polarization from M1 to M2 in response to adiponectin [38,39]. These activation mechanisms are illustrated in Figure 2.

"**microRNA**", also written as miRNA or miR, refers to any type of interfering RNAs, including but not limited to, endogenous microRNAs and artificial microRNAs. Endogenous microRNAs are small RNAs naturally present in the genome which are capable of modulation the

productive utilization of mRNA. An artificial microRNA can be any type of RNA sequence, other than endogeneous microRNA, which is capable of modulation the productive utilization of mRNA. For instance, it includes sequences previously identified as siRNA, regardless of the mechanism of down-stream processing of the RNA. A microRNA sequence can be an RNA molecule composed of any one or more of these sequences. Several types of agents are known that modulate microRNAs. These include, but are not limited to microRNA mimics and microRNA inhibitors.

A "**miRNA mimic**" is an agent used to increase the expression and/or function of a miRNA. The miRNA mimic can also increase, supplement, or replace the function of a natural miRNA. In one embodiment, the miRNA mimic may be a polynucleotide comprising the mature miRNA sequence. In another embodiment, the miRNA mimic may be a polynucleotide comprising the pri-miRNA or pre-miRNA sequence. The miRNA mimic may contain chemical modifications, such as locked nucleic acids, peptide nucleic acids, sugar modifications, such as 2′-O-alkyl (e.g. 2′-O-methyl, 2′-β-methoxyethyl), 2′-fluoro, and 4′ thio modifications, and backbone modifications, such as one or more phosphorothioate, morpholino, or phosphonocarboxylate linkages.

A "**miRNA inhibitor**" is an agent that inhibits miRNA function in a sequence-specific manner. In one embodiment, the miRNA inhibitor is an antagomir. "Antagomirs" are single-stranded, chemically-modified ribonucleotides that are at least partially complementary to the miRNA sequence. Antagomirs may comprise one or more modified nucleotides, such as 2′-O-methyl-sugar modifications. In some embodiments, antagomirs comprise only modified nucleotides. Antagomirs may also comprise one or more phosphorothioate linkages resulting in a partial or full phosphorothioate backbone. To facilitate in vivo delivery and stability, the antagomir may be linked to a cholesterol moiety at its 3′ end. Antagomirs suitable for inhibiting miRNAs may be about 15 to about 50 nucleotides in length, more preferably about 18 to about 30 nucleotides in length, and most preferably about 20 to about 25 nucleotides in length. "Partially complementary" refers to a sequence that is at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to a target

polynucleotide sequence. The antagomirs may be at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to a mature miRNA sequence. In some embodiments, the antagomirs are 100% complementary to the mature miRNA sequence.

"Sample" or **"biological sample"** as used herein can be any organ, tissue, cell, or cell extract isolated from a subject, a cell-derived vesicle, such as a sample isolated from a mammal having a metabolic syndrome disorder or at risk for a metabolic syndrome disorder (e.g., based on family history or personal history). For example, a sample can include, without limitation, cells or tissue (e.g., from a biopsy or autopsy), peripheral blood, whole blood, red cell concentrates, platelet concentrates, leukocyte concentrates, blood cell proteins, blood plasma, platelet-rich plasma, a plasma concentrate, a precipitate from any fractionation of the plasma, a supernatant from any fractionation of the plasma, blood plasma protein fractions, purified or partially purified blood proteins or other components, serum, tissue or fine needle biopsy samples, or any other specimen, or any extract thereof, obtained from a patient (human or animal), test subject, healthy volunteer, or experimental animal. A subject can be a human, rat, mouse, non-human primate, etc. A sample may also include sections of tissues such as frozen sections taken for histological purposes. A "sample" may also be a cell or cell line created under experimental conditions, that is not directly isolated from a subject.

In a particular embodiment the sample is selected from the group consisting of (a) a liquid containing cells; (b) a tissue-sample; (c) a cell-sample; (d) a cell-derived vesicle; (e) a cell biopsy; more in particular the sample comprises hematopoietic cells or blood cells; even more in particular the sample comprises at least one myeloid cell or debris thereof. In an even further embodiment the sample comprises at least one of monocytes or peripheral blood mononuclear cells or debris thereof.

In addition, a sample can also be a blood-derived sample, like plasma or serum. In another particular embodiment, the miRNAs of the present invention can be quantified or qualified on isolated microvesicles, particularly on monocyte-derived microvesicles.

A "**control**" or "**reference**" includes a sample obtained for use in determining base-line expression or activity. Accordingly, a control sample may be obtained by a number of means including from subjects not having a metabolic syndrome disorder; from subjects not suspected of being at risk for developing a metabolic syndrome disorder; or from cells or cell lines derived from such subjects. A control also includes a previously established standard, such as a previously characterized pool of RNA or protein extracts from monocytes of at least 20 subjects without any of the metabolic syndrome components as defined above. Accordingly, any test or assay conducted according to the invention may be compared with the established standard and it may not be necessary to obtain a control sample for comparison each time.

The **inflammatory state** of a cell can be measured by determining well-known inflammatory parameters associated with said cell. These parameters include certain chemokines and cytokines, including but not limited to IFN-γ, IL-1, IL-6, IL-8, and TNF-α. An increased inflammatory state of a cell refers to an increased amount of inflammatory parameters associated with said cell compared to a control cell. Similarly a normal or decreased inflammatory state of a cell refers to a similar or decreased amount, respectively, of inflammatory parameters associated with said cell compared to a control cell.

Similarly, the **oxidative stress state** of a cell can be meausured by determining well-known oxidative stress parameters, such as e.g. the amount of reactive oxygen species (ROS). An increased, normal or decreased oxidative stress state of a cell refers, respectively, to an increased, similar or decreased amount of oxidative stress parameters associated with said cell compared to a control cell.

Insulin signaling

Special transporter proteins in cell membranes allow glucose from the blood to enter a cell. These transporters are, indirectly, under blood insulin's control in certain body cell types (e.g., muscle cells). Low levels of circulating insulin, or its absence, will prevent glucose from

entering those cells (e.g., in type 1 diabetes). More commonly, however, there is a decrease in the sensitivity of cells to insulin (e.g., the reduced insulin sensitivity characteristic of type 2 diabetes), resulting in decreased glucose absorption. In either case, there is 'cell starvation' and weight loss, sometimes extreme. In a few cases, there is a defect in the release of insulin from the pancreas. Either way, the effect is the same: elevated blood glucose levels.

Activation of insulin receptors leads to internal cellular mechanisms that directly affect glucose uptake by regulating the number and operation of protein molecules in the cell membrane that transport glucose into the cell. The genes that specify the proteins that make up the insulin receptor in cell membranes have been identified, and the structures of the interior, transmembrane section, and the extra-membrane section of receptor have been solved.

Two types of tissues are most strongly influenced by insulin, as far as the stimulation of glucose uptake is concerned: muscle cells (myocytes) and fat cells (adipocytes). The former are important because of their central role in movement, breathing, circulation, etc., and the latter because they accumulate excess food energy against future needs. Together, they account for about two-thirds of all cells in a typical human body.

Insulin binds to the extracellular portion of the alpha subunits of the insulin receptor. This, in turn, causes a conformational change in the insulin receptor that activates the kinase domain residing on the intracellular portion of the beta subunits. The activated kinase domain autophosphorylates tyrosine residues on the C-terminus of the receptor as well as tyrosine residues in the IRS-1 protein.

1. phosphorylated IRS-1, in turn, binds to and activates phosphoinositol 3 kinase (PI3K)
2. PI3K catalyzes the reaction PIP2 + ATP → PIP3
3. PIP3 activates protein kinase B (PKB)
4. PKB phosphorylates glycogen synthase kinase (GSK) and thereby inactivates GSK
5. GSK can no longer phosphorylate glycogen synthase (GS)
6. unphosphorylated GS makes more glycogen
7. PKB also facilitates vesicle fusion, resulting in an increase in GLUT4 transporters in the plasma membrane. [40,41]

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton et al., Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, N.Y. 1994), Microarrays in Clinical Diagnostics (© 2005 Humana Press Inc.) provide one skilled in the art with a general guide to many of the terms used in the present application.

For purposes of the present invention, the following terms are defined below.

The term "**array**" or "**microarray**" in general refers to an ordered arrangement of hybridizable array elements such as polynucleotide probes on a substrate. An "array" is typically a spatially or logically organized collection, e.g., of oligonucleotide sequences or nucleotide sequence products such as RNA or proteins encoded by an oligonucleotide sequence. In some embodiments, an array includes antibodies or other binding reagents specific for products of a candidate library. The array element may be an oligonucleotide, DNA fragment, polynucleotide, or the like, as defined below. The array element may include any element immobilized on a solid support that is capable of binding with specificity to a target sequence such that gene expression may be determined, either qualitatively or quantitatively.

When referring to a pattern of expression, a "**qualitative**" difference in gene expression refers to a difference that is not assigned a relative value. That is, such a difference is designated by an "all or nothing" valuation. Such an all or nothing variation can be, for example, expression above or below a threshold of detection (an on/off pattern of expression). Alternatively, a qualitative difference can refer to expression of different types of expression products, e.g., different alleles (e.g., a mutant or polymorphic allele), variants (including sequence variants as well as post-translationally modified variants), etc. In contrast, a "quantitative" difference, when referring to a pattern of gene expression, refers to a difference in expression that can be assigned a value on a graduated scale, (e.g., a 0-5 or 1-10 scale, a + +++ scale, a grade 1 grade 5 scale, or the like; it will be understood that the numbers selected for illustration are entirely arbitrary and in no-way are meant to be interpreted to limit the

invention). Microarrays are useful in carrying out the methods disclosed herein because of the reproducibility between different experiments. DNA microarrays provide one method for the simultaneous measurement of the expression levels of large numbers of genes. Each array consists of a reproducible pattern of capture probes attached to a solid support. Labeled RNA or DNA is hybridized to complementary probes on the array and then detected for instance by laser scanning. Hybridization intensities for each probe on the array are determined and converted to a quantitative value representing relative gene expression levels. See the patent publications Nos. US6040138, US5800992 and US6020135, US6033860, US 6344316, US7439346, US7371516, US7353116, US7348181, US7347921, US7335762 , US7335470, US7323308, US7321829, US7302348, US7276592, US7264929, US7244559, US7221785, US7211390, US7189509, US7138506, US7052842, US7047141 and US7031845 which are incorporated herein by reference. High-density oligonucleotide arrays are particularly useful for determining the gene expression profile for a large number of RNA's in a sample.

A "**DNA fragment**" includes polynucleotides and/or oligonucleotides and refers to a plurality of joined nucleotide units formed from naturally-occurring bases and cyclofuranosyl groups joined by native phosphodiester bonds. This term effectively refers to naturally-occurring species or synthetic species formed from naturally-occurring subunits. "DNA fragment" also refers to purine and pyrimidine groups and moieties which function similarly but which have no naturally-occurring portions. Thus, DNA fragments may have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other sulfur containing species. They may also contain altered base units or other modifications, provided that biological activity is retained. DNA fragments may also include species that include at least some modified base forms. Thus, purines and pyrimidines other than those normally found in nature may be so employed. Similarly, modifications on the cyclofuranose portions of the nucleotide subunits may also occur as long as biological function is not eliminated by such modifications.

The term "**polynucleotide**," when used in singular or plural generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritiated bases, are included within the term "polynucleotides" as defined herein. In general, the term "polynucleotide" embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of cells, including simple and complex cells.

The term "**oligonucleotide**" refers to a relatively short polynucleotide, including, without limitation, single-stranded deoxyribonucleotides, single- or double-stranded ribonucleotides, RNA: DNA hybrids and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA oligonucleotides, are often synthesized by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available. However, oligonucleotides can be made by a variety of other methods, including in vitro recombinant DNA-mediated techniques and by expression of DNAs in cells.

The terms "**differentially expressed gene**," "differential gene expression" and their synonyms, which are used interchangeably, refer to a gene whose expression is activated to a higher or lower level in a

subject, relative to its expression in a normal or control subject. A differentially expressed gene may be either activated or inhibited at the nucleic acid level or protein level, or may be subject to alternative splicing to result in a different polypeptide product. Such differences may be evidenced by a change in mRNA levels, surface expression, secretion or other partitioning of a polypeptide, for example. Differential gene expression may include a comparison of expression between two or more genes, or a comparison of the ratios of the expression between two or more genes, or even a comparison of two differently processed products of the same gene, which differ between normal subjects and subjects suffering from a disease, or between various stages of the same disease. Differential expression includes both quantitative, as well as qualitative, differences in the temporal or cellular expression pattern in a gene or its expression products. As used herein, "differential gene expression" can be present when there is, for example, at least an about a one to about two-fold, or about two to about four-fold, or about four to about six-fold, or about six to about eight-fold, or about eight to about ten-fold, or greater than about 11-fold difference between the expression of a given gene in a patient of interest compared to a suitable control. However, folds change less than one is not intended to be excluded and to the extent such change can be accurately measured, a fold change less than one may be reasonably relied upon in carrying out the methods disclosed herein.
In some embodiments, the fold change may be greater than about five or about 10 or about 20 or about 30 or about 40.

The phrase "**gene expression profile**" as used herein, is intended to encompass the general usage of the term as used in the art, and generally means the collective data representing gene expression with respect to a selected group of two or more genes, wherein the gene expression may be upregulated, downregulated, or unchanged as compared to a reference standard A gene expression profile is obtained via measurement of the expression level of many individual genes. The expression profiles can be prepared using different methods. Suitable methods for preparing a gene expression profile include, but are not limited to reverse transcription loop-mediated amplification (RT-LAMP), for instance one-step RT-LAMP, quantitative

RT-PCR, Northern Blot, in situ hybridization, slot-blotting, nuclease protection assay, nucleic acid arrays, and immunoassays. The gene expression profile may also be determined indirectly via measurement of one or more gene products (whether a full or partial gene product) for a given gene sequence, where that gene product is known or determined to correlate with gene expression.

The phrase "**gene product**" is intended to have the meaning as generally understood in the art and is intended to generally encompass the product(s) of RNA translation resulting in a protein and/or a protein fragment. The gene products of the genes identified herein may also be used for the purposes of diagnosis or treatment in accordance with the methods described herein.

A "'**reference gene expression profile**" as used herein, is intended to indicate the gene expression profile, as defined above, for a pre selected group which is useful for comparison to the gene expression profile of a subject of interest. For example, the reference gene expression profile may be the gene expression profile of a single individual known to not have an metabolic syndrome disorder phenotype or a propensity thereto (i.e. a "normal" subject) or the gene expression profile represented by a collection of RNA samples from '"normal" individuals that has been processed as a single sample. The "reference gene expression profile' ' may vary and such variance will be readily appreciated by one of ordinary skill in the art.

The phrase "**reference standard**" as used herein may refer to the phrase "**reference gene expression profile**" or may more broadly encompass any suitable reference standard which may be used as a basis of comparison with respect to the measured variable. For example, a reference standard may be an internal control, the gene expression or a gene product of a "healthy" or "'normal" subject, a housekeeping gene, or any unregulated gene or gene product. The phrase is intended to be generally non-limiting in that the choice of a reference standard is well within the level of skill in the art and is understood to vary based on the assay conditions and reagents available to one using the methods disclosed herein.

"Gene expression profiling" as used herein, refers to any method that can analyze the expression of selected genes in selected samples.
The phrase "gene expression system" as used herein, refers to any system, device or means to detect gene expression and includes diagnostic agents, candidate libraries, oligonucleotide sets or probe sets.

The terms "**diagnostic oligonucleotide**" or "**diagnostic oligonucleotide set**" generally refers to an oligonucleotide or to a set of two or more oligonucleotides that, when evaluated for differential expression their corresponding diagnostic genes, collectively yields predictive data.

Such predictive data typically relates to diagnosis, prognosis, selection of therapeutic agents, monitoring of therapeutic outcomes, and the like. In general, the components of a diagnostic oligonucleotide or a diagnostic oligonucleotide set are distinguished from oligonucleotide sequences that are evaluated by analysis of the DNA to directly determine the genotype of an individual as it correlates with a specified trait or phenotype, such as a disease, in that it is the pattern of expression of the components of the diagnostic oligonucleotide set, rather than mutation or polymorphism of the DNA sequence that provides predictive value. It will be understood that a particular component (or member) of a diagnostic oligonucleotide set can, in some cases, also present one or more mutations, or polymorphisms that are amenable to direct genotyping by any of a variety of well known analysis methods, e.g., Southern blotting, RFLP, AFLP, SSCP, SNP, and the like.
The phrase "gene amplification" refers to a process by which multiple copies of a gene or gene fragment are formed in a particular cell or cell line. The duplicated region (a stretch of amplified DNA) is often referred to as "amplicon." Usually, the amount of the messenger RNA (mRNA) produced, i.e., the level of gene expression, also increases in the proportion of the number of copies made of the particular gene expressed.

A "**gene expression system**" refers to any system, device or means to detect gene expression and includes diagnostic agents, candidate

libraries oligonucleotide, diagnostic gene sets, oligonucleotide sets, array sets, or probe sets.

As used herein, a "**gene probe**" refers to the gene sequence arrayed on a substrate.

As used herein, a "**nucleotide probe**" refers to the oligonucleotide, DNA fragment, polynucleotide sequence arrayed on a substrate.
The terms "splicing" and "RNA splicing" are used interchangeably and refer to RNA processing that removes introns and joins exons to produce mature mRNA with continuous coding sequence that moves into the cytoplasm of a eukaryotic cell.

"**Stringency**" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures.
Hybridization generally depends on the ability of denatured DNA to re-anneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence the higher is the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995) and in Current Protocols in Molecular Biology Copyright © 2007 by John Wiley and Sons, Inc., 2008.

As used herein, a "**gene target**" refers to the sequence derived from a biological sample that is labeled and suitable for hybridization to a gene probe affixed on a substrate and a "nucleotide target" refers to the sequence derived from a biological sample that is labeled and suitable for hybridization to a nucleotide probe affixed on a substrate.

The term "**treatment**" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology and biochemistry, which are within the skill of the art.

"Adiponectin" is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism [42]. Adiponectin is exclusively secreted from adipose tissue into the bloodstream and is very abundant in plasma relative to many hormones. Levels of the hormone are inversely correlated with body fat percentage in adults, [43] while the association in infants and young children is less clear. The hormone plays a role in the suppression of the metabolic derangements that may result in type 2 diabetes, [42,43] non-alcoholic fatty liver disease (NAFLD) and an independent risk factor for metabolic syndrome [44]. Adiponectin is secreted into the bloodsteam where it accounts for approximately 0.01% of all plasma protein at around 5-10 µg/mL. Plasma concentrations reveal a sexual dimorphism, with females having higher levels than males. Levels of adiponectin are reduced in diabetics compared to non-diabetics. Weight reduction significantly increases circulating levels [45]. Adiponectin automatically self-associates into larger structures. Initially, three adiponectin molecules form together a homotrimer. The trimers continue to self-associate and form hexamers or dodecamers. Like the plasma concentration, the relative levels of the higher-order structures are sexually dimorphic, where females have increased proportions of the high-molecular weight forms. Adiponectin exerts some of its weight reduction effects via the brain. This is similar to the action of leptin, [46] but the two hormones perform complementary actions, and can have additive effects.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Monocytes/macrophages in obesity and obesity-associated metabolic disorders. Our target cells are monocytes

since they are readily accessible (blood) and their activation constitutes a reservoir of inflammatory cells that infiltrate in tissues (adipose, aortic and cardiac tissues) where they actively induce oxidative stress, inflammation, and cell death and thereby induce insulin resistance, atherosclerosis, and heart failure.

Figure 2: An overview of miRNAs deregulated in monocytes of obese subjects and predicted to be involved in regulating key molecules in the IRAK3-related pathway associated with increased inflammation and oxidative stress and impaired insulin signaling and sensitivity. miR-30a, -101, -103, -126, -130b, -146b-5p, -151-5p, -181a, -181b, -181d, and -335 are all decreased in circulating monocytes of obese subjects. Flow of the pathways at the protein interaction level is indicated by black arrows. Blunted arrows indicate inhibition and dashed arrows indicate translocation of transcription factors NFκB and FOXO3A to the nucleus. Phosphorylation is indicated by a circled P. Abbreviations: miR, microRNA; ROS, reactive oxygen species.

Figure 3: Expression profiles of 31 candidate miRNAs in circulating monocytes of obese and lean subjects. Left 2 bars of each panel show miRNA levels determined by miRNA microarray in 6 lean controls and 10 obese subjects. Right 2 bars show levels of the same miRNA now validated by qRT-PCR in an extended population (14 lean controls and 21 obese subjects). Data shown are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ obese compared with lean controls.

Figure 4: miRNAs differentially expressed in monocytes of obese persons after short-term weight loss. miRNA levels as determined by qRT-PCR in 14 lean controls and 21 obese subjects before and after weight loss. Data shown are means ± SEM. $^{**}P < 0.01$ obese compared with lean controls; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ obese after weight loss compared with before.

Figure 5: miRNAs differentially expressed in inflammation associated cell experiments. miRNA levels as determined by qRT-PCR in **(A)** *IRAK3*-depleted THP-1 cells (n = 4), **(B)** THP-1 cells exposed to 10 ng/ml IL-6 (n = 6) and **(C)** THP-1 cells exposed to 1

μg/ml gAcrp30 (n = 6). Data shown are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with THP-1 cells transfected with negative control siRNA, THP-1 control cells or THP-1 cells exposed to 10 μg/ml gAcrp30. Abbreviation: gAcrp30, globular adiponectin.

Figure 6: miRNAs differentially expressed in oxidative stress associated cell experiments. miRNA levels as determined by qRT-PCR in THP-1 cells exposed to 10 μg/ml ox-LDL (n = 6). Data shown are means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with THP-1 control cells. Abbreviation: ox-LDL, oxidized LDL.

Figure 7: miRNAs differentially expressed in insulin resistance associated cell experiments. miRNA levels as determined by qRT-PCR in THP-1 exposed to 10^{-7} M insulin and 15 mM glucose (n = 6). Data shown are means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with THP-1 control cells.

Figure 8: miRNA decision tree. Microarray analysis identified a total of 133 miRNAs that were differentially expressed in circulating monocytes of obese patients compared with lean controls. To gain insight into this miRNA expression profile, a bioinformatic analysis was performed. The *in silico* analysis identified 31 miRNAs with potential targets in the *IRAK3*-related gene cluster. The expressions of 18 miRNAs were validated by qRT-PCR in an extended population. The expression profiles of the 18 miRNAs-of-interest were determined after short-term weight loss and in inflammation, oxidative stress and insulin resistance associated cell experiments. We selected 11 miRNAs based on these expression profiles (depicted in bold). Two of the 11 miRNAs were associated with the occurrence of the metabolic syndrome, one miRNA was associated with the occurrence of cardiovascular risk equivalents (being a Framingham cardiovascular risk score above 10% per 10 years or type 2 daibetes) and 3 miRNAs were associated with angiographically documented coronary artery disease.
*miRNAs are still associated with occurrence of metabolic syndrome, cardiovascular risk equivalents or coronary artery disease even after adjusted for other risk factors

Figure 9: Effect of miRNA inhibitors on inflammation, oxidative stress and insulin resistance *in vitro*. Gene expression was analyzed using qRT-PCR and mROS production was determined by flow cytometry in THP-1 cells (n = 6) transfected with miRNA inhibitors against **(A)** miR-130b, **(B)** miR-151-5p, **(C)** miR-103, **(D)** miR-156b-5p, **(E)** miR-335, **(F)** miR-181d, **(G)** miR-126 and **(H)** miR-181b. Data shown are means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with THP-1 cells transfected with negative control miRNA. Abbreviation: mROS, mitochondrial reactive oxygen species.

Figure 10: Effect of miRNA mimics on inflammation, oxidative stress and insulin resistance *in vitro*. (A) *IRAK3*, **(B)** *TNFα* and **(C)** *IRS1* expression was analyzed using qRT-PCR and **(D)** iROS production was determined by flow cytometry in THP-1 cells (n = 6) transfected with miR-30a, miR-151-5p, miR-181d and miR-335 mimics. Data shown are means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with THP-1 cells transfected with negative control miRNA. Abbreviation: iROS, intracellular reactive oxygen species.

Figure 11: miR-146b-5p is an essential mediator of the anti-inflammatory, antioxidative stress and insulin-sensitizing actions of globular adiponectin. THP-1 cells were exposed to 10 μg/ml gAcrp30 with (n = 4) and without (n = 6) inhibition of miR-146b-5p. Gene expression was analyzed by measuring relative *RNA* levels using qRT-PCR and ROS production was determined by flow cytometry. Data shown are means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with THP-1 cells exposed to 10 μg/ml gAcrp30. Abbreviations: gAcrp30, globular adiponectin, iROS, intracellular ROS; mROS, mitochondrial ROS; ROS, reactive oxygen species.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The following detailed description of the invention refers to the accompanying drawings. Also, the following detailed description does not limit the invention. Instead, the scope of the invention is defined by the appended claims and equivalents thereof.

The risk for developing heart disease is directly related to the concomitant burden of obesity-related cardiovascular risk factors clustered in the metabolic syndrome (MetSyn) [8]: dyslipidemia (i.e. high triglycerides and low HDL-cholesterol), hypertension, and type 2 diabetes.

Persons with the metabolic syndrome (MetSyn) are at increased risk of developing coronary heart diseases (CHD) as well as increased mortality from CHD and any other cause [47,48].

The Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in adults (ATPIII), draws attention to the importance of MetSyn and provides a working definition of this syndrome[49].

Findings from the Third National Health and Nutrition Examination Survey showed that MetSyn is highly prevalent in the United States. Its prevalence has increased from 6.7% among participants aged 20 to 29 years, to 43.5% and 42.0% for participants aged 60 to 69 years and aged at least 70 years, respectively [3].

Over 75% of hypertension cases are reported to be directly attributable to obesity, and the risk of developing hypertension is five to six times greater in obese adult Americans age 20 to 45 compared to non–obese individuals of the same age. Obesity and insulin resistance, and the interaction between these two components, are associated with a high cardiovascular risk [50,51]. As many as 90% of individuals with type 2 diabetes are overweight or obese. Obesity-related type 2 diabetes is a leading cause of morbidity and mortality in western societies, and is quickly approaching pandemic proportions [52]. In addition to heart disease, obesity is reported to increase the risk of ischemic stroke independent of other risk factors, including age and systolic blood pressure. The incidence of osteoarthritis increases with BMI and is associated with arthritis of the hand, hip, back and, in particular, the knee. Increased weight adds stress to bones and joints due to increased load. Lastly, there is evidence that some cancers (endometrial, breast and colon) are associated with obesity.

Although obesity and insulin resistance, and the interaction between these two components, are associated with a high cardiovascular risk [50,51], the severity of insulinemia and glycaemia during the diabetic phase can only to a minor extent explain this increased cardiovascular risk.

A possible pathogenic mechanism which links obesity with type-2 diabetes and with cardiovascular risk is monocyte activation. Indeed, obesity is associated with increased infiltration in the adipose tissue of activated monocytes/macrophages that also produce inflammatory chemokines [23].
Increased oxidative stress causes increased activated monocyte infiltration and is an early instigator of MetSyn. Several findings support this hypothesis. As an example, we demonstrated that MetSyn is associated with elevated levels of circulating oxidized LDL (oxLDL), a marker of oxidative stress. High triglycerides, low HDL-cholesterol, and high glucose and insulin predicted elevated levels of oxLDL independent of LDL-cholesterol levels. The association between MetSyn and elevated levels of oxLDL has been confirmed in European and Japanese cohorts [53-55]. Persons with high oxLDL levels showed a greater disposition to myocardial infarction, adjusting for all established cardiovascular risk factors [18-21,56]. Two other studies confirmed that elevated levels of circulating oxLDL predict future cardiovascular events even after adjustment for traditional cardiovascular risk factors and C-reactive protein [57,58]. Recently, we have shown that persons with high oxLDL showed a 4.5-fold greater disposition to future MetSyn after 5 years follow-up, adjusted for age, gender, race, study centre, cigarette smoking, BMI, physical activity, and LDL-cholesterol, little changed by further adjustment for C-reactive protein, and adiponectin. In particular, oxLDL predicted the development of obesity, dyslipidemia and pre-diabetes. Several studies showed that oxLDL can induce the activation of monocytes as evidenced by increased capacity of monocytes to infiltrate vascular tissues in response to oxLDL-induced monocyte chemoattractant protein-1 by endothelial cells, by the oxLDL-induced activation of toll-like repceptor (TLR)-2 and 4-mediated pro-inflammatory response resulting in production of inflammatory cytokines, by the oxLDL-

induced NF-κB activation and by the oxLDL-induced mitochondrial dysfunction resulting in a further enehancement of ROS production [59]. We hypothesized that the identification of a cluster of genes and associated proteins which are associated with monocyte/macrophage activation, as evidenced by their inflammation and oxidative stress state, and of which the expression pattern is improved by weight loss that significantly reduces cardiovascular risk could lead to a better estimate of the risk for cardiovascular disease for obese persons. We started from the observation in obese miniature pigs on an atherogenic diet that toll-like receptor 2 (TLR2) was over expressed in plaque macrophages isolated by laser capture micro dissection and correlated with atherosclerotic plaque complexity [60]. Then, we performed micro array analysis of RNA extracted from monocytes of obese women. Because we found that TLR2 was over expressed, we searched for genes that correlated with TLR2. Structural modeling predicted a cluster of genes that besides TLR2 contains the following genes and associated proteins: IL1 receptor-associated kinase 3 (IRAK3), Tumor Necrosis Factor (TNF)-Associated Factor 6 (TRAF6), the myeloid differentiation marker MYD88, TNF-alpha-induced protein 3 and 6 (TNFAIP3; TNFAIP6), the Insulin Receptor Substrate 2 (IRS2), mitogen-activated protein kinase 13 (MAPK13), the Forkhead Box O3A (FOXO3A), and superoxide dismutase 2 (SOD2). These genes and associated proteins form the backbone of a pathway that links the toll-like receptor-mediated inflammation with the protection against oxidative stress by means of SOD2. Earlier (WO2009121152) we presented evidence that some of these predicted molecules indeed are novel (bio)markers of cardiovascular risk in association with obesity, lipid homeostasis disorder related cardiovascular disease and/or an impaired glucose tolerance condition and that some are even causal biomarkers. Especially, low expression was found to be associated with high prevalence of metabolic syndrome and high cardiovascular risk.

Description of the molecules

Toll-like receptors, nuclear factor NF-kappa-B, tumor necrosis factor alpha, *and IL1 receptor-associated kinases*

The Toll like /Interleukin 1 receptor family consists of a large number of transmembrane proteins which are involved in host defense and

have conserved intracellular domains. This superfamily is divided into 2 subgroups based on the components of the extracellular domains: the Toll like receptors (TLRs) with leucine-rich repeats, and the Interleukin-1 receptors (IL1Rs) with immunoglobulin-like motifs.

5 **TOLL-LIKE RECEPTOR 2; TLR2**

Aliprantis et al. (1999) found that bacterial lipoproteins induced apoptosis in THP-1 monocytic cells through human TLR2 [61]. In addition, bacterial lipoproteins also initiated apoptosis in an epithelial cell line transfected with TLR2. Bacterial lipoproteins stimulated NF-
10 kappa-B, a transcriptional activator of multiple host defense genes, and activated the respiratory burst through TLR2. Thus, TLR2 is a molecular link between microbial products, apoptosis, and host defense mechanisms. Aderem and Ulevitch (2000) reviewed the role of TLRs in innate immunity [62]. Whereas lipopolysaccharide (LPS) activates
15 cells through TLR4, gram-positive cell-wall components, including peptidoglycan and lipoteichoic acid, as well as mycobacterial cell-wall components such as lipoarabinomannan and mycolylarabinogalactan, and yeast cell-wall zymosan, activate cells via TLR2. Takeuchi et al. (2000) showed that Tlr2- and, particularly, Myd88-deficient mice are
20 highly susceptible, in terms of growth in blood and kidney and decreased survival, to infection with Staphylococcus aureus compared to wildtype mice [63]. In vitro, Tlr2-deficient macrophages produced reduced TNF and IL6 in response to S. aureus compared to wildtype or Tlr4-deficient macrophages, whereas Myd88-deficient macrophages
25 produced no detectable TNF or IL6. The authors concluded that TLR2 and MYD88 are critical in the defense against gram-positive bacteria. Shishido et al. (2003) found that Tlr2-deficient mice survived longer than wildtype mice after induced myocardial infarction [64]. There was no difference in inflammation or infarct size between knockout mice
30 and wildtype mice. They concluded that TLR2 plays an important role in ventricular remodeling after myocardial infarction. In atherosclerosis-susceptible Ldlr (606945)-null mice, Mullick et al. (2005) demonstrated that complete deficiency of Tlr2 led to a reduction in atherosclerosis whereas intraperitoneal injection of a
35 synthetic TLR2/TLR1 agonist dramatically increased atherosclerosis [65]. In Ldlr-null mice, transplantation of Tlr2 -/- bone marrow (BM) cells had no effect on atherosclerosis, suggesting the presence of an

endogenous TLR2 agonist activating TLR2 in cells that were not of BM cell origin. In Ldlr-null mice, complete deficiency of Tlr2 as well as a deficiency of Tlr2 only in BM-derived cells led to striking protection against agonist-mediated atherosclerosis, suggesting a role for BM-derived cell expression of TLR2 in transducing the effects of an exogenous TLR2 agonist. They stated that these findings support the concept that chronic or recurrent microbial infections contribute to atherosclerotic disease and also suggest the presence of host-derived endogenous TLR2 agonists.

The Homo sapiens toll-like receptor (TLR2) mRNA has been deposited in the NCBI database under the accession number NG_016229 (28803 bp mRNA linear PRI 05-NOV-2010) with the nucleotide sequence as in sequence ID 1.
The Homo sapiens toll-like receptor 2 (TLR2) protein has been deposited in the NCBI database under the accession number AAH33756 VERSION AAH33756.1 (784 aa PRI 07-OCT-2003) with the amino acid sequence depicted in sequence ID 2.

Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1)

NFKB has been detected in numerous cell types that express cytokines, chemokines, growth factors, cell adhesion molecules, and some acute phase proteins in health and in various disease states. NFKB is activated by a wide variety of stimuli such as cytokines, oxidant-free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products. Inappropriate activation of NFKB has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, and AIDS. In contrast, complete and persistent inhibition of NFKB has been linked directly to apoptosis, inappropriate immune cell development, and delayed cell growth. For reviews, see Chen et al. (1999) [66] and Baldwin (1996) [67]. Yang et al. (2004) observed that after hyperoxic exposure, neonatal mice showed increased Nfkb binding, whereas adult mice did not [68]. Neonatal Nfkb/luciferase transgenic mice demonstrated enhanced in vivo Nfkb activation after hyperoxia. Inhibition of Nfkbia resulted in decreased Bcl2 protein levels in neonatal lung homogenates and decreased cell

viability in lung primary cultures after hyperoxic exposure. In addition, neonatal Nfkb1-null mice showed increased lung DNA degradation and decreased survival in hyperoxia compared with wildtype mice. They concluded that there are maturational differences in lung NFKB activation and that enhanced NFKB may serve to protect the neonatal lung from acute hyperoxic injury via inhibition of apoptosis. In transgenic mice deficient for the LDL receptor (Ldlr) and with a macrophage-restricted deletion of Ikbkb, an activator of NFKB, Kanters et al. (2003) found an increase in atherosclerosis, as characterized by increased lesion size, more lesions, and necrosis [69]. In vitro studies showed that Ikbkb deletion in macrophages resulted in a reduction of TNF and the antiinflammatory cytokine Il10. The findings suggested that inhibition of the NFKB pathway affects the pro- and antiinflammatory balance that controls the development of atherosclerosis.

The mRNA of Homo sapiens nuclear factor NF-kappa-B p105 subunit (NFKB1), isoform 1has been deposited in the NCBI database under the accession number NM_003998 4093 bp mRNA linear (PRI 23-JAN-2011) with the mRNA nucleotide sequence as in sequence ID 3. The protein of NFKB1, isoform 1 has been deposited in the NCBI database under the accession number NP_003989 969 aa linear (PRI 23-JAN-2011) with the amino acid sequence depicted in sequence ID 4. The mRNA of Homo sapiens nuclear factor NF-kappa-B p105 subunit (NFKB1), isoform 2 has been deposited in the NCBI database under the accession number NM_001165412 4090 bp mRNA linear (PRI 22-JAN-2011) with the mRNA nucleotide sequence as in sequence ID 5. The protein of NFKB1, isoform 2 has been deposited in the NCBI database under the accession number NP_001158884 968 aa linear (PRI 22-JAN-2011) as depicted in sequence ID 6.

Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKBIA or IκBα)

The NFKB complex, a master regulator of proinflammatory responses, is inhibited by NFKBIA proteins, which inactivate NFKB by trapping it in the cytoplasm. Phosphorylation of serine residues on the NFKBIA proteins by kinases (IKK1 or IKK2) marks them for destruction via the ubiquitination pathway, thereby allowing activation of the NFKB

complex. Nenci et al. (2007) demonstrated that the transcription factor NFKB functions in gut epithelial cells to control epithelial integrity and the interaction between the mucosal immune system and gut microflora [70]. Intestinal epithelial-specific inhibition of NFKB through conditional ablation of NEMO or both IKK1 and IKK2, IKK subunits essential for NFKB activation, spontaneously caused severe chronic intestinal inflammation in mice. NFKB deficiency led to apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides, and translocation of bacteria into the mucosa. Concurrently, this epithelial defect triggered a chronic inflammatory response in the colon, initially dominated by innate immune cells but later also involving T lymphocytes. Deficiency of the gene encoding the adaptor protein MyD88 prevented the development of intestinal inflammation, demonstrating that Toll-like receptor activation by intestinal bacteria is essential for disease pathogenesis in this mouse model. Furthermore, NEMO deficiency sensitized epithelial cells to TNF-induced apoptosis, whereas TNF receptor-1 inactivation inhibited intestinal inflammation, demonstrating that TNFR1 signaling is crucial for disease induction. They concluded that a primary NFKB signaling defect in intestinal epithelial cells disrupts immune homeostasis in the gastrointestinal tract, causing an inflammatory bowel disease-like phenotype. Their results further identified NFKB signaling in the gut epithelium as a critical regulator of epithelial integrity and intestinal immune homeostasis and have important implications for understanding the mechanisms controlling the pathogenesis of human inflammatory bowel disease. Cytokine signaling is thought to require assembly of multicomponent signaling complexes at cytoplasmic segments of membrane-embedded receptors, in which receptor-proximal protein kinases are activated. Matsuzawa et al. (2008) reported that, upon ligation, CD40 formed a complex containing adaptor molecules TRAF2 and TRAF3, ubiquitin-conjugating enzyme UBC13, cellular inhibitor of apoptosis protein-1 and -2, IKK-gamma, and MEKK1. TRAF2, UBC13, and IKK-gamma were required for complex assembly and activation of MEKK1 and MAP kinase cascades [71]. However, the kinases were not activated unless the complex was translocated from the membrane to the cytosol upon CIAP1/CIAP2-induced degradation of TRAF3. They proposed that this 2-stage signaling mechanism may apply to other

innate immune receptors and may account for spatial and temporal separation of MAPK and IKK signaling

The mRNA of Homo sapiens NF-kappa-B inhibitor alpha (NFKBIA) has been deposited in the NCBI database under the accession number NG_007571.1 1579 bp mRNA linear (PRI 25-DEC-201) with the mRNA nucleotide sequence as in sequence ID 7. The protein of NFKBIA has been deposited in the NCBI database under the accession number NP_065390 317 aa linear (PRI 25-DEC-2010) with the amino acid sequence depicted in sequence ID 8.

Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor (TNF) is a multifunctional proinflammatory cytokine secreted predominantly by monocytes/macrophages that has effects on lipid metabolism, coagulation, insulin resistance, and endothelial function. TNF was originally identified in mouse serum after injection with Mycobacterium bovis strain bacillus Calmette-Guerin (BCG) and endotoxin. Serum from such animals was cytotoxic or cytostatic to a number of mouse and human transformed cell lines and produced hemorrhagic necrosis and in some instances complete regression of certain transplanted tumors in mice [72,73]. Kamata et al. (2005) found that TNF-α-induced reactive oxygen species (ROS), whose accumulation could be suppressed by mitochondrial superoxide dismutase (SOD2; 147460), caused oxidation and inhibition of JNK (see 601158)-inactivating phosphatases by converting their catalytic cysteine to sulfenic acid [74]. This resulted in sustained JNK activation, which is required for cytochrome c release and caspase-3 cleavage, as well as necrotic cell death. Treatment of cells or experimental animals with an antioxidant prevented H2O2 accumulation, JNK phosphatase oxidation, sustained JNK activity, and both forms of cell death. Antioxidant treatment also prevented TNF-α-mediated fulminant liver failure without affecting liver regeneration. Zinman et al. (1999) studied the relationship between TNF-α and anthropometric and physiologic variables associated with insulin resistance and diabetes in an isolated Native Canadian population with very high rates of NIDDM (125853) [75]. Using the homeostasis assessment (HOMA) model to

estimate insulin resistance, they found moderate, but statistically significant, correlations between TNF-α and fasting insulin, HOMA insulin resistance, waist circumference, fasting triglycerides, and systolic blood pressure; in all cases, coefficients for females were stronger than those for males. The authors concluded that in this homogeneous Native Canadian population, circulating TNF-α concentrations are positively correlated with insulin resistance across a spectrum of glucose tolerance. The data suggested a possible role for TNF-α in the pathophysiology of insulin resistance.

The Homo sapiens tumor necrosis factor alpha (TNF-α) mRNA has been deposited in the NCBI database under the accession number _ NG_007462 (9763 bp bp mRNA linear PRI 13-NOV-2010) with the nucleotide sequence as in sequence ID 9. The Homo sapiens tumor necrosis factor alpha (TNF-α) protein has been deposited in the NCBI database under the accession number ACO37640 VERSION ACO37640.1 (232 aa PRI 06-APR-2009) with the amino acid sequence depicted in sequence ID 10.

IL1 Receptor-Associated Kinase 1 (IRAK1)

Signal transduction pathways in these receptor families ultimately lead to activation of members of the Rel and AP1family of transcription factors. An important mediator in this pathway is **I**L1 **R**eceptor-**A**ssociated **K**inase **1** (IRAK1). Using a murine EST sequence encoding a polypeptide with significant homology to IRAK1 to screen a human phytohemagglutinin-activated peripheral blood leukocyte (PBL) cDNA library, Wesche et al. [76] isolated a full-length cDNA clone encoding a 596-amino acid protein. Sequence analysis revealed an overall sequence similarity of 30 to 40% with IRAK1 and IRAK2 as well as structural similarity in an N-terminal death domain and a central kinase domain. Kanakaraj et al. [77] determined that Irak-deficient mice had impaired responses to interleukin-18 (IL18, as measured by JNK and NFKB activation. They also noted a severe impairment in gamma-interferon (IFNG) production and the induction of natural killer cell cytotoxicity by IL18. Infection with murine cytomegalovirus showed that IRAK is essential for IFNG production but not for IL18 expression or NK cell cytotoxicity, which may be compensated for by IFNA/IFNB. Thomas et al. [78] noted that Irak-deficient mice were viable and fertile.

They observed diminished NFKB activation in fibroblasts from Irak knockout mice when stimulated with IL1. NFKB activation in response to TNF was unimpaired. Treatment of splenocytes with IL12 alone or in combination with IL18, but not with IL18 alone, resulted in the production of normal amounts of IFNG. On the other hand, Irak deletion did not impair delayed-type hypersensitivity responses or cell-mediated immunity to infection with the intracellular bacterium Listeria monocytogenes. Jacob et al. [79] found that absence of Irak1 in mice significantly attenuated the serologic and cellular immunologic phenotypes independently attributed to the Sle1 and Sle3 susceptibility loci for systemic lupus erythematosus (SLE) in mice.

The Homo sapiens interleukin-1 receptor-associated kinase 1 (IRAK1) mRNA has been deposited in the NCBI database as under the accession number ACCESSION NM_001025242.1 (LOCUS: NM_001025242 3499bp mRNA linear PRI 04-AUG-2010) with the nucleotide sequence as in sequence ID 11. The Homo sapiens interleukin-1 receptor-associated kinase 1 (IRAK1) protein has been deposited in the NCBI database as under the accession number ACCESSION AAH54000 VERSION AAH54000.1 (LOCUS AAH54000 693 aa linear PRI 07-OCT-2003) with the amino acid sequence depicted in sequence ID 12.

IL1 Receptor-Associated Kinase 3 (IRAK3)

Another IRAK was found to be predominantly expressed in PBL and the monocytic cell lines U937 and THP-1, in contrast to the other IRAKs that are expressed in most cell types. Because of the restriction of expression of this IRAK to monocytic cells, the authors termed the protein **IRAKM**, now called **IRAK3**. The IRAK3 (or IRAKM or Interleukin-1 Receptor-Associated Kinase 3 or Interleukin-1 Receptor-Associated Kinase M) gene consists of 12 exons spanning a region of approximately 60 kb in chromosome 12q14.3 [80]. Like IRAK2, the expression of IRAK3 in THP-1 cells is upregulated in the presence of phorbol ester and ionomycin, which also induce differentiation of these cells into more mature macrophages [76]. IRAK-3 (IRAK-M) is a member of the interleukine-1 receptor-associated kinase (IRAK) family. The IRAK family is implicated in the Toll-like receptor (TLR) and Il-1R signaling pathway. IRAK3 interacts with the myeloid differentiation

(MYD) marker MYD88 and TRAF6 signaling proteins in a manner similar to the other IRAKs. However, Kobayashi et al. [81] showed that IRAK3, in contrast to other IRAKs, is induced upon TLR stimulation but negatively regulates TLR signaling. IRAK3 -/- cells exhibited increased cytokine production upon TLR/IL1 stimulation and bacterial challenge, and Irakm -/- mice showed increased inflammatory responses to bacterial infection. Endotoxin tolerance, a protection mechanism against endotoxin shock, was significantly reduced in IRAKM -/- cells. Thus, the authors concluded that IRAK3 regulates TLR signaling and innate immune homeostasis. Data with IRAK-M knockout mice have revealed that IRAK-M serves as a negative regulator of IL-1R/TLR signaling. Moreover IRAK-M expression is mainly restricted to cells of a myeloid origin.

The Homo sapiens interleukin-1 receptor-associated kinase 3 (IRAK3) mRNA has been deposited in the NCBI database as under the accession number ACCESSION NM_007199, VERSION NM_007199.2 (LOCUS: NM_007199 8351 bp mRNA linear PRI 03-AUG-2010) with the nucleotide sequence as in sequence ID 13. The Homo sapiens interleukin-1 receptor-associated kinase 3 (IRAK3) protein has been deposited in the NCBI database as under the accession number NP_009130 ACCESSION VERSION NP_009130.2 GI:216547519 (LOCUS NP_009130 596 aa linear PRI 03-AUG-2010) with the amino acid sequence as in sequence ID 14.

IL1 Receptor-Associated Kinase 4 (IRAK4)

IRAK4 is another kinase that activates NF-kappaB in both the Toll-like receptor (TLR) and T-cell receptor (TCR) signaling pathways [82-85]. The protein is essential for most innate immune responses. Mutations in this gene result in IRAK4 deficiency and recurrent invasive pneumococcal disease. Multiple transcript variants encoding different isoforms have been found for this gene. The transcript variant 1 represents the longest transcript. Variants 1 and 2 both encode the same isoform A. Variants 3, 4, and 5 all encode the same isoform B.

This variant (4) lacks two alternate exons and uses a downstream start codon, compared to variant 1. The resulting isoform B, also known as the short form, has a shorter N-terminus, compared to isoform A.

The Homo sapiens interleukin-1 receptor-associated kinase 4 (IRAK4) mRNA has been deposited in the NCBI database as under the accession number ACCESSION M_001114182, VERSION NM_001114182.2 (LOCUS: NM_001114182 4351 bp mRNA linear PRI 05-AUG-2010) with the nucleotide sequence as in sequence ID 15. The Homo sapiens interleukin-1 receptor-associated kinase 4 (IRAK4) protein has two isoforms A and B. Isoform A been deposited in the NCBI database as under the accession number NP_057207 ACCESSION VERSION NP_057207.2 GI:166795293 (LOCUS NP_057207 460 aa linear PRI 01-AUG-2010) with the amino acid sequence as in sequence ID 16. Isoform B been deposited in the NCBI database as under the accession number NP_001138729 ACCESSION VERSION NP_001138729.1 (336 aa linear PRI 05-AUG-2010) with the amino acid sequence as in sequence ID 17.

TNF-alpha-induced protein 6 (TNFAIP6)

Lee et al. [86] described a gene, which they designated TSG6 (current name TNFAIP6), that is transcribed in normal fibroblasts and activated by binding of TNF-alpha and IL1 at AP-1 and NF-IL6 sites in its promoter. The cDNA was isolated from a library made from TNF-treated human fibroblasts. TNFAIP6 is a member of the hyaluronan-binding protein family, which includes cartilage link protein, proteoglycan core protein, and the adhesion receptor CD44. The predicted polypeptide is 277 amino acids long and includes a typical cleavage signal peptide. TNFAIP6 is highly homologous to CD44, particularly in the hyaluronic acid-binding domain. Western blots with antibodies made to a TNFAIP6 fusion protein detected a 39-kD glycoprotein in TNF-treated cells, and hyaluronate binding was shown by co-precipitation. TNFAIP6 expression is rapidly activated by TNF-alpha, IL1, and lipopolysaccharide in normal fibroblasts, peripheral blood mononuclear cells, synovial cells, and chondrocytes.

The mRNA of Homo sapiens tumour necrosis factor, alpha-induced protein 6 (TNFAIP6) has been deposited in the NCBI database under the accession number NM 007115 VERSION NM_007115.3 (1439 bp bp PRI 27-DEC-2010) with the mRNA nucleotide sequence as in sequence ID 18. The protein of Homo sapiens tumour necrosis factor, alpha-induced protein 6 (TNFAIP6) has been deposited in the NCBI database under the accession number CAD13434 VERSION CAD13434.1 (277 bp PRI 07-OCT-2008) as depicted in Sequence ID 19.

Insulin Receptor Substrate 1 and 2 (IRS1 and IRS2)

The Insulin Receptor Substrate 1 (IRS1) acts as an interface between signalling proteins with Src homology-2 domains (SH2 proteins) and the receptors for insulin, IGF2, growth hormone, several interleukins, and other cytokines. It regulates gene expression and stimulates mitogenesis and appears to mediate insulin/IGF1-stimulated glucose transport. Thus, the finding that the homozygous Irs1 KO mouse survives with only mild resistance to hypertension was surprising. This dilemma was provisionally resolved by the discovery by Sun et al. [87] of a second IRS signalling protein in mouse. They purified and cloned a likely candidate from mouse myeloid progenitor cells and, because of its resemblance to IRS1, they designated it IRS2. Withers et al. [88] demonstrated that homozygous absence of the Irs2 gene results in type II diabetes in mice. Heterozygous and wild type animals were unaffected. The authors demonstrated profound insulin resistance in both skeletal muscle and liver in the homozygous Irs2 -/- mice. Male mice lacking the Irs2 locus showed polydypsia and polyuria without ketosis and died from dehydration and hyperosmolar coma. A similar disease progression was observed in female mice, with the exception that the females rarely died. The authors concluded that dysfunction of IRS2 may contribute to the pathophysiology of human type II diabetes. Tobe et al. [89] observed that Irs2-deficient mice showed increased adiposity with increased serum leptin level, suggesting leptin resistance before the mice developed diabetes. Using oligonucleotide microarray and Northern blot analyses to analyze gene expression they detected increased expression of SREBP1, a downstream target of insulin, in Irs2-deficient mouse liver. Using high dose leptin administration, they provided evidence that leptin resistance in Irs2-

deficient mice is causally related to SREBP1 gene induction. The authors concluded that Irs2 gene disruption results in leptin resistance, causing SREBP1 gene induction, obesity, fatty liver, and diabetes. Taguchi et al. [90] showed that, in mice, less Irs2 signalling throughout the body or only in brain extended life span up to 18%. At 22 months of age, brain-specific Irs2 knockout mice were overweight, hyperinsulinemic, and glucose intolerant; however, compared with control mice, they were more active and displayed greater glucose oxidation, and during meals they displayed stable SOD2 concentrations in the hypothalamus. Thus, they concluded that less Irs2 signalling in aging brains can promote healthy metabolism, attenuate meal-induced oxidative stress, and extend the life span of overweight and insulin-resistant mice.

The mRNA of Homo sapiens insulin receptor substrate 1 (IRS1) has been deposited in the NCBI database under the accession number ACCESSION NG_015830 VERSION NG_015830.1 (74474 bp mRNA linear PRI 04-NOV-2010) with the mRNA nucleotide sequence as in sequence ID 20. The protein of IRS1 has been deposited in the NCBI database under the accession number AAH53895 VERSION AAH53895.1 (1242bp PRI 15-JUL-2006) with the amino acid sequence depicted in sequence ID 21. The mRNA of Homo sapiens insulin receptor substrate 1 (IRS2) has been deposited in the NCBI database under the accession number NG_008154 VERSION NM_ NG_008154.1 (39731 bpbp mRNA PRI 10-NOV-2010) with the mRNA nucleotide sequence as in sequence ID 22. The protein of IRS2 has been deposited in the NCBI database under the accession number Q9Y4H2 VERSION Q9Y4H2.2 (1338 bp PRI11-JAN-2011) as depicted in sequence ID 23.

FOXO3A

Survival factors can suppress apoptosis in a transcription-independent manner by activating the serine/threonine kinase AKT1, which then phosphorylates and inactivates components of the apoptotic machinery, including BAD and caspase-9. Brunet et al. [91] demonstrated that AKT1 also regulates the activity of FKHRL1 (current name FOXO3A). In the presence of survival factors, AKT1 phosphorylates FKHRL1, leading to the association of FKHRL1 with 14-

3-3 proteins and its retention in the cytoplasm. Survival factor withdrawal leads to FKHRL1 dephosphorylation, nuclear translocation, and target gene activation. Within the nucleus, FKHRL1 most likely triggers apoptosis by inducing the expression of genes that are critical for cell death, such the TNF ligand superfamily 6 (TNFSF6). Nemoto and Finkel [92] observed that exposure to intracellular ROS induced an increase in phosphorylated Fkhrl1 and a shift from a nuclear to a cytosolic localization. They found that serum starvation, a stimulus that increases oxidative stress, resulted in lower levels of hydrogen peroxide in Shc1 -/- cells or in cells expressing a ser36-to-ala (S36A) Shc1 mutant compared with wild type cells. Serum starvation also increased Fkhrl1-dependent transcriptional activity, which was further augmented in the Shc1-deficient cells. Increased ROS exposure failed to induce increased Fkhrl1 phosphorylation in the mutant cells. Essers et al. [93] reported an evolutionarily conserved interaction of beta-catenin with FOXO transcription factors, which are regulated by insulin and oxidative stress signalling. In mammalian cells, beta-catenin binds directly to FOXO and enhances FOXO transcriptional activity. In C. elegans, loss of the beta-catenin BAR1 reduces the activity of the FOXO ortholog DAF16 in dauer formation and life span. Association of beta-catenin with FOXO was enhanced in cells exposed to oxidative stress. Furthermore, BAR1 was required for the oxidative stress-induced expression of the DAF16 target gene sod3 and for resistance to oxidative damage. They concluded that their results demonstrated a role for beta-catenin in regulating FOXO function that is particularly important under conditions of oxidative stress.

The mRNA of Homo sapiens forkhead box O3 (FOXO3), transcript variant 1, has been deposited in the NCBI database under the accession number NM_001455 VERSION NM_001455.3 (7341 bp PRI 14-JAN-2011) with the mRNA nucleotide sequence as in sequence ID 24. The mRNA of Homo sapiens forkhead box O3 (FOXO3), transcript variant 2, has been deposited in the NCBI database as under the accession number NM_201559 VERSION NM_201559.2 (7314 bp PRI 16-JAN-2011) with the mRNA nucleotide sequence as in sequence ID 25. The protein of Homo sapiens forkhead box O3 (FOXO3), transcript 1, as presented in CDS344..2365 is depicted in sequence ID

26. The protein of Homo sapiens forkhead box O3 (FOXO3), transcript 2, as presented in CDS317..2338 is depicted in sequence 27.

Adiponectin

Adiponectin (ADPN or ACRP30) is a hormone secreted by adipocytes that regulates energy homeostasis and glucose and lipid metabolism. Adipocytes also produce and secrete proteins such as leptin (LEP), adipsin (factor D), various other complement components (e.g., properdin) and C3a), and TNF), suggesting a possible link to the immune system. Adiponectin, an adipose tissue-specific plasma protein, has antiinflammatory effects on the cellular components of the vascular wall [94,95].

By constructing and screening an adipose tissue cDNA library for novel genes, Maeda et al. [96] isolated a cDNA encoding APM1, an adipose tissue-specific collagen-like factor. Sequence analysis predicted that the 244-amino acid secretory protein has a signal peptide but no transmembrane hydrophobic stretch, and a short N-terminal noncollagenous sequence followed by a short collagen-like motif of G-X-Y repeats. APM1 shares significant similarity to collagen X), collagen VIII, and complement protein C1q within the C terminus. Northern blot analysis detected a 4.5-kb APM1 transcript in adipose tissue but not in muscle, intestine, placenta, uterus, ovary, kidney, liver, lung, brain, or heart.

Saito et al. [97] cloned an adipose tissue-specific gene they termed GBP28. They stated that the GBP28 protein is encoded by the APM1 mRNA identified by [96]. By genomic sequence analysis, Saito et al. [97] and Schaffler et al. [98] determined that the GBP28 gene spans 16 kb and contains 3 exons, and that the promoter lacks a TATA box. By Southern blot and genomic sequence analyses, Das et al. [99] determined that the mouse gene, which they termed Acrp30 (adipocyte complement-related protein, 30-kD), contains 3 exons and spans 20 kb. Using FISH, Saito et al. [97] mapped the APM1 gene to chromosome 3q27. However, also by FISH, Schaffler et al. [98] mapped the APM1 gene to 1q21.3-q23. By radiation hybrid analysis, Takahashi et al. [100] confirmed that the APM1 gene maps to 3q27. Using FISH, Das

et al. [99] mapped the mouse Acrp30 gene to chromosome 16 in a region showing homology of synteny with human 3q27. By RNase protection and Western blot analysis, Schaffler et al. [98] showed that APM1 is expressed by differentiated adipocytes as a 33-kD protein that is also detectable in serum. By sequence comparisons, they found links between APM1 and TNF family ligands as well as to cytokines expressed by T cells.Using cell ELISA analysis, Ouchi et al. [101] determined that the APM1 gene product, which they termed adiponectin, suppressed TNF-induced monocyte adhesion to aortic endothelial cells (HAECs), as well as expression of vascular cell adhesion molecule-1 (VCAM1), selectin E (SELE), and intercellular adhesion molecule-1 (ICAM1) on HAECs, in a dose-dependent manner. These results indicated that adiponectin may attenuate the inflammatory response associated with atherogenesis. In addition, Ouchi et al. [95] found that plasma adiponectin values were significantly lower in patients with coronary artery disease compared with those of subjects matched for age and body mass index. By immunoblot analysis, Ouchi et al. [102] extended these studies to show that adiponectin suppresses TNF-induced I-kappa-B-alpha (IKBA) phosphorylation and nuclear factor kappa-B (NFκB) activation without affecting the interaction of TNF and its receptors or other TNF-mediated phosphorylation signals. The inhibitory effect was accompanied by cAMP accumulation, which could be blocked by adenylate cyclase or protein kinase A (PKA) inhibitors. These results, together with a finding by Arita et al. [103] that plasma adiponectin values are low in obese subjects, suggested that adiponectin levels may be helpful in assessing the risk for coronary artery disease.Using hematopoietic colony formation assays, Yokota et al. [104] showed that adiponectin inhibited myelomonocytic progenitor cell proliferation, at least in part due to apoptotic mechanisms, at physiologic concentrations of the protein (approximately 2.0 to 17 micrograms/ml in plasma). Analysis of colony formation from CD34-positive stem cells in the presence of a combination of growth factors showed that CFU-GM (myelomonocytic) but not BFU-E (erythrocytic) colony formation was inhibited by adiponectin and by complement factor C1q. Proliferation of lymphoid cell lines was not inhibited by adiponectin. Northern blot analysis revealed that adiponectin-treated cells had reduced expression of the antiapoptotic BCL2 gene but not of

apoptosis-inducing factors such as BAX. Analysis of macrophage function established that adiponectin suppresses phagocytic activity as well as lipopolysaccharide (LPS)-induced TNF, but not interleukin-1B (IL1B) or interleukin-6 (IL6), production and expression. Blockade of C1QRP, a C1q receptor on macrophages, abrogated the suppression of phagocytic function but not the inhibition of TNF production or myelomonocytic cell proliferation mediated by adiponectin. Yokota et al. {Yokota, 2000 623 /id} suggested that adiponectin is an important regulator of hematopoiesis and inflammatory responses that acts through C1QRP and other receptors. Yamauchi et al. [105] demonstrated that phosphorylation and activation of the 5-prime-AMP-activated protein kinase (AMPK) are stimulated with globular and full-length adiponectin in skeletal muscle and only with full-length adiponectin in the liver. In parallel with its activation of AMPK, adiponectin stimulates phosphorylation of acetyl coenzyme A carboxylase (ACC1), fatty acid oxidation, glucose uptake and lactate production in myocytes, phosphorylation of ACC and reduction of molecules involved in gluconeogenesis in the liver, and reduction of glucose levels in vivo. Blocking AMPK activation by a dominant-negative mutant inhibits each of these effects, indicating that stimulation of glucose utilization and fatty acid oxidation by adiponectin occurs through activation of AMPK. Yamauchi et al. [105] concluded that their data provided a novel paradigm, that an adipocyte-derived antidiabetic hormone, adiponectin, activates AMPK, thereby directly regulating glucose metabolism and insulin sensitivity in vitro and in vivo. Yokota et al. [106] found that brown fat in normal human bone marrow contains adiponectin and used marrow-derived preadipocyte lines and long-term cultures to explore potential roles of adiponectin in hematopoiesis. Recombinant adiponectin blocked fat cell formation in long-term bone marrow cultures and inhibited the differentiation of cloned stromal preadipocytes. Adiponectin also caused elevated expression of COX2 by these stromal cells and induced release of prostaglandin E2. A COX2 inhibitor prevented the inhibitory action of adiponectin on preadipocyte differentiation, suggesting involvement of stromal cell-derived prostanoids. Furthermore, adiponectin failed to block fat cell generation when bone marrow cells were derived from COX2 heterozygous mice. Yokota et al. [106] concluded that preadipocytes represent direct targets for adiponectin action,

establishing a paracrine negative feedback loop for fat regulation. They also linked adiponectin to the COX2-dependent prostaglandins that are critical in this process.

Using SDS-PAGE to analyze human and mouse adiponectin from serum or adipocytes and recombinant adiponectin expressed in mammalian cells, Waki et al. [107,108] detected 3 different molecular mass species and characterized them as low-molecular weight (LMW) trimers (67 kD), middle-molecular weight (MMW) hexamers (136 kD), and high-molecular weight 12- to 18-mers (greater than 300 kD). A disulfide bond through an N-terminal cysteine was required for the formation of multimers larger than a trimer. Noting that Arita et al. [103] had found total adiponectin concentrations to be higher in females than in males, Waki et al. [107,108] analyzed serum samples from healthy young Japanese volunteers and found that HMW multimers, but not MMW or LMW multimers, were significantly less abundant in males than females. Sivan et al. [109] sought to determine if adiponectin is present in human fetal blood, to define its association with fetal birth weight, and to evaluate whether dynamic changes in adiponectin levels occur during the early neonatal period. Cord blood adiponectin levels were extremely high compared with serum levels in children and adults and were positively correlated with fetal birth weights. No significant differences in adiponectin levels were found between female and male neonates. Cord adiponectin levels were significantly higher compared with maternal levels at birth, and no correlation was found between cord and maternal adiponectin levels. Sivan et al. [109] concluded that adiponectin in cord blood is derived from fetal and not from placental or maternal tissues. Kumada et al. [110] incubated human monocyte-derived macrophages with physiologic concentrations of recombinant human adiponectin to determine the effect of adiponectin on matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Adiponectin treatment increased TIMP1mRNA levels in a dose-dependent manner without affecting MMP9 mRNA. Adiponectin also augmented TIMP1 secretion into the media. Adiponectin significantly increased IL10 mRNA expression and protein secretion. Cotreatment of cells with adiponectin and anti-IL10 monoclonal antibodies abolished adiponectin-induced TIMP1 mRNA expression. Kumada et al. [110] concluded that adiponectin acts as an

antiinflammatory signal by selectively increasing TIMP1 expression through IL10 induction. Biochemical, genetic, and animal studies established a critical role for Acrp30/adiponectin in controlling whole-body metabolism, particularly by enhancing insulin sensitivity in muscle and liver, and by increasing fatty acid oxidation in muscle. Wong et al. [111] described a widely expressed and highly conserved family of adiponectin paralogs. They focused particularly on the mouse paralog most similar to adiponectin, CTRP2. At nanomolar concentrations, bacterially produced CTRP2 rapidly induced phosphorylation of AMP-activated protein kinase, acetyl-coA carboxylase, and mitogen-activated protein kinase in cultured myotubes, which resulted in increased glycogen accumulation and fatty acid oxidation. The authors suggested that the discovery of the family of adiponectin paralogs has implications for understanding the control of energy homeostasis and could provide new targets for pharmacologic intervention in metabolic diseases such as diabetes and obesity. To study how the biologic activities of adiponectin are transmitted, Hug et al. [112] performed a series of expression cloning studies to identify cell surface molecules capable of binding adiponectin, using a magnetic-bead panning method that may present higher-valency forms of the adiponectin ligand. Specifically, they transduced a C2C12 myoblast cDNA retroviral expression library into Ba/F3 cells and panned infected cells on recombinant adiponectin linked to magnetic beads. They identified T-cadherin (see 601364) as a receptor for the hexameric and high molecular weight species of adiponectin but not for the trimeric or globular species. Only eukaryotically expressed adiponectin bound to T-cadherin, implying that posttranslational modifications of adiponectin are critical for binding. T-cadherin is expressed in endothelial and smooth muscle cells, where it is positioned to interact with adiponectin. Because T-cadherin is a glycosylphosphatidylinositol-anchored extracellular protein, it may act as a coreceptor for a signaling receptor through which adiponectin transmits metabolic signals. Iwabu et al. [113] provided evidence that adiponectin induces extracellular calcium influx by adiponectin receptor-1 (ADIPOR1), which was necessary for subsequent activation of calcium/calmodulin-dependent protein kinase kinase-beta (CaMKK-beta; CAMKK2), AMPK, and SIRT1, increased expression and decreased acetylation of PGC1-alpha (604517), and

increased mitochondria in myocytes. Moreover, muscle-specific disruption of AdipoR1 suppressed the adiponectin-mediated increase in intracellular calcium concentration, and decreased the activation of CaMkk, AMPK, and SIRT1 by adiponectin. Suppression of AdipoR1 also resulted in decreased PGC1-alpha expression and deacetylation, decreased mitochondrial content and enzymes, decreased oxidative type I myofibers, and decreased oxidative stress-detoxifying enzymes in skeletal muscle, which were associated with insulin resistance and decreased exercise endurance.

Yang et al. [114] studied the changes of plasma adiponectin levels with body weight reduction among 22 obese patients who received gastric partition surgery. A 46% increase of mean plasma adiponectin level was accompanied by a 21% reduction in mean BMI. The authors concluded that body weight reduction increased the plasma levels of a protective adipocytokine, adiponectin. In addition, they inferred that the increase in plasma adiponectin despite the reduction of the only tissue of its own synthesis suggests that the expression of adiponectin is under feedback inhibition in obesity. Lindsay et al. [115] found that 70 Pima Indian patients who later developed type II diabetes had, at baseline, lower concentrations of adiponectin than did controls. Those individuals with high concentrations of the protein were less likely to develop type II diabetes than those with low concentrations. Stefan et al. [116] measured fasting plasma adiponectin and insulin concentrations and body composition in 30 5-year-old and 53 10-year-old Pima Indian children. Cross-sectionally, plasma adiponectin concentrations were negatively correlated with percentage body fat and fasting plasma insulin concentrations at both 5 and 10 years of age. At age 10 years, percentage body fat ($p = 0.03$), but not fasting plasma insulin, was independently associated with fasting plasma adiponectin concentrations. Longitudinally, plasma adiponectin concentrations decreased with increasing adiposity. Longitudinal analyses indicated that hypoadiponectinemia is a consequence of the development of obesity in childhood. Tagami et al [117] studied adiponectin levels in 31 female patients with anorexia nervosa and in 11 with bulimia nervosa. Serum adiponectin concentrations in anorexia nervosa and bulimia nervosa were significantly lower than those in normal-weight controls. These results were unexpected in light of reports that circulating

adiponectin levels are downregulated in obesity [103] and that weight reduction increases plasma adiponectin levels [114] levels were high in constitutionally thin subjects and low in obese subjects, which provided a negative correlation with body mass index (BMI) and body fat mass. In contrast, serum leptin levels correlated very well with BMI and fat mass among all the patients and controls. The concentrations of adiponectin after weight recovery increased to the normal level despite a relatively small increase in BMI. The authors suggested that abnormal feeding behavior in patients with eating disorders may reduce circulating adiponectin levels, and that weight recovery can restore it. Williams et al. [118] determined the extent to which low maternal plasma adiponectin is predictive of gestational diabetes mellitus (GDM), a condition that is biochemically and epidemiologically similar to type II diabetes, using a prospective, nested case-control study design to compare maternal plasma adiponectin concentrations in 41 cases with 70 controls. Adiponectin concentrations were statistically significantly lower in women with GDM than controls (4.4 vs 8.1 microg/ml, P less than 0.001). Approximately 73% of women with GDM, compared with 33% of controls, had adiponectin concentrations less than 6.4 microg/ml. After adjusting for confounding, women with adiponectin concentrations less than 6.4microg/ml experienced a 4.6-fold increased risk of GDM, as compared with those with higher concentrations (95% confidence interval, 1.8-11.6). The authors concluded that their findings were consistent with other reports suggesting an association between hypoadiponectinemia and risk of type II diabetes.Using Spearman univariate analysis, Liu et al. [119] demonstrated that both total and high molecular weight adiponectin levels were inversely associated with body mass index (BMI), fasting glucose, homeostasis model of assessment of insulin resistance, triglycerides, and alanine aminotransferase (ALT), with the high molecular weight isoform also positively correlated with high-density lipoprotein cholesterol ($r = 0.19$; $p = 0.036$). They concluded that high molecular weight adiponectin, but not hexameric or trimeric, tracks with the metabolic correlates of total adiponectin and that an independent inverse association exists between ALT and high molecular weight adiponectin.

The Homo sapiens adiponectin (ADPQ or ACRP30) has to transcript variants. The longer one (variant 1) mRNA has been deposited in the NCBI database as under the accession number NM_001177800 VERSION NM_001177800.1 (4629 bp mRNA linear PRI 01-AUG-2010) with the nucleotide sequence as in sequence ID 28. The second variant differs in the 5' UTR compared to variant 1. Both variants 1 and 2 encode the same protein. It has been deposited in the NCBI database as under the accession NM_004797 VERSION NM_004797.3 (LOCUS: NM_004797 4578 bp mRNA linear PRI 02-AUG-2010) with the nucleotide sequence as in sequence ID 29.

The Homo sapiens adiponectin (ADPQ or ACRP30) protein has been deposited in the NCBI database as under the accession number ABZ10942 ACCESSION VERSION ABZ10942.1 GI:167077467 (LOCUS ABZ10942.1 GI:167077467 244 aa linear PRI 11-FEBR-2008) with the amino acid sequence as in sequence ID 30.

Isolation of monocyte-derived microvesicles from plasma samples

Plasma samples from patients are easy to collect and contain miRNAs [120-123], which have diagnostic potential in metabolic syndrome and cardiovascular disease [124,125]. The main physiological carrier of plasma miRNAs are microvesicles (MVs) which are small vesicles shed from almost all cell types under both normal and pathological conditions [126,127]. Interestingly, MVs bear surface receptors/ligands of the original cells and have the potential to selectively interact with specific target cells. They are involved in cell-to-cell communication including the communication between adipocytes and macrophages and between circulating monocytes and vascular endothelial cells [123]. Due to the presence of specific surface receptors/ligands, peripheral blood MVs can be divided in origin-based subpopulations which can be used to determine miRNA expression profiles in MVs derived from one specific cell type. In detail, peripheral blood MVs derived from mononuclear phagocyte cell lineage can be detected with anti-CD14, anti-CD16, anti-CD206, anti-CCR2, anti-CCR3 and anti-CCR5 antibodies [122]. By labeling the antibodies with a fluorescent group or magnetic particles, these cell-specific MVs can be isolated using FACS or magnetic cell separation technology.

Methods to determine the gene expression / activity

Preparation of Reagents Using Biomarkers

The biomarkers described herein may be used to prepare oligonucleotide probes and antibodies that hybridize to or specifically bind the biomarkers mentioned herein, and homologues and variants thereof.

Probes and Primers

A "probe" or "primer" is a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence (the target). The stability of the resulting hybrid molecule depends upon the extent of the base pairing that occurs, and is affected by parameters such as the degree of complementarity between the probe and target molecule, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as the temperature, salt concentration, and concentration of organic molecules, such as formamide, and is determined by methods that are known to those skilled in the art. Probes or primers specific for the nucleic acid biomarkers described herein, or portions thereof, may vary in length by any integer from at least 8 nucleotides to over 500 nucleotides, including any value in between, depending on the purpose for which, and conditions under which, the probe or primer is used. For example, a probe or primer may be 8, 10, 15, 20, or 25 nucleotides in length, or may be at least 30, 40, 50, or 60 nucleotides in length, or may be over 100, 200, 500, or 1000 nucleotides in length. Probes or primers specific for the nucleic acid biomarkers described herein may have greater than 20-30% sequence identity, or at least 55-75% sequence identity, or at least 75-85% sequence identity, or at least 85-99% sequence identity, or 100% sequence identity to the nucleic acid biomarkers described herein. Probes or primers may be derived from genomic DNA or cDNA, for example, by amplification, or from cloned DNA segments, and may contain either genomic DNA or cDNA sequences representing all or a portion of a single gene from a single individual. A probe may have a unique sequence (e.g., 100% identity to a nucleic acid biomarker) and/or have a known sequence. Probes or

primers may be chemically synthesized. A probe or primer may hybridize to a nucleic acid biomarker under high stringency conditions as described herein.

Diagnostic Use

In a preferred embodiment, the invention involves methods to assess quantitative and qualitative aspects of the biomarker gene expression(s), e.g. miRNAs. of which the increased or decreased expression as provided by the present invention is indicative for the combination of oxidative stress and inflammation and insulin resistance related to the progression of a metabolic syndrome disorder in a subject or the increased risk to develop related cardiovascular diseases in said subject. Techniques well known in the art, e.g., quantitative or semi-quantitative RT PCR for instance real time RT PCR, for instance mRNA analysis by the fluorescence-based real-time reverse transcription polymerase chain reaction (qRT-PCR or RT-qPCR) or reverse transcription loop-mediated amplification (RT-LAMP), for instance one-step RT-LAMP, or real-time NASBA for detection, quantification and differentiation of the RNA and DNA targets [128], or Northern blot, can be used.

In a particular embodiment, the analyzing techniques include the application of detectably-labeled probes or primers. The probes or primers can be detectably-labeled, either radioactively or non-radioactively, by methods that are known to those skilled in the art, and their use in the methods according to the invention, involves nucleic acid hybridization, such as nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction (e.g., RT-PCR), single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, northern hybridization, in situ hybridization, electrophoretic mobility shift assay (EMSA), fluorescent in situ hybridization (FISH), and other methods that are known to those skilled in the art.

By "detectably labeled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labeling a molecule are well known in the art and

include, without limitation, radioactive labeling (e.g., with an isotope such as 32P or 35S) and nonradioactive labeling such as, enzymatic labeling (for example, using horseradish peroxidase or alkaline phosphatase), chemiluminescent labeling, fluorescent labeling (for example, using fluorescein), bioluminescent labeling, or antibody detection of a ligand attached to the probe. Also included in this definition is a molecule that is detectably labeled by an indirect means, for example, a molecule that is bound with a first moiety (such as biotin) that is, in turn, bound to a second moiety that may be observed or assayed (such as fluorescein-labeled streptavidin). Labels also include digoxigenin, luciferases, and aequorin.

Therefore, in a first aspect, the present invention provides an in vitro method to determine activation of a monocyte in a sample, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335 in said sample.

In another embodiment, the present invention provides the in vitro method of the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; in particular wherein the one or more microRNAs are selected from the group consisting of miR-103, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335.

In yet another embodiment, the present invention provides the in vitro method of the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, miR-151-5p, miR-181d, and miR-335.

In a further embodiment, the invention provides the in vitro method of the invention wherein the activation of the monocyte is indicative for the inflammatory state of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group

consisting of let-7c, let-7g, miR-18a, miR-30a, miR-30b, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335 in said sample.

In another embodiment, the invention provides the in vitro method of the invention, wherein the activation of the monocyte is indicative for the inflammatory state of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-30a, miR-30b, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181b, and miR-335 in said sample.

In yet another embodiment, the invention provides the in vitro method of the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR130b, miR-151-5p, miR-181b, and miR-335; in particular, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, miR-151-5p, and miR-335.

In an even further embodiment, the invention provides the in vitro method of the invention, wherein the activation of the monocyte is indicative for the oxidative stress state of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-151-5p, miR-181a, and miR-181b in said sample; in particular wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-151-5p, miR-181a, and miR-181b; more in particular wherein the one or more microRNAs are selected from the group consisting of miR-30a, and miR-151-5p.

In a still further embodiment, the invention provides the in vitro method of the invention, wherein the activation of the monocyte is indicative for insulin signalling deregulation of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group consisting of miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR181b, miR-181d, and miR-335

in said sample; in particular wherein the activation of the monocyte is indicative for insulin signalling deregulation of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group consisting of miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, and miR181b in said sample; more in particular wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, and miR-151-5p.

It is also an object of the invention to provide an in vitro method to predict if a subject will respond to adiponectin or adiponectin mimetic treatment, said method comprising determining the activation of monocytes in a sample by measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-126, miR-130b, miR-146b-5p, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; in particular miR-30a and miR-146b-5p; in said sample, whereby
a) the absence of activated monocytes is an indication that said subject will respond to said adiponectin or adiponectin mimetic treatment, and
b) the presence of activated monocytes is an indication that said subject will not respond to said adiponectin or adiponectin mimetic treatment.

The present invention also provides an in vitro method to predict if a subject will respond to adiponectin or adiponectin mimetic treatment, said method comprising determining the activation of monocytes according to the invention, whereby
a) the absence of activated monocytes is an indication that said subject will respond to said adiponectin or adiponectin mimetic treatment, and
b) the presence of activated monocytes is an indication that said subject will not respond to said adiponectin or adiponectin mimetic treatment.

In a particular embodiment, the present invention provides diagnosis, treatment and/or monitoring methods for a subject that suffers from or is at risk of suffering from at least one disease or disorder selected from the group comprising obesity, metabolic syndrome, type 2

diabetes mellitus, hyperglycemia, low glucose tolerance, insulin resistance, a lipid disorder, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis, and a cardiovascular disease.

It is a further object of the invention to provide an in vitro method of the invention wherein the activation of the monocyte is indicative for a cardiovascular disease, in particular a coronary artery disease, in a person, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of miR-30a, miR-101, miR-130b, and miR-181a in said sample; in particular one or more microRNAs selected from the group consisting of miR-30a, miR-101, and miR-181a.

In another embodiment, the invention provides an in vitro method of the invention wherein the activation of the monocyte is indicative for a cardiovascular disease, in particular a coronary artery disease, in a person, said method comprising measuring the expression level of one or more microRNAs selected from miR-30a and miR-130b.

In another further embodiment, the invention provides an in vitro method of the invention wherein the activation of the monocyte is indicative for the cardiovascular risk of a person, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of miR-101, miR-130b, miR-181a, miR-181b, miR-181d, and miR-335 in said sample; in particular one or more microRNAs selected from miR-130b and miR-181b.

In yet another embodiment, the invention provides an in vitro method of diagnosing the cardiovascular risk of a person, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of miR-130b, miR-181d, and miR-335 in a monocyte obtained from said person.

It is also a further object of the invention to provide an in vitro method of the invention wherein the activation of the monocyte is indicative for metabolic syndrome in a person, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of miR-30a, miR-130b, and miR-181a in said

sample; in particular one or more microRNAs selected from miR-130b and miR-181a.

In a different embodiment, the present invention provides an in vitro method of the invention wherein the activation of the monocyte is indicative for metabolic syndrome in a person, said method comprising measuring the expression level of one or more microRNAs selected from miR-30a and miR-130b.

The in vitro methods of the invention may further comprise analysing the expression level and/or activity of one or more members selected from the group consisting of miR-146b-5p, IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα in said sample; in particular the expression level and/or activity of IRAK3 and optionally one or more members selected from the group consisting of SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα in said sample.

In a particular embodiment, a sample consists of one or more cells, tissues, or parts thereof. For example, in another particular embodiment, said sample is a blood-derived sample; more in particular plasma, serum, or a fraction thereof. The samples used in the present invention can also comprise tissues containing activated monocytes and/or macrophages that originate from activated monocytes; examples of such tissues can include artheroslerotic plaques, cardiac tissues, liver tissues, and pancreatic tissues.

In another particular embodiment, said sample consists essentially of monocytes or monocyte-derived material, in particular monocytes or monocyte-derived microvesicles.

The methods of the present invention to determine activation of at least one monocyte can also be used in a method of monitoring the progression of the treatment of a disease associated with activated monocytes. In particular of at least one disease or disorder selected from the group comprising obesity, metabolic syndrome, type 2 diabetes mellitus, hyperglycemia, low glucose tolerance, insulin resistance, a lipid disorder, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis, and a cardiovascular disease in a person. In

different particular embodiments, the methods of monitoring the progression of treatment comprise determining monocyte activation, the inflammatory state of a monocyte, the oxidative stress state of a monocyte, insuling signalling deregulation of a monocyte, or determining the cardiovascular risk of a subject according to any one of the different embodiments of the present invention.

Methods of treatment

Detection of the biomarkers described herein may enable a medical practitioner to determine the appropriate course of action for a subject (e.g., further testing, drug or dietary therapy, surgery, no action, etc.) based on the diagnosis. Detection of the biomarkers described herein may also help determine the presence or absence of a syndrome or disorder associated with activated monocytes, early diagnosis of such a syndrome or disorder, prognosis of such a syndrome or disorder, or efficacy of a therapy for such a syndrome or disorder. In alternative aspects, the biomarkers and reagents prepared using the biomarkers may be used to identify therapeutics for such a syndrome or disorder. The methods according to the invention allow a medical practitioner to monitor a therapy for a syndrome or disorder associated with activated monocytes in a subject, enabling the medical practitioner to modify the treatment based upon the results of the test.

In said aspect of the present invention, it has for example been found that a syndrome or disorder associated with activated monocytes can be treated by administering to a subject in need thereof an effective amount of a therapeutic or a combination of therapeutics that increase(s) or decrease(s) the expression of miRNAs in the monocytes or macrophages or any white blood cell. Said therapeutic may include an agent that increases the expression of IRAK3.

Syndromes or disorders associated with activated monocytes include (1) non-insulin dependent Type 2 diabetes mellitus (NIDDM), (2) hyperglycemia, (3) low glucose tolerance, (4) insulin resistance, (6) a lipid disorder, (7) dyslipidemia, (8) hyperlipidemia, (9) hypertriglyceridemia, (10) hypercholesterolemia, (11) low HDL levels,

(12) high LDL levels, (13) atherosclerosis, and (14) metabolic syndrome.

The effective amount of a compound, which is required to achieve a therapeutic effect will be, of course, vary with the type of therapeutic component, such as small molecules, peptides, etc ; the route of administration; the age and condition of the recipient; and the particular disorder or disease being treated. In all aspects of the invention, the daily maintenance dose can be given for a period clinically desirable in the patient, for example from 1 day up to several years (e.g. for the mammal's entire remaining life); for example from about (2 or 3 or 5 days, 1 or 2 weeks, or 1 month) upwards and/or for example up to about (5 years, 1 year, 6 months, 1 month, 1 week, or 3 or 5 days). Administration of the daily maintenance dose for about 3 to about 5 days or for about 1 week to about 1 year is typical. Nevertheless, unit doses should preferably be administered from twice daily to once every two weeks until a therapeutic effect is observed.

Adiponectin is an adipocytokine, which is mainly produced by the adipose tissue. Although it is the most abundantly produced protein of the fat cell, plasma levels are reduced in obese patients. There is growing evidence that reduced adiponectin concentrations indicate an increased cardiovascular risk because hypoadiponectinemia is associated with the components of the metabolic syndrome, in particular with insulin resistance, elevated triglycerides, and low HDL Apart from this, adiponectin possesses anti-inflammatory properties and exerts direct antiatherosclerotic and cardioprotective effects [129,130]. Therefore, it was suggested that low adiponectin concentrations are a cardiovascular risk factor and that therapeutic strategies that enhance the secretion or action or mimetic the action of this adipocytokine might reduce the incidence of cardiovascular diseases (CVDs). However, several recently published studies on the prospective association between adiponectin and CVD events/mortality showed inconsistent results. Five studies reported that adiponectin was not independently associated with future CVD [131-135]. Low adiponectin concentrations turned out as a risk factor for future CVD in some studies [136-142], whereas others showed that high adiponectin levels were associated with an increased risk of CVD and/or mortality [143-149]. The underlying mechanisms for these contradictory results are still

unclear but may be due to differences in the study populations. Toward this, it was speculated that low adiponectin predicts cardiovascular events in low-risk populations for CVD, whereas in high-risk populations, a counter-regulatory increase of adiponectin occurs that is responsible for the elevated cardiovascular risk associated with high adiponectin levels. In this application, we present a method to identify persons that will be vulnerable to adiponectin or adiponectin mimetics and ways to overcome this vulnerability.

Therefore, in a second aspect, the invention provides an agent that modulates one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; in particular from the group consisting of miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181b, miR-181d, and miR-335; for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

In a particular embodiment, the present invention provides agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; in particular wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, miR-151-5p, miR-181d, and miR-335.

In another particular embodiment, the present invention provides agents of the invention, wherein the one or more microRNAs are selected from the group consisting of miR-103, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335.

In a further embodiment, the present invention provides agents of the invention, wherein the one or more microRNAs are selected from the group consisting of let-7c, let-7g, miR-18a, miR-30a, miR-30b, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d and miR-335; in particular let-7c, let-7g, miR-18a, miR-30a, miR-30b, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-

181b, and miR-335; and wherein the activated monocyte is characterized by an increased inflammatory state.

In another further embodiment, the invention provides agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-103, miR-130b, miR-151-5p, miR-181d, and miR-335; and wherein the activated monocyte is characterized by an increased inflammatory state.

In another further embodiment, the invention provides agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR130b, miR-151-5p, miR-181b, and miR-335; in particular miR-30a, miR-130b, miR-151-5p, and miR-335; and wherein the activated monocyte is characterized by an increased inflammatory state.

In another embodiment, the present invention provides agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of let-7c, let-7g, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-151-5p, miR-181a, and miR-181b; in particular from miR-30a, miR-101, miR-103, miR-151-5p, miR-181a, and miR-181b; more in particular from miR-30a, and miR-151-5p; and wherein the activated monocyte is characterized by an increased oxidative stress state.

In yet another embodiment, the present invention provides agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of miR-126, miR-130b, miR-151-5p, and miR-181b and wherein the activated monocyte is characterized by an increased oxidative stress state.

In a particular embodiment, the present invention provides agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; in particular from miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, and miR181b; more in particular from miR-30a,

miR-130b, and miR-151-5p; and wherein the activated monocyte is characterized by insulin signalling deregulation.

In another particular embodiment, the present invention provides agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of miR-103, miR-130b, miR-151-5p, miR-181d, and miR-335 and wherein the activated monocyte is characterized by insulin signalling deregulation.

In a particular embodiment, the agents according to the different embodiments of the present invention can be used for the treatment of activated monocytes or the prevention of monocyte activation in a subject that suffers from at least one disease or disorder selected from the group comprising obesity, metabolic syndrome, type 2 diabetes mellitus, hyperglycemia, low glucose tolerance, insulin resistance, a lipid disorder, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis, and a cardiovascular disease.

It is furthermore an aim of the invention to provide agents according to the invention, wherein the one or more microRNAs are selected from the group consisting of miR-101, miR-130b, miR-181a, miR-181b, miR-181d, and miR-335; in particular one or more microRNAs selected from miR-130b and miR-181b; and wherein the treatment of said activated monocyte or the prevention of activation of said monocyte in said subject leads to a decreased cardiovascular risk of said subject.

In a different embodiment, the one or more microRNAs are selected from the group consisting of miR-130b, miR-181d, and miR-335; and the treatment of said activated monocyte or the prevention of activation of said monocyte in said subject leads to a decreased cardiovascular risk of said subject.

In yet another embodiment, the present invention provides agents of the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR130b, and miR-181a; in

particular one or more microRNAs selected from the group consisting of miR-30a, miR-101, and miR-181a;
and wherein the treatment of said activated monocyte or the prevention of activation of said monocyte treats and/or prevents a cardiovascular disease, in particular a coronary artery disease, in said person.

In a particular embodiment, the present invention provides agents of the invention, wherein the one or more microRNAs are selected from miR-30a and miR-130b; and wherein the treatment of said activated monocyte or the prevention of activation of said monocyte treats and/or prevents a cardiovascular disease, in particular a coronary artery disease, in said person.

In another particular embodiment, the present invention pvoides agents of the invention, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, and miR-181a in said sample; in particular one or more microRNAs selected from miR-130b and miR-181a, or from miR-30a and miR-130b; and wherein the treatment of said activated monocyte or the prevention of activation of said monocyte treats and/or prevents metabolic syndrome in said person.

In a further embodiment, the agent of the invention that modulates one or more microRNAs is a microRNA mimic, such as for example an antagomir.

As also described elsewhere in the present application, the agents of the present invention can also be used in combination with other agents that are known to reduce, prevent or treat monocyte activation or that are known to reduce, prevent or treat diseases associated with monocyte activation. It is therefore also an object of the present invention to provide a combination comprising

- an agent according to the invention, and
- adiponectin or an adiponectin mimetic,

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

In a different embodiment, the inventions provides a combination comprising

- an agent according to the invention, and
- a modulator of one or more members selected from the group consisting of IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

In particular, such a combination comprises

- an agent according to the invention, and
- a modulator of IRAK3, and
- optionally a modulator of one or more members selected from the group consisting of SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

In a futher embodiment, the present inventions provides a combination comprising

- an agent according to the invention and/or an agent that modulates miR-146b-5p, and
- adiponectin or an adiponectin mimetic,

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

In another embodiment, the present invention provides a combination comprising

- an agent according to the invention and/or an agent that modulates miR-146b-5p, and
- a modulator of one or more members selected from the group consisting of IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject; in particular a combination comprising

- an agent according to the invention and/or an agent that modulates miR-146b-5p, and
- a modulator of IRAK3, and

- optionally a modulator of one or more members selected from the group consisting of SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

In a particular embodiment, the invention provides a combination according to the invention further comprising adiponectin or an adiponectin mimetic, for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

Furthermore, the present invention provides the use of an agent according to the different embodiments of the invention to treat and/or prevent one or more elements selected from the group comprising monocyte activation, the oxidative stress state of a monocyte, the inflammatory state of a monocyte, and insulin signaling deregulation in a monocyte.

In addition, it is the object of the present invention to provide a method of treating and/or prevention a disease or disorder associated with activated monocytes in a subject. It is therefore another aspect of the invention to provide a method of treating and/or preventing a disease or disorder associated with activated monocytes in a subject in need thereof, said method comprising modulating one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335 in a monocyte cell in said subject. In particular, the method wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335, in particular from the group consisting of miR-103, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; more in particular from miR-30a, miR-130b, miR-151-5p, miR-181d, and miR-335.

In another embodiment, the activated monocytes in the method according to the invention have an increased inflammatory state and

the one or more microRNAs in the method of treating and/or preventing are selected from the group consisting of let-7c, let-7g, miR-18a, miR-30a, miR-30b, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; in particular from let-7c, let-7g, miR-18a, miR-30a, miR-30b, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181b, and miR-335; more in particular from miR-30a, miR-103, miR-130b, miR-151-5p, miR-181d, and miR-335.

In yet another embodiment, the present invention provides a method of treating and/or preventing as hereinbefore, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR130b, miR-151-5p, miR-181b, and miR-335; in particular wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, miR-151-5p, and miR-335.

The invention further provides a method of treating and/or prevention as hereinbefore, wherein the activated monocytes have an increased oxidative stress state and wherein the one or more microRNAs are selected from the group consisting of let-7c, let-7g, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-151-5p, miR-181a, and miR-181b; in particular from miR-126, miR-130b, miR-151-5p, and miR-181b. In a different embodiment, the present invention provides said method, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-151-5p, miR-181a, and miR-181b; in particular from miR-30a, and miR-151-5p.

In a further embodiment, the present invention provides a method of treating and/or preventing as described hereinbefore, wherein the activated monocytes have deregulated insulin signalling and wherein the one or more microRNAs selected from the group consisting of miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335; in particular from miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, and miR181b. In a different embodiment, the present invention provides said method, wherein the one or more microRNAs are selected from the group consisting of miR-

103, miR-130b, miR-151-5p, miR-181d, and miR-335; in particular from miR-30a, miR-130b, and miR-151-5p.

In another particular embodiment, the disease or disorder associated with activated monocytes that have deregulated insulin signalling is selected from the group comprising obesity, metabolic syndrome, type 2 diabetes mellitus, hyperglycemia, low glucose tolerance, insulin resistance, a lipid disorder, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis, and a cardiovascular disease.

In a particular embodiment, the method of the invention is characterized in that the disease or disorder associated with activated monocytes is a cardiovascular disease, in particular a coronary artery disease, and that the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR130b, and miR-181a; in particular one or more microRNAs selected from the group consisting of miR-30a, miR-101, and miR-181a; more in particular miR-30a and/or miR-130b.

In another particular embodiment, the method of the invention is characterized in that the disease or disorder associated with activated monocytes is metabolic syndrome, and that the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, and miR-181a; in particular one or more microRNAs selected from miR-130b and miR-181a; more in particular miR-30a and/or miR-130b.

In a particular embodiment, the downregulation of a microRNA of the invention is indicative for an activated monocyte. In another particular embodiment, the downregulation of a microRNA of the invention is indicative for a monocyte with an increased inflammatory state. In yet another particular embodiment, the downregulation of a microRNA is indicative for a monocyte with an increased oxidative stress state, with the exception for miR-151-5p, for which an upregulation is indicative for a monocyte with an increased oxidative stress state. In another particular embodiment, the downregulation of a microRNA of the

invention is indicative for a monocyte with deregulated insulin signalling.

It is furthermore an object of the present invention to provide methods, agents, us of agents, and compositions according to the different embodiments of the invention wherein one or more of the following restrictions apply:

- let-7c can not be selected
- let-7g can not be selected
- miR-18a can not be selected
- miR-27b can not be selected
- miR-30a can not be selected
- miR-30b can not be selected
- miR-30d can not be selected
- miR-101 can not be selected
- miR-103 can not be selected
- miR-107 can not be selected
- miR-126 can not be selected
- miR-130b can not be selected
- miR-151-5p can not be selected
- miR-181a can not be selected
- miR-181b can not be selected
- miR-181d can not be selected
- miR-335 can not be selected

Compositions

It is also an object of the present invention to provide a composition comprising the above mentioned components. In particular, suitable for use in treating and/or preventing activation of a monocyte and accordingly useful in a metabolic syndrome or a disease associated with the activation of said monocytes, condition or disorder selected from the group consisting of (1) non-insulin dependent Type 2 diabetes mellitus (NIDDM), (2) hyperglycemia, (3) low glucose tolerance, (4) insulin resistance, (6) a lipid disorder, (7) dyslipidemia, (8) hyperlipidemia, (9) hypertriglyceridemia, (10)

hypercholesterolemia, (11) low HDL levels, (12) high LDL levels, (13) atherosclerosis, in a subject in need thereof.

Therefore, it is an object of the present invention to provide a pharmaceutical composition comprising an agent or a combination according to the invention, for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.
In addition, the present invention provides the use of an agent according to any one of the different embodiments of the invention in the preparation of a pharmaceutical composition.

The compositions of the present invention, for use in the methods of the present invention, can be prepared in any known or otherwise effective dosage or product form suitable for use in providing topical or systemic delivery of the therapeutic compounds, which would include both pharmaceutical dosage forms as well as nutritional product forms suitable for use in the methods described herein.

The above mentioned components may be administrated to induce an increase or a decrease of microRNAs in myeloid cells in particular in blood monocytes. Such administration can be in any form by any effective route, including, for example, oral, parenteral, enteral, intraperitoneal, topical, transdermal (e.g., using any standard patch), ophthalmic, nasally, local, non-oral, such as aerosal, spray, inhalation, subcutaneous, intravenous, intramuscular, buccal, sublingual, rectal, vaginal, intra-arterial, and intrathecal, etc. Oral administration is prefered. Such dosage forms can be prepared by conventional methods well known in the art, and would include both pharmaceutical dosage forms as well as nutritional products.

Pharmaceutical compositions

The pharmaceutical compositions of the present invention can be prepared by any known or otherwise effective method for formulating or manufacturing the selected product form. For example, the above mentioned components can be formulated along with common excipients, diluents, or carriers, and formed into oral tablets, capsules,

sprays, mouth washes, lozenges, treated substrates (e. g. oral or topical swabs, pads, or disposable, non-digestible substrate treated with the compositions of the present invention); oral liquids (e. g. suspensions, solutions, emulsions), powders, or any other suitable dosage form.

Non-limiting examples of suitable excipients, diluents, and carriers include: fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose and other cellulose derivatives, alginates, gelatin, and polyvinyl pyrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as acetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; carriers such as propylene glycol and ethyl alcohol, and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

Antagomirs are one of a novel class of chemically engineered oligonucleotides. Antagomirs are used to silence endogenous miRNA. An antagomir is a small synthetic RNA that is perfectly complementary to the specific miRNA target with either mispairing at the cleavage site of Ago2 or some sort of base modification to inhibit Ago2 cleavage. Usually, antagomirs have some sort of modification, such as 2' methoxi groups and phosphothioates, to make it more resistant to degradation. It is unclear how antagomirization (the process by which an antagomir inhibits miRNA activity) operates, but it is believed to inhibit by irreversibly binding the miRNA. Antagomirs are now used as a method to constitutively inhibit the activity of specific miRNAs [150,151]. Understanding the miRNA signature in susceptible individuals may facilitate the partitioning of patients into distinct subpopulations for targeted therapy with antagomirs [152].

In addition to the antimiRs, there is also the opportunity to **mimic or reexpress miRNAs** by using synthetic RNA duplexes designed to mimic the endogenous functions of the miRNA of interest, with modifications for stability and cellular uptake. The "guide strand" is

identical to the miRNA of interest, whereas the "passenger strand" is modified and typically linked to a molecule such as cholesterol for enhanced cellular uptake. Another way to increase the level of a miRNA is by the use of adeno-associated viruses (AAVs). Delivered in viral vectors, the miRNA of interest can be continually expressed, resulting in robust replacement expression of miRNAs downregulated during disease. Additionally, the availability of a number of different AAV serotypes allows for the potential of tissue-specific expression because of the natural tropism toward different organs of each individual AAV serotype, as well as the different cellular receptors with which each AAV serotype interacts. The use of tissue-specific promoters for expression allows for further specificity in addition to the AAV serotype. Furthermore, AAV is currently in use in a number of clinical trials for gene therapy, of which the safety profiles have looked quite positive. In line with this, Kota et al recently showed AAV-mediated delivery of miR-26a blunts tumor genesis in a mouse model of liver cancer [153]. Although systemic viral delivery of miRNAs to the heart during disease has not been performed yet, there have been a number of studies using AAV9 to successfully deliver RNA interference to cardiac tissue and effectively restore cardiac function during disease in rodents [154].

To deliver **miRNA mimics *in vivo*** effectively and into specific cells and organs, one can explore encapsulation technologies. In this approach, inhibitors/mimics are sequestered in various kinds of liposomes/nanoparticles to further protect them from degradation and to direct them to the appropriate tissues [155,156]. Another technique involves conjugating a cationic protein (carrier) with a monoclonal antibody targeting a specific cell surface receptor. The antibody only binds to the cells expressing the surface antigen that it recognizes. Oftentimes, the antigen is a receptor. Prior to administration to the animal, the "conjugate" is loaded with miRNA inhibitors/mimics. The carrier binds the miRNA through electrostatic forces. The inhibitor/mimic is negatively charged and the carrier is positively charged. Upon binding to the cell receptor, the conjugate complex is internalized and the inhibitor/mimic released into the cytoplasm to silence or mimic the desired miRNA [157,158].

Cell surface markers have been used for targeting cargo to mouse monocytes. Sialoadhesin (CD169 or Siglec-1) is an endocytic receptor expressed on monocytes and macrophages [159]. Sialoadhesin-specific immunoconjugates have been used for targeting toxin and antigens to these specific cell types [160]. Dectin-1, a major receptor that recognized b-glucans, is highly expressed on monocytes and macrophages. Complexes of antisense ODN with b-1,3-glucans, have been shown to be incorporated into macrophages via dectin-1 [161]. Recently a rabies virus glycoprotein (RVG) peptide was used for delivery of siRNA payload to macrophages and microglial cells in the brain. Here the 29-mer RVG peptide that binds specifically to acetylcholine receptor expressed on myeloid cells are linked to a positively-charged nona-D-arginine (9R) residues for binding of siRNA for efficient delivery in vitro and in vivo [162]. Dendrimers are branched, synthetic polymers with layered architectures that have recently shown considerable promise in several therapeutic applications. In a recent report by Hayder M *et al.* [163], a phosphorus-containing dendrimer with an N3P3 (cyclotriphosphazene) core and phenoxymethyl-methylhydrazone (PMMH) branches, and capped with anionic azabisphosphonate (ABP)-end groups was chemically synthesized and shown to be able to selectively target monocytes. Rapid internalization of monocytes with the dendrimer is followed by anti-inflammatory activation in monocytes as has been shown in mouse model of arthritis (also see review [164]).

The **zebrafish** has proven to be a powerful vertebrate model in genetics and developmental biology. Nowadays it has been emerging as a model for human disease and therapy. The relatively low cost, availability of transgenic lines, external and rapid development of the embryo and transparency during development makes the zebrafish a popular and attractive model. The zebrafish is an excellent model for the functional validation of miRNAs *in vivo* [165]. It can also be used as screening model to identify therapeutic agents that influence the miRNA expression during inflammatory diseases. The myeloid lineage- which shows close homology structurally, biochemically and functionally to their mammalian counterparts- appears already after 12-16 hours post fertilization (hpf) and by 24 hpf a functional cardiovascular system has been formed [166,167]. Until now 415 zebrafish miRNAs have been identified demonstrating the conservation of this

important mechanism in vertebrates [168]. Gain- and loss of function experiments can be performed to unravel the miRNA biology in relation to inflammation, oxidative stress and insulin resistance. Fertilized eggs of the zebrafish are easily manipulated by microinjections of small oligonucleotide fragments inhibiting or mimicking a specific miRNA [169,170]. The monocyte/macrophage population can be tracked in the transparent embryos by using the *Tg(fms:GAL4.VP16)i186* transgenic line in which the monocytes/macrophages are fluorescently labeled [171]. Crossing this line with the *Fli1-eGFP* line makes it possible to evaluate the interaction of the monocytes/macrophages with the endothelial layer of the blood vessels. The behavior, number and interaction of the monocytes/macrophages with blood vessels and surrounding tissue can be monitored by simple *in vivo* time lapse microscopy [165,171-174].

Besides the zebrafish screening model, **mice** can be applied for long term *in vivo* research. Several mouse models of obesity, metabolic syndrome and atherosclerosis are available in which the therapeutic potential of miRNAs can be evaluated [175-182]. RNA-analogs have been developed to achieve silencing or mimicking of endogenous miRNAs and can be systemically administered in mice to study the miRNAs of interest [158,183,184].

Drawing and Table Description

BRIEF DESCRIPTION OF THE DRAWINGS AND TABLES

The present invention will become more fully understood from the detailed description given herein below and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein:

Figure 1: Monocytes/macrophages in obesity and obesity-associated metabolic disorders.

Figure 2: An overview of miRNAs deregulated in monocytes of obese subjects and predicted to be involved in regulating key molecules in the IRAK3-related pathway associated with increased inflammation and oxidative stress and impaired insulin signaling and sensitivity.

Figure 3: Expression profiles of 31 candidate miRNAs in circulating monocytes of obese and lean subjects.

Figure 4: miRNAs differentially expressed in monocytes of obese persons after short-term weight loss.

Figure 5: miRNAs differentially expressed in inflammation associated cell experiments.

Figure 6: miRNAs differentially expressed in oxidative stress associated cell experiments.

Figure 7: miRNAs differentially expressed in insulin resistance associated cell experiments.

Figure 8: miRNA decision tree

Figure 9: Effect of miRNA inhibitors on inflammation, oxidative stress and insulin resistance *in vitro*

Figure 10: Effect of miRNA mimics on inflammation, oxidative stress and insulin resistance *in vitro*.

Figure 11: miR-146b-5p is an essential mediator of the anti-inflammatory, antioxidative stress and insulin-sensitizing actions of globular adiponectin.

Table 1: Characteristics and gene expressions before and after weight loss in obese patients

Table 2: List of differentially expressed miRNAs in obese patients ($P < 0.05$)

Table 3: List of differentially expressed microRNAs-of-interest in obese patients with their theoretical targets as determined by *in silico* target prediction ($P < 0.05$)

Table 4: Characteristics of patients in the second cohort

Table 5: AUC of ROC curves regarding diagnostic power to distinguish CAD patients from healthy controls

Table 6: Association of miRNA expressions in monocytes with occurrence of angiographically documented CAD

Table 7: Primers used in qRT-PCR

Examples

Study design, materials and methods

Materials

All chemicals were obtained from Sigma-Aldrich unless stated otherwise. Human THP-1 monocytic cells (TIB-202) were obtained from ATCC.

Study cohort

This study complies with the Declaration of Helsinki, and the locally appointed Ethics Committee approved the study protocol. All human participants gave written informed consent and underwent coronary angiography for (suspected) stable or unstable coronary artery disease (CAD). For practical purposes, the presence of significant CAD was defined in this study as at least one stenosis of 50% in a major coronary artery (left main, LAD, Cx or RCA), while absence of CAD was defined as having no detectable lesions on angiography, including luminal irregularities. Patients with a history of cardiovascular disease, including coronary, peripheral artery or cerebrovascular disease, where excluded from the CAD negative group.

The first cohort comprised 14 lean control (27% male; BMI < 30 kg/m^2) and 21 obese individuals (33% male; BMI > 30 kg/m^2). These 21 morbidly obese subjects were referred to our hospital for bariatric surgery. Before they were included, patients were evaluated by an endocrinologist, an abdominal surgeon, a psychologist and a dietician. Only after multidisciplinary deliberation the selected patients received a laparoscopic Roux-en-Y gastric bypass. A 30 ml fully divided gastric pouch is created and the jejunum, 30 cm distal of the ligament of Treitz, is anastomosed to it with a circular stapler of 25 mm. To restore intestinal transit, a fully stapled entero-entero anastomose is constructed 120 cm distal on the alimentary limb. In this way the food passage is derived away from almost the whole stomach, the duodenum and the proximal jejunum [185-187]. All participants in the first cohort were without symptoms of clinical cardiovascular disease. The samples were collected between March 29th, 2005 and May 30th, 2006.

The second cohort comprised 126 subjects (83% male, BMI = 28±1 kg/m^2, mean±SEM) of which 39% had the metabolic syndrome and 65% had a positive angiogram. This population was used for ROC and

regression analysis. The samples of the second cohort were collected between June 8th, 2010 and January 31st, 2011.

Monocyte isolation

Blood samples were collected, and PBMCs were prepared from the anti-coagulated blood using gradient separation on Histopaque-1077 after removal of the plasma fraction. Cells were washed three times in Ca^{2+}- and Mg^{2+}-free Dulbecco's (D)-PBS. PBMCs were incubated for 15 min at 4°C with CD14 microbeads at 20 µl/1 × 10^7 cells. The cells were washed once, re-suspended in 500 µl Ca^{2+}- and Mg^{2+}-free DPBS containing 0.5% BSA/1 × 10^8 cells. The suspension was then applied to an LS column in a MidiMACS Separator (Miltenyi) [188,189]. We selected $CD14^+$ monocytes because CD14 intensity expression on circulating monocytes was found to be associated with increased inflammation in patients with diabetes [190].

Blood analysis

Blood samples were centrifuged to prepare plasma samples for analysis. Total and HDL-cholesterol and triglyceride levels were determined with enzymatic methods (Boehringer Mannheim). LDL-cholesterol levels were calculated with the Friedewald formula. Plasma glucose was measured with the glucose oxidase method (on Vitros 750XRC, Johnson & Johnson), and insulin with an immunoassay (Biosource Technologies). Ox-LDL [191] and IL-6 were measured with ELISA (Mercodia and R&D Systems). Hs-CRP (Beckman Coulter) was measured on an Immage 800 Immunochemistry System. Blood pressure was taken three times with the participant in a seated position after 5 minutes quiet rest. The average of the last two measurements was used for systolic and diastolic blood pressure.

Microvesicle isolation, total RNA isolation and quantitative RT-PCR analysis

Microvesicles (MV) were isolated from cell culture medium by differential centrifugation according to previous publications [120,121]. Briefly, after removing cells and other debris by centrifugation at 300*g*, and 16,500*g*, the supernatant was centrifuged at 100,000*g* for 70 min (all steps were performed at 4°C). MVs were collected from the pellet and resuspended in RNase-free water. The presence of MVs after

ultracentrifugation was determined with flow cytometry. To confirm that microvesicles were the correct size, flow cytometry gates were set using 1 micron beads (Invitrogen).

Total RNA was extracted with the TRIzol reagent (Invitrogen) and purified on a miRNeasy Mini Kit column (Qiagen). The RNA quality was assessed with the RNA 6000 Nano assay kit using the Agilent 2100 Bioanalyzer.

For mRNA expression analysis, first-strand cDNA was generated from total RNA by reverse transcription using the VILO cDNA synthesis kit (Invitrogen). Quantitative (q)RT-PCR was performed on a 7500 Fast Real-Time PCR system using the Fast SYBR Green Master mix (Applied Biosystems) according to the manufacturer's instructions. Table 7 summarizes forward and reverse primers used in qRT-PCR analysis. RNA expression levels were expressed as the ratio compared to controls as previously described [60,192]. To make sure that primer sequences, used in qRT-PCR, target the right gene, their specificity was validated by Basic Local Alignment Search Tool (BLAST) [193]. Furthermore, cDNA clones (OriGene) for IRAK3 (and TNFAIP3 and -6, and SOD2) were used to double check the primer specificity. In addition, PCR fragments were validated for GC/AT ratio, length, and amplification specificity with dissociation curve analysis and agarose gel electrophoresis [194].

For miRNA expression analysis, first-strand cDNA was generated from total RNA by reverse transcription using the Universal cDNA synthesis kit (Exiqon). Quantitative (q)RT-PCR was performed on a 7500 Fast Real-Time PCR system using the Universal SYBR Green master mix and fully validated and optimized LNA PCR primer sets according to the manufacturer's instructions (Exiqon). We used *RNU5G* as housekeeping gene for normalization of the miRNA content in monocytes. However, no housekeeping miRNA has been established and validated to normalize for the miRNA content in plasma and MV samples. Therefore, we supplemented the plasma samples (after addition of TRIzol) with 10 fmol *Caenorhabditis elegans* miR-39 (cel-miR-39) as described previously [195]. The expression levels of miRNAs in MVs were directly normalized to the total protein content of MVs.

MiRNA Array Profiling and Target Prediction

The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. 600 ng total RNA from sample and reference was labelled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY™ LNA Array power labelling kit (Exiqon, Denmark) following the procedure described by the manufacturer. The Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY™ LNA *Array version 5th Generation* (Exiqon), which contains capture probes targeting all miRNAs for human, mouse or rat registered in the miRBASE version 15.0 at the Sanger Institute. The hybridization was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridization station (Tecan). After hybridization the microarray slides were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY™ LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery). The quantified signals were background corrected (Normexp with offset value)[196] and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. miRNAs, differentially expressed in monocytes of obese women compared to lean controls at a *P*-value < 0.05, were considered for further analysis. To gain insight into the functional significance of differential miRNA expression in monocytes of obese women, a bioinformatic analysis was performed, which determined predicted miRNAs for each of the members of the gene cluster using the DIANA-microT, miRanda, PicTar and TargetScan target prediction algorithms [197] and compared them with the list of differentially expressed miRNAs.

Cell culture

THP-1 monocytic cells were subcultured in RPMI 1640 as described previously in detail [60,192]. For globular adiponectin incubation experiments, cells were cultured at a density of 1 x 10^6 cells/ml in RPMI 1640 supplemented with 10% FBS and 5 µg/ml gentamicin. After 24 h, 1 or 10 µg/ml globular adiponectin (PeproTech) was added and the cells were incubated for 6 to 24 h. Globular adiponectin is a recombinant protein derived from human globular domain adiponectin

cDNA expressed in *Escherichia coli*. This protein was endotoxin free (<2 EU/µg) according to the manufacturer. The ox-LDL incubation experiments were performed like previously described [60]. For insulin/glucose incubation experiments, cells were cultured at a density of 1 x 10^6 cells/ml in glucose-free RPMI 1640 supplemented with 10% FBS, 5 µg/ml Gentamicin, and 5.5 mmol/l D-glucose in a 5% CO_2 incubator at 37°C. After 24 h, 10^{-7} mol/l insulin and 9.5 mmol/l D-glucose or 9.5 mmol/l D-mannitol (osmotic control) was added and incubated for 24 h under normal growth conditions. For IL-6 experiments, THP-1 cells were stimulated with 10 ng/ml recombinant IL-6 (PeproTech) for 24 h. Cell viability, as determined by trypan blue exclusion, was > 80%. mROS and iROS formation were measured with MitoSOX and CellROX (Invitrogen). Cells were incubated with PBS containing 5 µM MitoSOX or 2.5 µM CellROX for 10 or 30 minutes at 37°C and 5% CO_2. The labeled cells were washed twice with PBS and then suspended in warm PBS for analysis by flow cytometry (Becton, Dickinson and Company).

RNA interference

To deplete *IRAK3* mRNA, THP-1 cells were transiently transfected with chemical synthesized HP GenomeWide siRNAs (Qiagen; target sequence: 5′-CACATTCGAATCGGTATATTA-3′ (Hs_IRAK3_5) and 5′-CTGGATGTTCGTCATATTGAA-3′ (Hs_IRAK3_6)). To inhibit miR-30a, -103, -126, -130b, -146b-5p, -151-5p, 181b, -181d or -335 THP-1 cells were transiently transfected with mIRCURY LNA miRNA Power Inhibitors (Exiqon):
miR-30a: 5′-TTCCAGTCGAGGATGTTTAC-3′,
miR-103: 5′-CATAGCCCTGTACAATGCTGC-3′,
miR-126: 5′-GCATTATTACTCACGGTACG-3′,
miR-130b: 5′-TGCCCTTTCATCATTGCACT-3′,
miR-146b-5p: 5′-GCCTATGGAATTCAGTTCTC-3′,
miR-151-5p: 5′-CTAGACTGTGAGCTCCTCG-3′,
miR-181b: 5′-ACCCACCGACAGCAATGAATGT-3′,
miR-181d: 5′-CCACCGACAACAATGAATGT-3′,
miR-335: 5′-CATTTTTCGTTATTGCTCTTG-3′.
To overexpress miR-30a, -103, -130b, -146b-5p, -151-5p, 181b, -181d or -335 THP-1 cells were transiently transfected with synthetic miScript miRNA mimics (Qiagen):

miR-30a: 5′-UGUAAACAUCCUCGACUGGAAG-3′,
miR-103: 5′-AGCAGCAUUGUACAGGGCUAUGA-3′,
miR-130b: 5′-CAGUGCAAUGAUGAAAGGGCAU-3′,
miR-146b-5p: 5′-UGAGAACUGAAUUCCAUAGGCU-3′,
miR-151-5p: 5′-UCGAGGAGCUCACAGUCUAGU-3′,
miR-181b: 5′-AACAUUCAUUGCUGUCGGUGGGU-3′,
miR-181d: 5′-AACAUUCAUUGUUGUCGGUGGGU-3′,
miR-335: 5′-UUUUUCAUUAUUGCUCCUGACC-3′.

As a negative control, we used AllStars Negative Control siRNA (Qiagen) or miRCURY LNA miRNA Power Inhibitor Control (Exiqon; 5′-GTGTAACACGTCTATACGCCCA-3′); as a positive control, we used Mm/Hs_MAPK1 control siRNA (Qiagen; target sequence: 5′-AATGCTGACTCCAAAGCTCTG-3′). Cells were transfected with 50 nM of siRNA using HiPerfect reagent (Qiagen) according to the manufacturer's instructions with modifications as follows. THP-1 cells were seeded at density 1.5 x 10^5/well (24 well-plate) in 100 µl of culture medium. Next, complexes (3 pmol of siRNA/miRNA plus 6 µl of HiPerfect reagent) were formed in 0.1 ml of serum-free RPMI-1640 for 10 min at room temperature and then added to each well. After 6 hours of incubation under normal growth conditions, 400 µl of growth medium was added to each well and the cells were incubated for 42 hours. Gene silencing was monitored at the mRNA level by means of qRT-PCR.

Statistical analysis

Patient groups were compared with an unpaired t-test with Welch's correction; *in vitro* data were compared with the Mann–Whitney *U* test. Spearman rank correlation was determined with GraphPad Prism 5. Receiver operating characteristic curve (ROC) analysis was performed with MedCalc statistical software for biomedical research. Odds ratios were determined by Chi-square test with Yates' correction (GraphPad Prism 5). Regression analysis was performed with the Statistical Package for the Social Sciences (SPSS for Windows; release 16). A *P*-value of less than 0.05 was considered statistically significant.

Results

miRNAs differentially expressed in circulating monocytes of obese patients and involved in regulating the IRAK3-related gene cluster

Our target cells for the identification of novel biomarkers and/or therapeutic targets are monocytes since they are readily accessible (blood) and constitute a reservoir of inflammatory cells that infiltrate in tissues (adipose, aortic and cardiac tissues) during obesity where they actively induce oxidative stress, inflammation, and cell death and thereby induce insulin resistance, atherosclerosis, and heart failure [198] (Figure 1).

The first patient cohort used in this study comprised 14 lean controls (29% male; age: 33±3 years, mean±SEM) and 21 morbidly obese individuals (33% male; age: 39±3 years), without clinical symptoms of cardiovascular disease. Obese subjects in the first cohort had higher IL-6, hs-CRP, leptin and glucose levels, and lower adiponectin levels, indicating the presence of systemic inflammation. The higher levels of circulating ox-LDL indicated systemic oxidative stress. Furthermore, insulin and triglyceride concentrations were higher; HDL-cholesterol was lower. Obese individuals had higher systolic and diastolic blood pressure. Insulin resistance, calculated by a homeostasis model assessment (HOMA-IR) index, was 86% higher in obese subjects (Table 1A). A cluster of risk factors for cardiovascular disease and T2DM including raised blood pressure, dyslipidemia (elevated triglycerides and/or decreased HDL-cholesterol), raised fasting glucose, and central obesity have become known as the metabolic syndrome. A person qualifies for the metabolic syndrome with three abnormal findings out of five [199]. Four controls used in this study had 1 metabolic syndrome component; 1 had 2. Two obese patients had 1, 7 had 2, 5 had 3, and 7 had 4 metabolic syndrome components. Thus, 57% of the obese individuals had the metabolic syndrome. Finally, we also collected blood of the obese subjects three months after bariatric surgery. The blood characteristics after short-term weight loss are depicted in Table 1A. In aggregate, there was less systemic inflammation but no reduction in circulating ox-LDL. Triglycerides, HOMA-IR and adiponectin concentrations were restored to levels of lean persons (Table 1A). The numbers of leukocytes ($3.70 \pm 1.68 \times 10^6$ vs. $3.56 \pm 0.80 \times 10^6$ per ml blood) and $CD14^+$ monocytes (3.24±1.32

x 10^5 vs. 3.30±1.56 x 10^5 per ml blood) were similar in lean and obese subjects.

Our lab has previously identified a theoretical model (also referred to as the IRAK3-related pathway) in circulating monocytes of obese patients that links IRAK-mediated inflammation with oxidative stress and impaired insulin signaling and sensitivity. In detail, this model contains TLR2 as cell surface marker, NFκB and FOXO3A as transcription factors, TNFα as inflammatory output, SOD1, SOD2 and CAT as oxidative stress markers, IRAK3 and TNFAIP3 and -6 as putative inhibitors of the TLR2/NFκB inflammatory pathway and IRS1 and IRS2 as markers of insulin signaling (Figure 2 and Table 1B). *IRAK3*, predominantly expressed in monocytes/macrophages [81], was the only inhibitor of which the expression was decreased in obese patients compared to lean controls and was associated with increased inflammation, evidenced by increased expression of *TLR2* and *TNFα* (Table 1B). Low *IRAK3* and high *SOD2* was associated with a high prevalence of metabolic syndrome (odds ratio: 9.3; sensitivity: 91%; specificity: 77%). By comparison, the odds ratio of hs-CRP, a widely used marker of systemic inflammation, was 4.0 (sensitivity: 69%; specificity: 65%). Weight loss was associated with an increase in *IRAK3* and a decrease in *SOD2*, in association with a lowering of systemic inflammation and a decreasing number of metabolic syndrome components (Table 1). We identified the increase in reactive oxygen species in combination with obesity-associated low adiponectin and high glucose and IL-6 as cause of the decrease in IRAK3 in human monocytic THP-1 cells *in vitro*.

In this study, we identified miRNAs differentially expressed in circulating monocytes of obese patients and involved in modulating the IRAK3-related pathway. miRNA microarrays (Exiqon, miRBASE version 15.0) were performed on total *RNA* from isolated monocytes of 6 lean and 10 obese individuals. A total of 133 miRNAs were differentially expressed between obese and lean controls (Table 2). To gain insight into this miRNA expression profile, a bioinformatic analysis was performed, which determined predicted miRNAs for each of the members of the IRAK3-related gene cluster using the DIANA-microT, miRanda, PicTar and TargetScan target prediction algorithms [197] and compared them with the list of differentially expressed miRNAs (Table 3). The *in silico* analysis identified 31 miRNAs with potential targets in the presented gene cluster. Figure 2 illustrates the interactions

between the selected miRNAs and their potential targets in the IRAK3-related pathway associated with increased inflammation and oxidative stress and impaired insulin signaling and sensitivity.

Next, we validated the expressions of the 31 candidate miRNAs in 14 lean and 21 obese subjects. The expression levels of the miRNAs were assessed by qRT-PCR, and normalized by expression levels of *RNU5G*, identified as most stable reference gene (GeNorm [200]). The fold changes of miRNA levels for the obese individuals *vs.* lean controls are shown in Figure 3. Of the 31 miRNAs that were significantly deregulated in the microarray analysis, 18 miRNAs were validated with qRT-PCR in the extended population. For mature miRNA sequences, see sequence IDs 31-74.

Effect of short-term weight loss (in vivo intervention) that is associated with an increase in insulin sensitivity and a reduction of inflammation

The expression profile of the 18 selected miRNAs was also determined three months after bariatric surgery. The expressions of miR-103, miR-151-5p, miR-181a, miR-181b, miR-181d and miR-335, all decreased in obese subjects, were significantly increased after short-term weight loss (Figure 4). Interestingly, the expression profile of miR-103, miR-151-5p, miR-181a, miR-181b and miR-335 correlated negatively with BMI (r_s = -0.34, r_s = -0.44, r_s = -0.34, r_s = -0.34 and r_s = -0.44 respectively, all $P < 0.05$) and systemic markers of inflammation (hs-CRP, leptin and glucose). The expressions of miR-151-5p, miR-181a, miR-181b and miR-335 were also associated with the number of metabolic syndrome components (r_s = -0.42, r_s = -0.34, r_s = -0.31 and R_s = -0.36 respectively, all $P < 0.01$). In these obese persons (before and after weight loss), miR-181a, miR-181b and miR-335 correlated positively with the *IRAK3* expression (r_s = 0.40, r_s = 0.41 and r_s = 0.50 respectively, all $P < 0.01$); and miR-126, miR-151-5p and miR-335 correlated negatively with the *SOD2* expression (r_s = -0.35, r_s = -0.47 and r_s = -0.34 respectively, all $P < 0.05$).

miRNAs associated with inflammation (in vitro) and related oxidative stress

In order to establish the role of miRNAs in inflammation we followed three approaches: 1) measuring their expressions in response to silencing of IRAK3, an inhibitor of the TLR/TNFα-mediated inflammation; 2) measuring their expressions in the antioxidative and

anti-inflammatory response of cells exposed to the inflammatory cytokine IL-6; and 3) establishing their role in the maintenance of the anti-inflammatory (and antioxidative) response to adiponectin.

First, we depleted the expression of *IRAK3* in THP-1 cells by means of siRNAs. Knockdown of *IRAK3* (-71%, n = 10, $P < 0.001$) resulted in an increased inflammatory state of the cells as can be seen from an increased expression of *TNFα* (+38%, $P < 0.001$), and an increased oxidative stress state, evidenced by a higher production of mitochondrial ROS (mROS, +33%) and intracellular ROS (iROS, +62%, both $P < 0.05$). To prove that these effects were not caused by off-target effects, THP-1 cells were transfected with a second siRNA targeting *IRAK3* at a different location. The gene expressions were not different when we compared the effect of the two siRNAs (data not shown). *IRAK3*-depletion decreased the expressions of let-7c, let-7g, miR-18a, miR-30b, miR-101, miR-130b, miR-146b-5p, miR-181b and miR-335 (Figure 5A).

Next, we incubated THP-1 cells with high levels of IL-6 characteristic for obese patients (Table 1A). Exposure to IL-6 decreased the expression of *TNFα* (-29%%, $P < 0.01$). There was no effect on m- and iROS production. These protective effects of THP-1 cells were associated with an increased expression of miR-103, miR-126, miR-130b, miR-146b-5p and miR-151-5p indicating that these miRNAs can protect against inflammation and oxidative stress caused by IL-6 (Figure 5B).

Furthermore, we investigated the effect of high (as in lean controls and obese persons after weight loss) and low (as in obese persons) levels of globular adiponectin (gAcrp30) because this domain of the adiponectin protein appears to be responsible for the anti-inflammatory effects of adiponectin [108,201]. Exposure of THP-1 cells to low levels of adiponectin resulted in a decreased expression of IRAK3 (*RNA* and protein) compared to cells exposed to high levels of adiponectin. This decrease was associated with more *TNFα* (+75%, $P < 0.001$) and mROS production (+20%, $P < 0.01$), and less miR-30a and miR-146b-5p (Figure 5C).

miRNAs associated with response to oxidative stress (in vitro) and related inflammation

We measured the protective effects of THP-1 cells exposed to ox-LDL, evidenced by a decrease in *TNFa* expression (-88%; P<0.001); this resulted in a 13% (P<0.01) reduction of ROS. The increased expressions of let-7c, let-7g, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-146b-5p, miR-181a and miR-181b indicate that these miRNAs can protect against oxidative stress and inflammation induced by ox-LDL. In contrast, the expression of miR-151-5p was decreased in THP-1 cells exposed to ox-LDL (Figure 6).

miRNAs associated with insulin resistance (in vitro)

Insulin resistance in obese patients is characterized by high levels of insulin and glucose and thus an increased HOMA-IR index (Table 1A). Incubation of THP-1 cells with 10^{-7} M insulin and 15 mM glucose resulted in a decreased expression of *IRAK3* (-13%, $P < 0.01$), *IRS1* (-29%, $P < 0.05$) and *IRS2* (-29%, $P < 0.01$), and more *TNFa* (+18%, $P < 0.05$) and iROS (+5%, $P < 0.01$). Induction of insulin resistance in those cells, associated with increased inflammation and oxidative stress was associated with less miR-30a, miR-103, miR-126, miR-130b, miR-146b-5p, miR-151-5p, miR-181a and miR-181b (Figure 7).

Association of miRNA expressions in monocytes with occurrence of metabolic syndrome and angiographically documented CAD

Next, we determined the association of the miRNA expressions with the occurrence of the metabolic syndrome and CAD in the second independent cohort.
The characteristics of the 126 subjects are summarized in Table 4. In aggregate, 25% of the patients were obese and 65% were diagnosed with CAD. Moreover, 16 patients had 0; 24 had 1; 37 had 2; 28 had 3; 17 had 4; and 4 patients had 5 metabolic syndrome components. Thus, 39% of these individuals had the metabolic syndrome. MiR-130b and miR-181a predicted a higher number of metabolic syndrome components even after adjusting for smoking, insulin, adiponectin and IL-6. The R^2-value of the model with miR-130b was 0.277 ($P < 0.001$); that with miR-181a was 0.262 ($P < 0.001$). MiR-30a also correlated with a higher number of metabolic syndrome components, but its association was lost after adjusting for smoking, insulin, adiponectin and IL-6.
We used binary logistic regression analysis to determine the association of miRNAs with occurring cardiovascular risk equivalents, being a Framingham cardiovascular risk score above 10% per 10 years [202] or type 2 diabetes. MiR-101, miR-130b, miR-181a, miR-181b, miR-

181d, and miR-335 were all significantly ($P < 0.05$) associated with cardiovascular risk equivalents. Their respective *B*-values were -1.92±0.89, mean±SEM), -1.30±0.52, -1.49±0.75, -1.66±0.77, -1.28±0.56, and -1.90±0.84. When adjusting for age, gender, smoking and BMI, miR-130b and miR-181b remained significant predictors of cardiovascular risk equivalents. When adjusting for age, gender, smoking, BMI, HDL, triglycerides, glucose, insulin, and systolic blood pressure, miR-130b remained a significant predictor of occurring cardiovascular risk equivalents. As indicated above BMI (obesity), HDL and triglycerides (dyslipidemia), glucose (diabetes) and blood pressure (hypertension) are measures of the metabolic syndrome [199].
ROC analysis revealed that miR-30a, miR-101, miR-130b and miR-181a were associated with CAD (Table 5). Table 6 shows the odds ratios for CAD in relation to miRNA expressions in monocytes determined by Chi-square test with Yates' correction. Odd ratios of low miR-30a, low miR-101, low miR-130b and low miR-181a varied between 2 and 12. Binary logistic regression analysis showed that miR-30a, miR-101 and miR-181a, but not miR-130b were associated with CAD after adjusting for age gender, smoking and even BMI, suggesting that these miRNAs are associated with CAD even in the absence of obesity. Their respective *B*- and *P*-values were -3.42±1.57 ($P < 0.05$), -2.32±1.16 ($P < 0.05$), and -3.04±1.47 ($P < 0.05$). All 3 miRNAs remained associated with CAD even after adjustment for age, gender, smoking, BMI, HDL, triglycerides, glucose, insulin, and systolic blood pressure.

Overview of the followed selection procedure to identify miRNAs-of-interest

Figure 8 depicts an overview of the followed selection procedure to identify miRNAs-of-interest in activated monocytes. It summarizes the involvement of the selected miRNAs in processes related to inflammation, oxidative stress and insulin resistance, in said monocytes. Furthermore, this figure also illustrates miRNAs associated with the occurrence of the metabolic syndrome, cardiovascular risk equivalents and angiographically documented CAD. The selected miRNAs are depicted in bold.

miRNAs detectable in plasma samples and monocyte-derived microvesicles

Because circulating plasma miRNAs secreted by all kind of cell types (including monocytes) [120-123] may have diagnostic potential in

metabolic syndrome and cardiovascular disease [124,125], we measured the selected miRNAs (Figure 8) in plasma samples of obese patients and patients with angiographically documented CAD. All miRNAs, except miR-30a and miR-181d, were easily detectable in plasma samples of obese patients and patients with CAD: miR-30a and miR-181d had low expression levels.
The main physiological carrier of plasma miRNAs are microvesicles (MVs) which are derived from circulating blood cells and other tissues directly affected by disease [126]. To determine the presence of the selected miRNAs in monocyte-derived MVs, we isolated MVs from THP-1 culture medium by ultracentrifugation and measured the miRNA expressions by qRT-PCR. All miRNAs, discussed in Figure 8, were detectable in MVs secreted by THP-1 cells.

Association between miRNA expressions and expressions of IRAK3-related targets (illustrated in Figure 2)

MiR-30a (R = 0.20; $P < 0.05$), miR-151 (R = 0.22; $P < 0.01$), miR-181b (R = 0.27; $P < 0.001$), miR-181d (R = 0.17; $P < 0.05$) and miR-335 (R = 0.26; $P < 0.01$) correlated with the expression of *IRAK3* in monocytes of 126 lean and obese patients, characterized in Table 4. Mir-30a (R = 0.22; $P < 0.05$), miR-101 (R = 0.27; $P < 0.01$), miR-103 (R = 0.26; $P < 0.01$), miR-126 (R = 0.22; $P < 0.01$), miR-151-5p (R = 0.24; $P < 0.01$), miR-181b (R = 0.25; $P < 0.01$), miR-181d (R = 0.26; $P < 0.01$), and miR-335 (R = 0.25; $P < 0.05$) correlated with the expression of *TNFAIP3*. MiR-30a (R = 0.30; $P < 0.001$), miR-101 (R = 0.35; $P < 0.001$), miR-103 (R = 0.44; $P < 0.001$), miR-126 (R = 0.29; $P < 0.001$), miR-130b (R = 0.30; $P < 0.001$), miR-151-5p (R = 0.32; $P < 0.001$), miR-181a (R = 0.30; $P < 0.001$), miR-181b R = 0.39; $P < 0.001$), miR-181d (R = 0.36; $P < 0.001$), and miR-335 (R = 0.37; $P < 0.001$) correlated with the expression of *SOD1*. MiR-130b correlated with the expression of *SOD2*. In multiple regression analysis (using a model containing all miRNAs), miR-181d was the best predictor of *SOD1*, miR-103 was the best predictor of *TNFAIP3*, miR-181d was the best predictor of *SOD1*, and miR-335 was the best predictor of *SOD2*.

Effect of miRNA inhibition on monocyte activation as evidenced by inflammation, oxidative stress and insulin signaling in vitro

To confirm the functional role of selected miRNAs in regulating inflammation (evidenced by *TNF*α upregulation), oxidative stress (evidenced by mROS production) and insulin resistance (evidenced by *IRS1* downregulation) in monocytes, we transiently transfected THP-1

cells with a LNA-modified miRNA inhibitors targeting miR-103, miR-130b, miR-146b-5p, miR-151-5p, miR-181d and miR-335.
Sequestration of endogenous miR-130b (possible target: *TNFα*) and miR-151-5p (possible target: NFκB1) levels in THP-1 cells resulted in more *TNFα* and mROS production and less *IRS1* suggesting the involvement of these miRNAs in the protection against inflammation and oxidative stress (ROS production) and loss of insulin signaling (Figure 9A-B). Furthermore, depletion of miR-103 (possible target: *TNFα*), miR-146b-5p (targets: IRAK1 and TRAF6 [203]) and miR-335 (possible target: NFκBIa) increased the *TNFα* expression and decreased the *IRS1* expression suggesting the involvement of these miRNAs in the regulation of inflammation and subsequently the insulin signaling (Figure 9C-E). MiR-181d depletion (possible targets: *TNFAIP6* and *IRS2*) also resulted in more *TNFα* and less *IRS1*. However, miR-181d in contrast to miR-103, miR-146b-5p and miR-335 can target the inflammatory as well as the insulin signaling pathway directly (Figure 9F). Sequestration of miR-126 (possible target: *SOD2*) and miR-181b (possible targets: *TNFAIP6* and *IRS2*) levels in THP-1 cells resulted in more mROS production without increasing the *TNFα* expression or reducing the insulin sensitivity (Figure 9G-H).
In conclusion, several inhibitors of miRNAs of the invention increased inflammation, oxidative stress, and/or insulin resistance, which again underscores the importance of these miRNAs in monocyte activation.

Effect of miRNA mimics on inflammation, oxidative stress and insulin signaling in vitro

To determine wether mimics of the miRNAs of the invention are able to reduce monocyte activation parameters, we transiently transfected THP-1 cells with synthetic miRNA mimics and determined the effect of miRNA overexpression in monocytes.
Overexpression of miR-30a, miR-181d and miR-335 decreased inflammation in THP-1 cells: miR-30a by increasing *IRAK3*, miR-181d by decreasing *TNFα* and miR-335 by both increasing *IRAK3* and decreasing *TNFα* (Figure 10A-B). Furthermore, transfection of miR-130b increased the *IRS1* expression and miR-151-5p overexpression decreased the iROS production (Figure 10C-D).
In conclusion, several mimics of miRNAs of the invention indeed reduced one or more of the important monocyte activation parameters.

miR-146b-5p is required for the protective actions of adiponectin

The causal relation between decreased IRAK3 and miR-146b-5p and miR-30a levels in THP-1 cells (Figure 5C) suggest that these miRNAs are a mediator of the protective actions of adiponectin. To determine whether miR-146b-5p is an essential mediator of adiponectin-related anti-inflammatory and antioxidative stress properties, THP-1 cells were transfected with miR-146b-5p inhibitor and exposed to high levels of gAcrp30. The sequestration of miR-146b-5p and subsequent exposure to high gAcrp30 resulted in an increased expression of *TNFa*, more iROS production and decreased *IRS1* expression compared to cells exposed to high levels of gAcrp30 (Figure 11). Interestingly, there was no significantly decrease in *IRAK3* expression detectable suggesting that miR-146b-5p is an essential mediator of the anti-inflammatory, antioxidative stress and insulin-sensitizing actions of gAcrp30.

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Tables

Table 1: Characteristics and gene expressions before and after weight loss in obese patients.

	Lean controls (n = 14)	Obese patients (n = 21)	
		Before weight loss	After weight loss
A. Characteristics			
Age (years)	33±3	39±3	39±3
BMI (kg/m^2)	21±1	44±1***	36±1*** / $^{\$\$\$}$
Leptin (ng/ml)	8.7±1.4	65.6±8.0***	21.0±3.5** / $^{\$\$\$}$
Adiponectin (µg/ml)	10.9±1.8	3.9±0.6**	7.0±1.0$^{\$\$\$}$
Glucose (mg/dl)	83±2	111±7***	89±4$^{\$\$\$}$
Insulin (mU/l)	10.3±1.8	16.5±2.1*	6.3±0.8$^{\$\$\$}$
HOMA-IR	2.1±0.4	3.9±0.5**	1.8±0.2$^{\$\$\$}$
Triglycerides (mg/dl)	80±7	132±11***	99±8$^{\$\$}$
LDL-C (mg/dl)	110±9	85±6*	92±3
HDL-C (mg/dl)	64±4	49±3**	47±2**
SBP (mmHg)	120±3	136±3**	118±1$^{\$\$\$}$
DBP (mmHg)	75±3	86±2**	62±1*** / $^{\$\$\$}$
IL-6 (pg/ml)	1.8±0.2	4.8±0.4***	3.4±0.4*** / $^{\$}$
Hs-CRP (mg/l)	0.49±0.10	5.65±1.12***	3.45±0.82** / $^{\$}$
Ox-LDL (IU/l)	50±5	71±4**	69±4**
B. Gene expressions			
CAT	1.03±0.03	0.90±0.03**	0.99±0.04
FOXO3A	1.06±0.10	1.65±0.18**	1.00±0.06$^{\$\$}$
INSR	0.99±0.04	0.65±0.02***	0.75±0.04*** / $^{\$}$
IRAK1	0.99±0.04	1.21±0.04***	1.07±0.04$^{\$\$}$
IRAK3	0.98±0.04	0.49±0.03***	0.79±0.05** / $^{\$\$\$}$
IRAK4	1.03±0.07	0.86±0.03*	0.90±0.04
IRS2	1.05±0.16	1.01±0.06	0.96±0.08
MyD88	0.98±0.04	1.24±0.03***	1.12±0.03* / $^{\$\$}$
NFκB1	1.01±0.05	0.92±0.03	1.12±0.07$^{\$\$}$
NFκBIa	1.23±0.20	3.34±0.53**	2.67±0.47** / $^{\$}$
SOD1	1.01±0.04	0.81±0.02***	0.89±0.04* / $^{\$}$
SOD2	1.00±0.05	2.65±0.28***	1.91±0.19*** / $^{\$}$
TLR2	0.99±0.08	1.54±0.06***	1.02±0.09$^{\$\$\$}$
TNFAIP3	1.05±0.13	1.54±0.11**	1.03±0.11$^{\$\$}$
TNFAIP6	1.05±0.16	4.43±0.69***	2.32±0.34** / $^{\$\$\$}$
TNFα	1.05±0.09	2.18±0.31**	1.06±0.12$^{\$\$}$
TRAF6	0.98±0.32	2.26±0.25***	2.18±0.24***

Data shown are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ obese compared with lean controls; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ compared with before weight loss. Abbreviations: BMI, body mass index; C, cholesterol; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; hs-CRP, high sensitivity C-reactive protein; ox-LDL, oxidized LDL; SBP, systolic blood pressure.

Table 2: List of differentially expressed microRNAs in obese patients (*P* < 0.05)

	Obese (n = 10) vs. Lean (n = 6)	
miRNA	**p-value**	**Fold change**
hsa-miRPlus-F1026	0,0001	1,2
hsa-miR-1255a	0,0002	1,2
hsa-let-7c	0,0002	0,8
hsa-miRPlus-E1077	0,0003	1,4
hsa-miR-513a-5p	0,0003	1,8
hsa-miR-1275	0,0003	2,3
hsa-miR-361-5p	0,0004	0,8
hsa-miR-652	0,0005	0,8
hsa-miRPlus-E1151	0,0005	1,3
hsa-miR-30e*	0,0007	0,9
hsa-miR-1201	0,0007	0,9
hsa-miR-494	0,0008	2,3
hsa-miRPlus-E1097	0,0009	1,2
hsa-miR-18b	0,0009	0,8
hsa-miR-126	0,0010	0,8
hsa-miR-151-5p	0,0011	0,8
hsa-miRPlus-F1037	0,0012	0,9
hsa-miRPlus-E1101	0,0012	1,4
hsa-miR-3202	0,0014	1,3
hsa-miR-374a	0,0015	0,7
hsa-miR-765	0,0017	1,4
hsa-miR-374b	0,0017	0,8
hsa-miR-2116	0,0017	1,2
hsa-miR-30a	0,0017	0,8
hsa-miR-583	0,0018	1,3
hsa-miR-30b	0,0019	0,9
hsa-miR-602	0,0019	1,3
hsa-miRPlus-F1066	0,0019	1,2
hsa-miR-300	0,0019	1,9
hsa-miR-148b	0,0019	0,8
hsa-miRPlus-E1238	0,0019	1,3
hsa-miRPlus-E1209	0,0020	1,9
hsa-miR-186	0,0022	0,8
hsa-miR-101	0,0023	0,7
hsa-miR-30d	0,0027	0,9
hsa-miR-532-5p	0,0028	0,9
hsa-miR-32*	0,0029	1,3
hsa-miR-625*	0,0031	0,8

hsa-miR-199b-5p	0,0033	0,9
hsa-miR-195	0,0033	0,9
hsa-miR-146b-5p	0,0036	0,8
hsa-miR-32	0,0038	0,7
hsa-miR-184	0,0038	1,2
hsa-miR-1827	0,0041	1,2
hsa-let-7g	0,0044	0,9
hsa-miRPlus-E1200	0,0045	1,1
hsa-miR-17*	0,0047	0,9
hsa-miR-335*	0,0047	1,2
hsa-miRPlus-E1110	0,0049	1,4
hsa-miR-331-3p	0,0049	0,9
hsa-miRPlus-A1015	0,0050	0,9
hsa-miR-642	0,0052	1,3
hsa-miR-29c	0,0055	0,8
hsa-miR-320a	0,0055	0,8
hsa-miRPlus-A1072	0,0055	1,3
hsa-miR-146b-3p	0,0056	1,2
hsa-miR-27b	0,0056	0,9
hsa-miRPlus-E1232	0,0058	1,3
hsa-miR-140-5p	0,0061	0,9
hsa-miRPlus-F1225	0,0062	1,2
hsa-miR-18a	0,0062	0,8
hsa-miR-181a	0,0063	0,8
hsa-miRPlus-E1212	0,0064	1,2
hsa-miR-181b	0,0065	0,9
hsa-miR-107	0,0069	0,8
hsa-miRPlus-E1285	0,0071	1,1
hsa-miR-214	0,0071	1,2
hsa-miR-17	0,0072	0,9
hsa-miRPlus-F1147	0,0078	0,9
hsa-miR-340	0,0081	0,8
hsa-miR-106a	0,0081	0,9
hsa-miR-1299	0,0081	1,1
hsa-miR-27a	0,0083	0,9
hsa-miR-938	0,0091	1,2
hsa-miR-744	0,0093	0,9
hsa-miR-525-5p	0,0095	1,2
hsa-miR-103	0,0096	0,9
hsa-miR-19a	0,0096	0,8
hsa-miRPlus-F1086	0,0099	1,1

hsa-miR-196a*	0,0101	0,9
hsa-miR-574-5p	0,0103	1,1
hsa-miRPlus-F1074	0,0103	0,9
hsa-miR-660	0,0107	0,9
hsa-miR-920	0,0110	1,2
hsa-miR-500*	0,0111	0,9
hsa-miR-665	0,0111	1,3
hsa-miR-181d	0,0111	0,9
hsa-miR-505	0,0113	0,9
hsa-miRPlus-F1155	0,0123	1,1
hsa-miR-19b	0,0126	0,8
hsa-miR-485-3p	0,0126	1,2
hsa-miR-551b	0,0132	1,4
hsa-miR-142-5p	0,0134	0,8
hsa-miR-335	0,0138	0,8
hsa-miRPlus-F1166	0,0145	1,2
hsa-miR-92b	0,0146	0,9
hsa-miRPlus-E1098	0,0149	1,2
hsa-miR-34b	0,0152	0,9
hsa-miRPlus-E1072	0,0156	1,1
hsa-miR-221	0,0162	0,9
hsa-miR-425*	0,0164	0,9
hsa-let-7i	0,0165	0,9
hsa-miR-30e	0,0168	0,9
hsa-miRPlus-E1146	0,0168	1,2
hsa-miR-362-3p	0,0170	0,8
hsa-miR-620	0,0182	1,1
hsa-miR-1297	0,0194	0,9
hsa-let-7e	0,0198	1,2
hsa-miR-197	0,0211	0,9
hsa-miR-423-3p	0,0214	0,9
hsa-miR-340*	0,0216	1,3
hsa-miR-146a	0,0219	0,8
hsa-miR-1304	0,0222	1,1
hsa-miRPlus-E1175	0,0223	1,1
hsa-miRPlus-E1093	0,0226	1,1
hsa-miRPlus-E1090	0,0231	1,2
hsa-miR-342-3p	0,0237	0,8
hsa-miRPlus-F1064	0,0244	1,1
hsa-miR-502-3p	0,0252	0,9
hsa-miR-339-5p	0,0252	0,8

hsa-let-7f	0,0285	0,9
hsa-miR-2113	0,0294	1,4
hsa-miR-484	0,0329	0,9
hsa-miR-1285	0,0338	1,1
hsa-miR-143	0,0355	1,5
hsa-miRPlus-F1180	0,0357	0,9
hsa-miR-130a	0,0370	0,8
hsa-miR-223*	0,0396	0,9
hsa-miR-130b	0,0414	0,9
hsa-miR-320d	0,0421	0,8
hsa-let-7a	0,0440	0,9
hsa-miR-25	0,0469	0,9
hsa-miR-574-3p	0,0486	1,1

Table 3: List of differentially expressed microRNAs-of-interest in obese patients with their theoretical targets as determined by *in silico* target prediction ($P < 0.05$)

	Obese (n = 10) vs. Lean (n = 6)		
miRNA	**p-value**	**Fold change**	**Targets**
hsa-miR-25	0,0469	0,9	ABCA1, PAFAH1B1, IRS2
hsa-miR-30a	0,0017	0,8	CAT, FOXO3A, ZNF217, KLF9, IRS1, IRS2
hsa-miR-101	0,0023	0,7	TLR2, ZNF217, PAFAH1B1
hsa-miR-30d	0,0027	0,9	CAT, FOXO3A, INSR, IRS1, IRS2
hsa-miR-19a	0,0096	0,8	TLR2, TNFAIP3, TNFAIP6, TNF, ZNF217
hsa-miR-30b	0,0019	0,9	CAT, FOXO3A, IRS1, IRS2
hsa-miR-19b	0,0126	0,8	TLR2, TNFAIP3, TNFAIP6, ZNF217
hsa-miR-30e-5p	0,0168	0,9	CAT, FOXO3A, IRS1, IRS2
hsa-miR-126	0,0010	0,8	NFκBIa, SOD2, FOXO3A, IRS1
hsa-miR-151-5p	0,0011	0,8	TLR2, TLR4, NFκB1
hsa-let-7g	0,0044	0,9	TNFAIP3, MyD88, IRS1
hsa-miR-29c	0,0055	0,8	PAFAH1B1, NFκB1, IL13
hsa-miR-27b	0,0056	0,9	IRAK4, INSR, IRS1
hsa-miR-106b	0,0081	0,9	IRAK1, IKKγ, ZNF217
hsa-miR-142-3p	0,0134	0,8	TLR2, IRAK1, ZNF217
hsa-miR-425-3p	0,0164	0,9	NFκB1, CAT, IRS1
hsa-let-7f	0,0285	0,9	TNFAIP3, MyD88, IRS1
hsa-miR-130a	0,0370	0,8	TNFAIP6, TNF, ZNF217
hsa-miR-130b	0,0414	0,9	TNFAIP6, TNF, ZNF217, ABCA1, ESR1, IRF1, PAFAH1B1
hsa-let-7c	0,0002	0,8	TNFAIP3, IRS1
hsa-miR-30e-3p	0,0007	0,9	NFκB1, SOD2
hsa-miR-374a	0,0015	0,7	TNFAIP6, ZNF217
hsa-miR-374b	0,0017	0,8	TNFAIP6, ZNF217
hsa-miR-146b-5p	0,0036	0,8	IRAK1[$], TRAF6[$]
hsa-miR-18a	0,0062	0,8	TNFAIP3, IRS2
hsa-miR-181a	0,0063	0,8	TNFAIP6, IRS2
hsa-miR-181a*	0,0063	0,8	TLR4, IKKγ
hsa-miR-181b	0,0065	0,9	TNFAIP6, IRS2
hsa-miR-107	0,0069	0,8	IKKγ, TNF
hsa-miR-17	0,0072	0,9	IRAK1, ZNF217
hsa-miR-27a	0,0083	0,9	INSR, IRS1
hsa-miR-103	0,0096	0,9	IKKγ, TNF
hsa-miR-181d	0,0111	0,9	TNFAIP6, IRS2
hsa-let-7i	0,0165	0,9	TNFAIP3, IRS1
hsa-miR-620	0,0182	1,1	TLR2, TNFAIP6
hsa-let-7e	0,0198	1,2	TNFAIP3, IRS1

hsa-miR-146a	0,0219	0,8	IRAK1[$], TRAF6[$]
hsa-miR-143	0,0355	1,5	TLR2, IRS1
hsa-let-7a	0,0440	0,9	TNFAIP3, IRS1
hsa-let-7d	0,078	0,9	TNFAIP3, MyD88
hsa-miR-18b	0,0009	0,8	TNFAIP3
hsa-miR-765	0,0017	1,4	IKKγ
hsa-miR-602	0,0019	1,3	CAT
hsa-miR-532-5p	0,0028	0,9	NFκB1, IRS1
hsa-miR-195	0,0033	0,9	SOCS, PAFAH1B1
hsa-miR-32	0,0038	0,7	IRS2
hsa-miR-340	0,0081	0,8	IRS1
hsa-miR-27a	0,0083	0,9	IRAK4
hsa-miR-525-5p	0,0095	1,2	IRAK3
hsa-miR-660	0,0107	0,9	IRAK4
hsa-miR-551b	0,0132	1,4	IRAK4
hsa-miR-142-5p	0,0134	0,8	TNFAIP6
hsa-miR-335	0,0138	0,8	FOXO3A, NFκBIa
hsa-miR-92b	0,0146	0,9	IKKγ
hsa-miR-34b	0,0152	0,9	IKKγ
hsa-miR-197	0,0211	0,9	FOXO3A
hsa-miR-342-3p	0,0237	0,8	IRAK3
hsa-miR-502-3p	0,0252	0,9	IRAK1
hsa-miR-339-5p	0,0252	0,8	IRAK1

[$]targets are functionally validated [203]

Table 4: Characteristics of patients in the second cohort.

	Patients (n = 126)
Age (years)	51±1
Gender (% male)	66
Smoking (%)	25
Obesity (%)	25
T2DM (%)	10
MetS (%)	39
CAD (%)	65
BMI (kg/m^2)	28±1
Leptin (ng/ml)	11.1±1.0
Adiponectin (µg/ml)	9.4±0.5
Glucose (mg/dl)	110±3
Insulin (mU/l)	18.6±1.7
HOMA-IR	6.2±0.7
Triglycerides (mg/dl)	116±4
LDL-C (mg/dl)	98±3
HDL-C (mg/dl)	50±1
SBP (mmHg)	138±2
DBP (mmHg)	79±1
IL-6 (pg/ml)	3.7±0.2
Hs-CRP (mg/l)	2.98±0.32
Ox-LDL (IU/l)	51±2

Data shown are means ± SEM. Abbreviations: BMI, body mass index; C, cholesterol; CAD, coronary artery disease; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; hs-CRP, high sensitivity C-reactive protein; MetS, metabolic syndrome; ox-LDL, oxidized LDL; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus.

Table 5: AUC of ROC curves regarding diagnostic power to distinguish CAD patients from healthy controls.

microRNA	AUC	95%CI	*P*-value
miR-30a	0.64	0.54-0.74	0.011
miR-101	0.74	0.65-0.82	< 0.0001
miR-130b	0.72	0.63-0.80	0.0001
miR-181a	0.82	0.74-0.89	< 0.0001

Abbreviations: AUC, area under the curve; CI, confidence intervals.

Table 6: Association of miRNA expressions in monocytes with occurrence of angiographically documented CAD.

microRNA	Cut point*	OR	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Low miR-30a	≤ 0.195	9.6 (2.1-44.3)	35	95	91	48
Low miR-101	≤ 0.633	7.6 (2.1-44.3)	57	85	90	45
Low miR-130b	≤ 0.925	10.0 (4.1-24.8)	86	62	45	69
Low miR-181a	≤ 0.521	11.8 (4.5-31.0)	73	81	89	59

*Cut points were determined by ROC curve analysis. Data are means (and 95% confidence intervals). Abbreviations: OR, odds ratio; NPV, negative predictive value; PPV, positive predictive value.

Table 7: Primers used in qRT-PCR

	Forward primer	Reverse primer
ACTB	5'-GGACCTGACCGACTACCTCATG-3'	5'-CGACGTAGCAGAGCTTCTCCTT-3'
CAT	5'-CATCCAGAAGAAAGCGGTCAA-3'	5'-TCAGCATTGTACTTGTCCAGAAGAG-3'
FOXO3A	5'-CAACAAAATGAAATCCATAGAAGCA-3'	5'-AGTGTATGAGTGAGAGGCAATAGCA-3'
INSR	5'-TGTGTACCTCTTGTGGCGTTTC-3'	5'-CTCAGTGCACCTCTCTCTTACATTG-3'
IRAK1	5′-TCAGTCCTAGCAAGAAGCGAGAA-3'	5′-ACTGGCCCGAGGTTGGA-3'
IRAK3	5'-TGCAACGCGGGCAAA-3'	5'-TTTAGTGATGTGGGAGGATCTTCA-3'
IRAK4	5′-AGTGATGGAGATGACCTCTGCTTAG-3'	5′-TGAGCAATCTTGCATCTCATGTG-3'
IRS1	5'-CATCCATTTCAGTTTGTTTACTTTATCC-3'	5'-TTATTCTGGTGTCACAGTGCATTTT-3'
IRS2	5'-GCTTCCCCAGTGCCTATCTTC-3'	5'-AAACCAACAACTTACATCTCCAATGA-3'
MyD88	5'-TGCATATCTTTGCTCCACTTTCA-3'	5'-ATTCCCTCCCAAGATCCTAAGAA-3'
NFKB1	5'-CCCTGACCTTGCCTATTTGC-3'	5'-CGGAAGAAAAGCTGTAAACATGAG-3'
NFKBIA	5'-TGGCCACACGTGTCTACACTTAG-3'	5'-CAGCACCCAAGGACACCAA-3'
SOD1	5'-TTGGGCAAAGGTGGAAATGA-3'	5'-CACCACAAGCCAAACGACTTC-3'
SOD2	5'-TGGAAGCCATCAAACGTGACT-3'	5'-TTTGTAAGTGTCCCCGTTCCTT-3'
TLR2	5'-TGCAAGTACGAGCTGGACTTCTC-3'	5'-GTGTTCATTATCTTCCGCAGCTT-3'
TNFAIP3	5'-TCCCTGCTCCTTCCCTATCTC-3'	5'-ATGTTTCGTGCTTCTCCTTATGAA-3'
TNFAIP6	5'-GGCCATCTCGCAACTTACAAG-3'	5'-GCAGCACAGACATGAAATCCA-3'
TNFα	5'-CAAGCCTGTAGCCCATGTTGTA-3'	5'-TTGGCCAGGAGGGCATT-3'
TRAF6	5'-CATGAAAAGATGCAGAGGAATCAC-3'	5'-GAACAGCCTGGGCCAACAT-3'

CLAIMS

1. An in vitro method to determine activation of a monocyte in a sample, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335 in said sample.

2. An in vitro method according to claim 1, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335.

3. An in vitro method according to claim 1, wherein the one or more microRNAs are selected from the group consisting of miR-103, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335.

4. An in vitro method according to claim 1, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, miR-151-5p, miR-181d, and miR-335.

5. An in vitro method according to claim 1 wherein the activation of the monocyte is indicative for the inflammatory state of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-30a, miR-30b, miR-101, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335 in said sample.

6. An in vitro method according to claim 5 wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-126, miR130b, miR-151-5p, miR-181b, and miR-335.

7. An in vitro method according to claim 1 wherein the activation of the monocyte is indicative for the oxidative stress state of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g,

miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-151-5p, miR-181a, and miR-181b in said sample.

8. An in vitro method according to claim 7 wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-103, miR-151-5p, miR-181a, and miR-181b.

9. An in vitro method according to claim 1 wherein the activation of the monocyte is indicative for insulin signalling deregulation of said monocyte and comprises measuring the expression level of one or more microRNAs selected from the group consisting of miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, miR181b, miR-181d, and miR-335 in said sample.

10. An in vitro method according to claim 9 wherein the one or more miRNAs are selected from miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181a, and miR181b.

11. An in vitro method to predict if a subject will respond to adiponectin or adiponectin mimetic treatment, said method comprising determining the activation of monocytes in a sample by measuring the expression level of one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-126, miR-130b, miR-146b-5p, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335 in said sample, whereby
a) the absence of activated monocytes is an indication that said subject will respond to said adiponectin or adiponectin mimetic treatment, and
b) the presence of activated monocytes is an indication that said subject will not respond to said adiponectin or adiponectin mimetic treatment.

12. An in vitro method to predict if a subject will respond to adiponectin or adiponectin mimetic treatment, said method comprising determining the activation of monocytes according to the method of any one of claims 1 to 10, whereby

a) the absence of activated monocytes is an indication that said subject will respond to said adiponectin or adiponectin mimetic treatment, and
b) the presence of activated monocytes is an indication that said subject will not respond to said adiponectin or adiponectin mimetic treatment.

13. An in vitro method according to any one of claims 1 to 12, wherein the subject suffers from or is at risk of suffering from at least one disease or disorder selected from the group comprising obesity, metabolic syndrome, type 2 diabetes mellitus, hyperglycemia, low glucose tolerance, insulin resistance, a lipid disorder, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis, and a cardiovascular disease.

14. An in vitro method according to claim 1 wherein the activation of the monocyte is indicative for a cardiovascular disease, in particular a coronary artery disease, in a person, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of miR-30a, miR-101, miR-130b, and miR-181a in said sample; in particular one or more microRNAs selected from the group consisting of miR-30a, miR-101, and miR-181a.

15. An in vitro method according to claim 1 wherein the activation of the monocyte is indicative for the cardiovascular risk of a person, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of miR-101, miR-130b, miR-181a, miR-181b, miR-181d, and miR-335 in said sample; in particular one or more microRNAs selected from miR-130b and miR-181b.

16. An in vitro method according to claim 1 wherein the activation of the monocyte is indicative for metabolic syndrome in a person, said method comprising measuring the expression level of one or more microRNAs selected from the group consisting of miR-30a, miR-130b, and miR-181a in said sample; in particular one or more microRNAs selected from miR-130b and miR-181a.

17. An in vitro method according to any one of claims 1 to 16 further comprising analysing the expression level and/or activity of one or more members selected from the group consisting of miR-146b-5p, IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα in said sample.

18. A method according to any one of claims 1 to 17, wherein said sample consists of one or more cells, tissues, or parts thereof; in particular a blood-derived sample or a sample from a tissue that has been infiltrated by activated monocytes.

19. A method according to any one of claims 1 to 18, wherein said sample consists essentially of monocytes or monocyte-derived material, in particular monocytes or monocyte-derived microvesicles.

20. A method of monitoring the progression of treatment of at least one disease or disorder selected from the group comprising obesity, metabolic syndrome, type 2 diabetes mellitus, hyperglycemia, low glucose tolerance, insulin resistance, a lipid disorder, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis, and a cardiovascular disease in a subject; said method comprising determining the activation of at least one monocyte in a sample obtained from said subject according to any one of claims 1 to 19.

21. An agent that modulates one or more microRNAs selected from the group consisting of miR-30a, miR-103, miR-126, miR-130b, miR-151-5p, miR-181b, miR-181d, and miR-335 for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

22. An agent according to claim 21, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-103, miR-130b, miR-151-5p, miR-181d, and miR-335; and wherein the activated monocyte is characterized by an increased inflammatory state.

23. An agent according to claim 21, wherein the one or more microRNAs are selected from the group consisting of miR-126, miR-

130b, miR-151-5p, and miR-181b and wherein the activated monocyte is characterized by an increased oxidative stress state.

24. An agent according to claim 21, wherein the one or more microRNAs are selected from the group consisting of miR-103, miR-130b, miR-151-5p, miR-181d, and miR-335 and wherein the activated monocyte is characterized by insulin signalling deregulation.

25. An agent according to any one of claims 21 to 24, wherein the subject suffers from at least one disease or disorder selected from the group comprising obesity, metabolic syndrome, type 2 diabetes mellitus, hyperglycemia, low glucose tolerance, insulin resistance, a lipid disorder, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis, and a cardiovascular disease.

26. An agent according to claim 21, wherein the one or more microRNAs are selected from the group consisting of miR-101, miR-130b, miR-181a, miR-181b, miR-181d, and miR-335; in particular one or more microRNAs selected from miR-130b and miR-181b; and wherein the treatment of said activated monocyte or the prevention of activation of said monocyte in said subject leads to a decreased cardiovascular risk of said subject.

27. An agent according to claim 21, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-101, miR-130b, and miR-181a; in particular one or more microRNAs selected from the group consisting of miR-30a, miR-101, and miR-181a;
and wherein the treatment of said activated monocyte or the prevention of activation of said monocyte treats and/or prevents a cardiovascular disease, in particular a coronary artery disease, in said person.

28. An agent according to claim 21, wherein the one or more microRNAs are selected from the group consisting of miR-30a, miR-130b, and miR-181a in said sample; in particular one or more microRNAs selected from miR-130b and miR-181a; and wherein the

treatment of said activated monocyte or the prevention of activation of said monocyte treats and/or prevents metabolic syndrome in said person.

29. An agent according to any one of claims 21 to 28, wherein the agent that modulates one or more microRNAs is a microRNA mimic, such as for example an antagomir.

30. A combination comprising
- an agent according to any one of claims 21 to 29, and
- adiponectin or an adiponectin mimetic,

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

31. A combination comprising
- an agent according to any one of claims 21 to 30, and
- a modulator of one or more members selected from the group consisting of IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

32. A combination comprising
- an agent according to any one of claims 21 to 29 and/or miR-146b-5p, and
- adiponectin or an adiponectin mimetic,

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

33. A combination comprising
- an agent according to any one of claims 21 to 29 and/or miR-146b-5p, and
- a modulator of one or more members selected from the group consisting of IRAK3, SOD2, TNFAIP6, TNFAIP3, TLR2, and TNFα

for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

34. A combination according to claims 32 and 33, further comprising adiponectin or an adiponectin mimetic, for use in the treatment of at

least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

35. A pharmaceutical composition comprising an agent according to any one of claims 21 to 29, or a combination according to any one of claims 31 to 34 for use in the treatment of at least one activated monocyte or the prevention of activation of at least one monocyte in a subject.

36. A method of treating and/or preventing a disease or disorder associated with activated monocytes in a subject in need thereof, said method comprising modulating one or more microRNAs selected from the group consisting of let-7c, let-7g, miR-18a, miR-27b, miR-30a, miR-30b, miR-30d, miR-101, miR-103, miR-107, miR-126, miR-130b, miR-151-5p, miR-181a, miR-181b, miR-181d, and miR-335 in a monocyte cell in said subject.

Figure 1

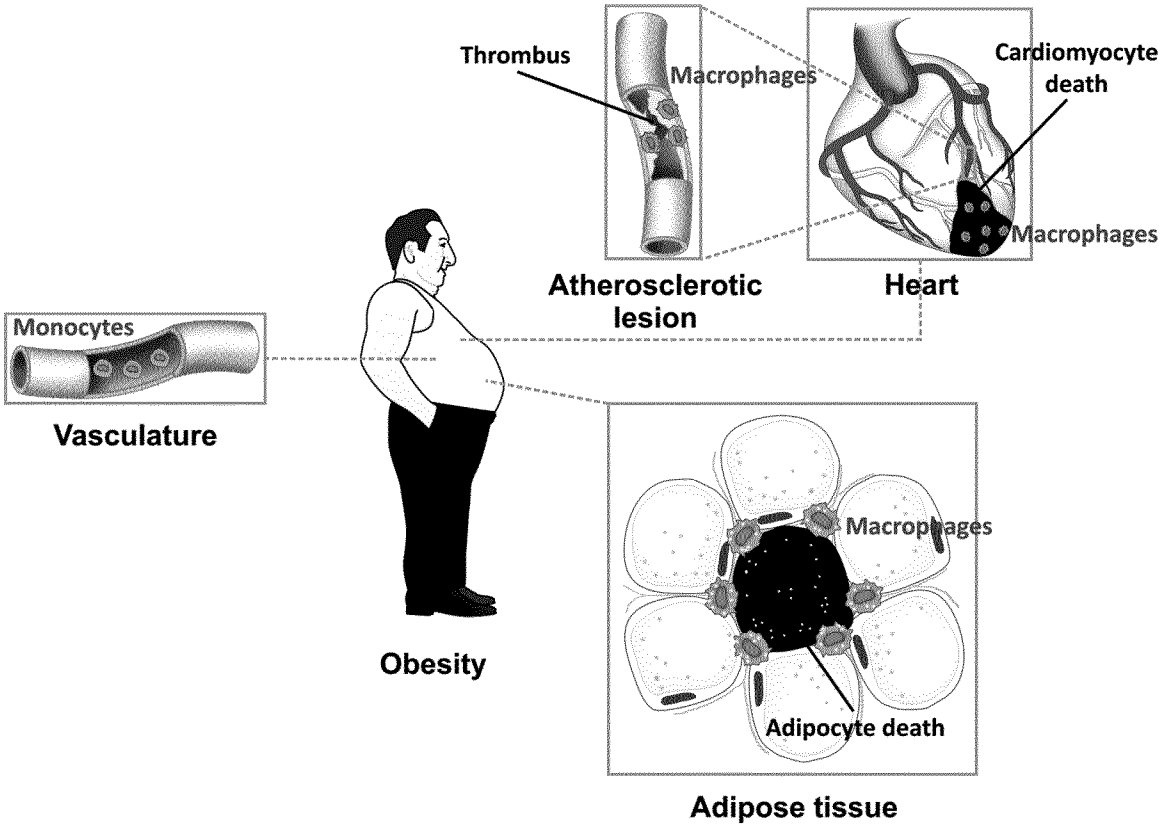

Thrombus
Macrophages
Cardiomyocyte death
Macrophages
Atherosclerotic lesion
Heart
Monocytes
Vasculature
Obesity
Macrophages
Adipocyte death
Adipose tissue

Figure 2

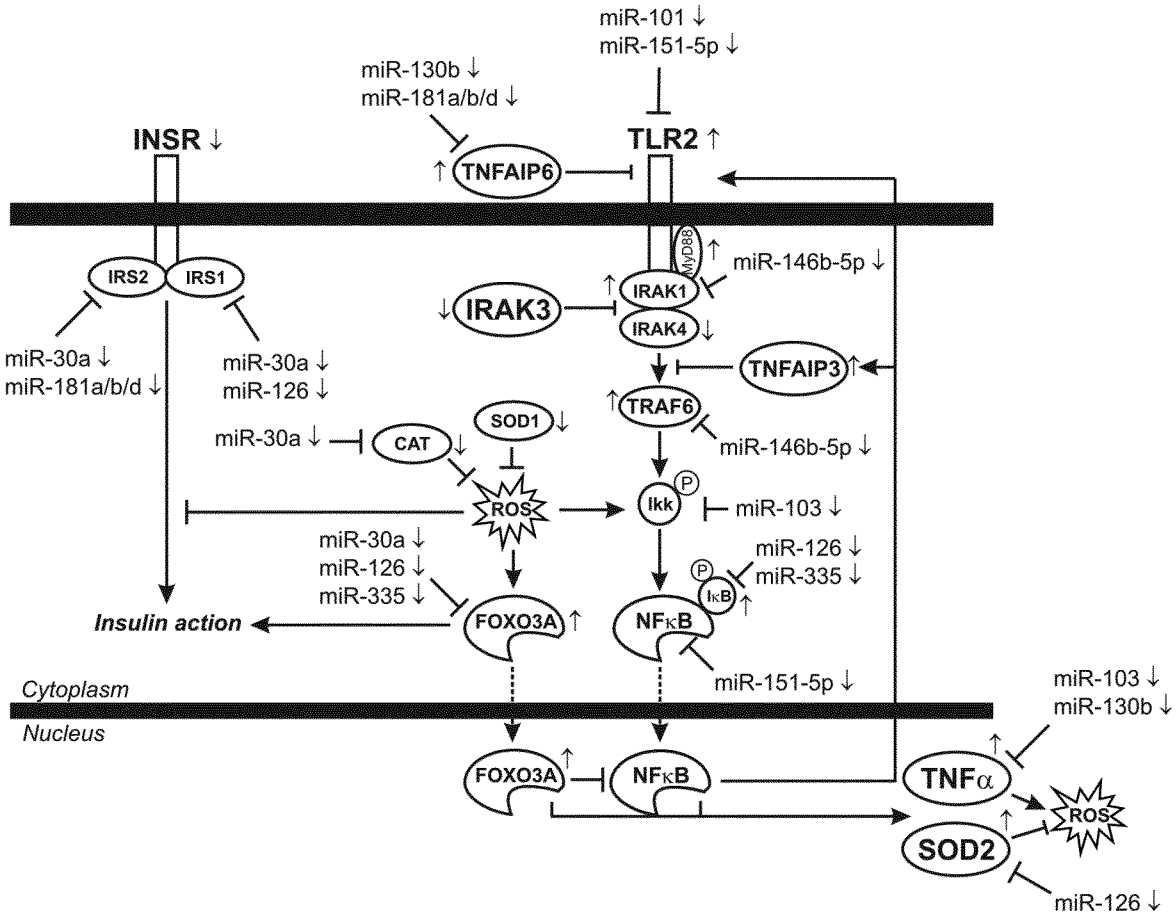

miR-101 ↓
miR-151-5p ↓
miR-130b ↓
miR-181a/b/d ↓
INSR ↓
TLR2 ↑
↑ TNFAIP6
MyD88
↑ miR-146b-5p ↓
IRS2
IRS1
↑ IRAK1
↓ IRAK3
IRAK4 ↓
miR-30a ↓
miR-181a/b/d ↓
miR-30a ↓
miR-126 ↓
TNFAIP3 ↑
↑ TRAF6
miR-30a ↓
CAT ↓
SOD1 ↓
miR-146b-5p ↓
ROS
Ikk
P
miR-103 ↓
miR-30a ↓
miR-126 ↓
miR-335 ↓
miR-126 ↓
miR-335 ↓
P
IκB ↑
Insulin action
FOXO3A ↑
NFκB
miR-151-5p ↓
Cytoplasm
Nucleus
miR-103 ↓
miR-130b ↓
FOXO3A ↑
NFκB
TNFα ↑
ROS
SOD2 ↑
miR-126 ↓

Figure 3

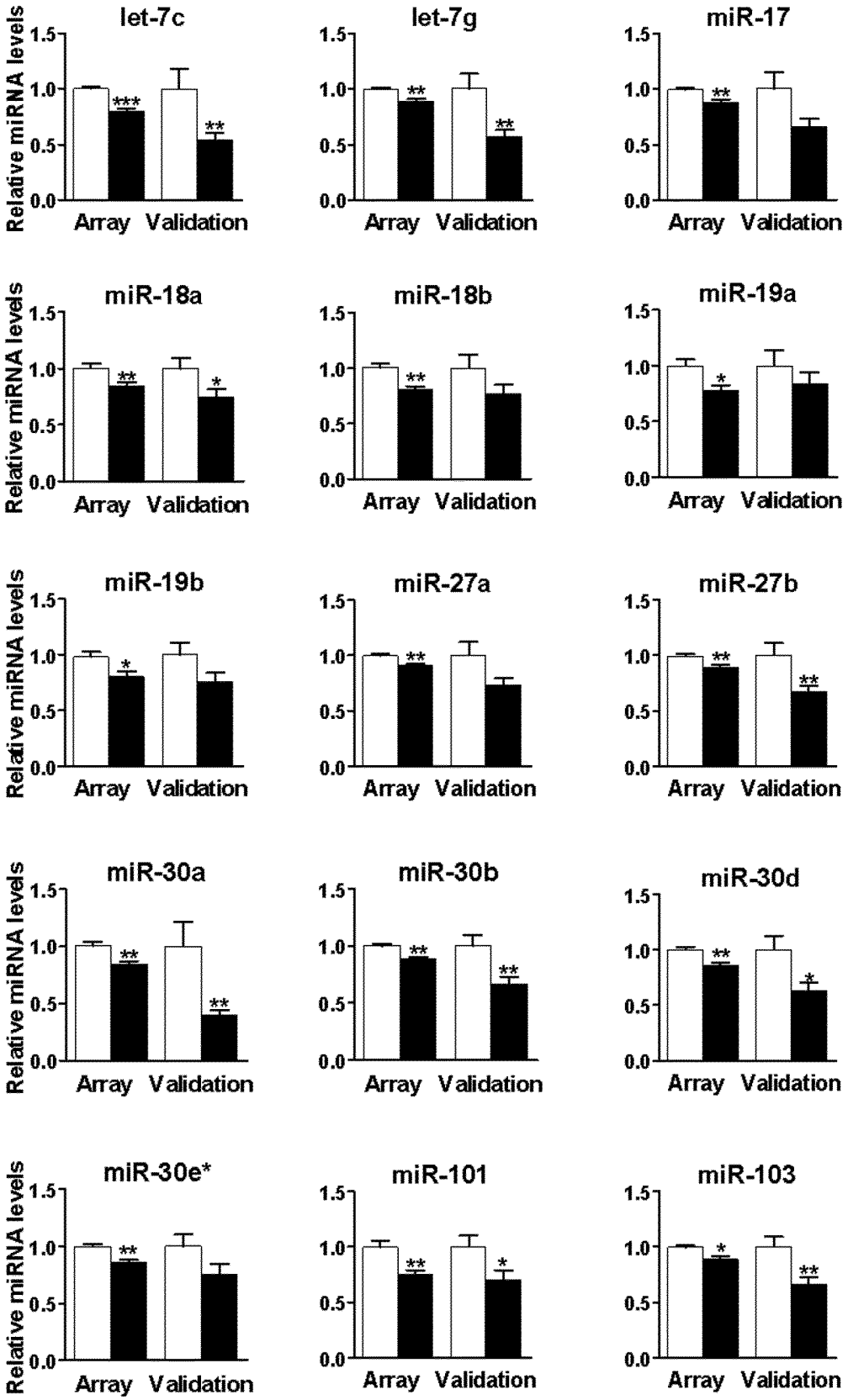

let-7c
let-7g
miR-17
miR-18a
miR-18b
miR-19a
miR-19b
miR-27a
miR-27b
miR-30a
miR-30b
miR-30d
miR-30e*
miR-101
miR-103
Relative miRNA levels
1.5
1.0
0.5
0.0
Array
Validation
***
**
*

Figure 3 (continued)

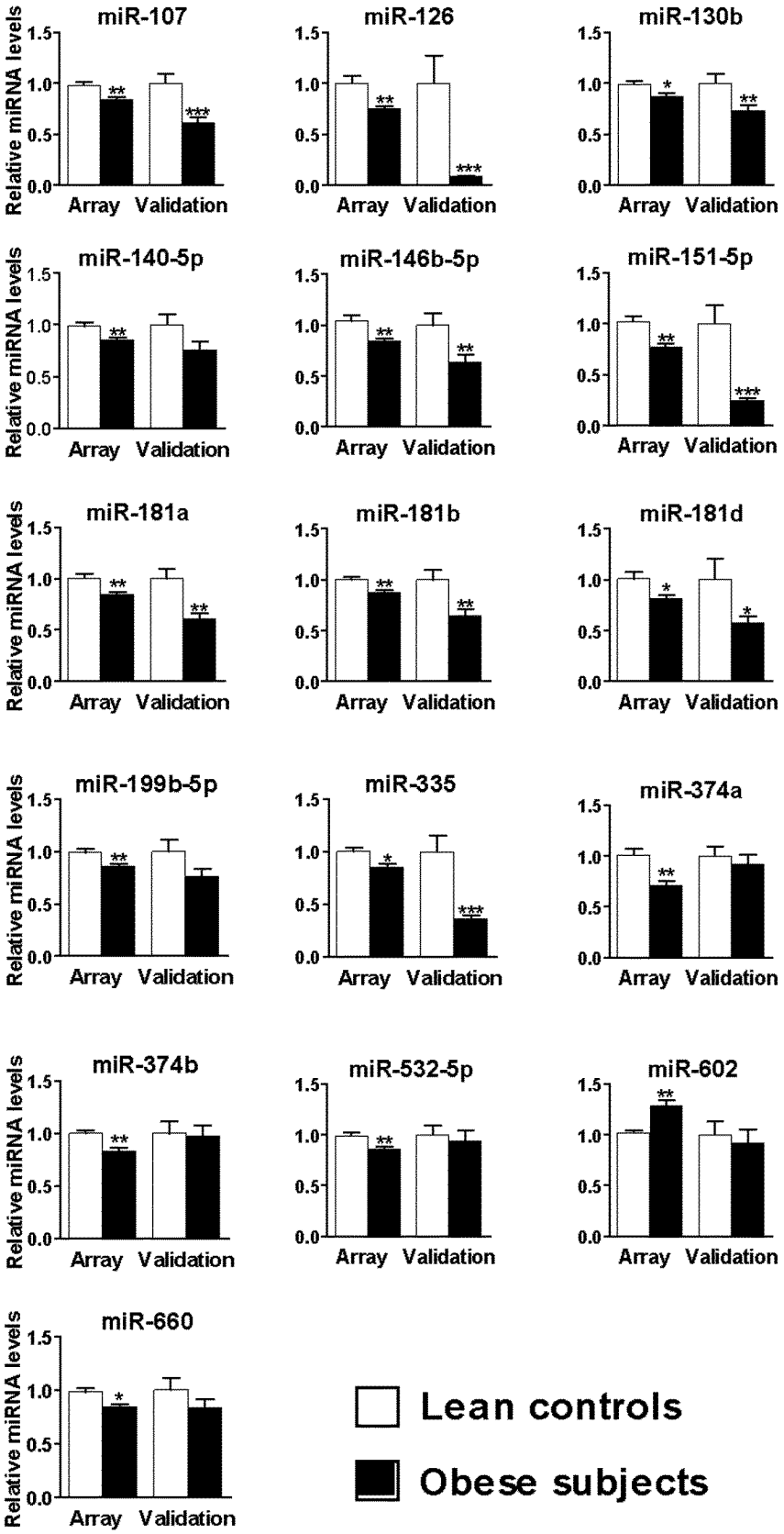


Figure 4

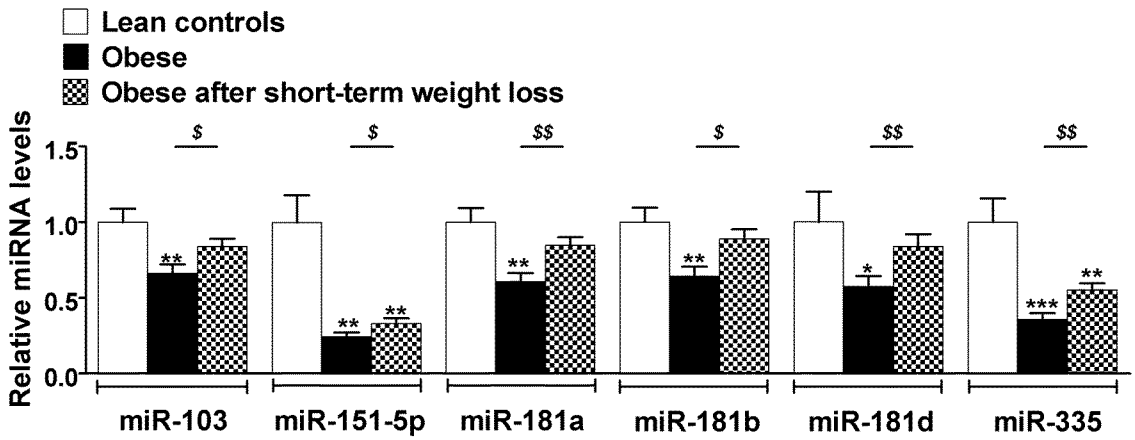

Lean controls
Obese
Obese after short-term weight loss
Relative miRNA levels
1.5
1.0
0.5
0.0
$
$
$$
$
$$
$$
**
**
**
**
**
*
***
**
miR-103
miR-151-5p
miR-181a
miR-181b
miR-181d
miR-335

Figure 5

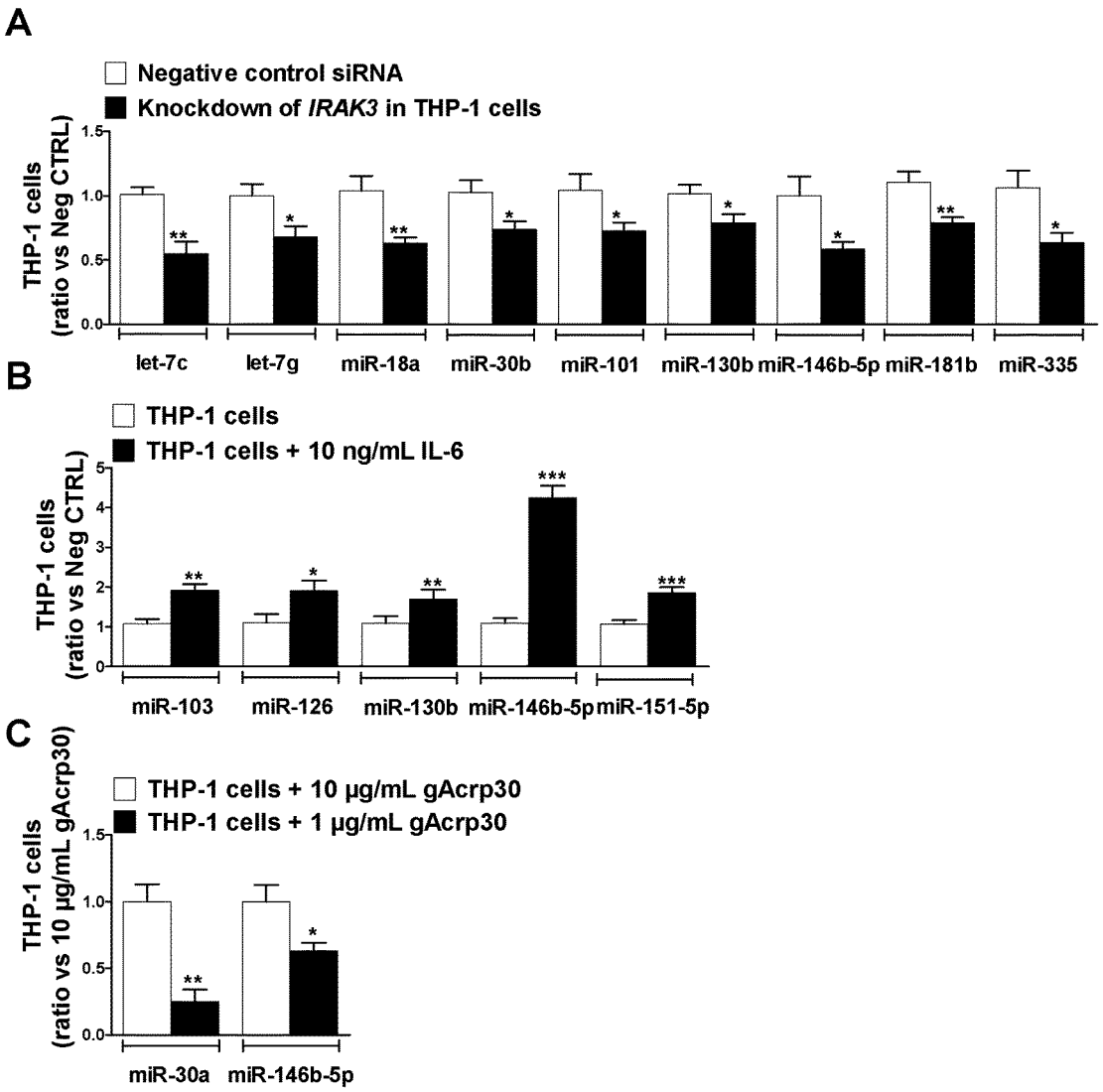

A
Negative control siRNA
Knockdown of IRAK3 in THP-1 cells
THP-1 cells
(ratio vs Neg CTRL)
1.5
1.0
0.5
0.0
**
*
**
*
*
*
*
**
*
let-7c
let-7g
miR-18a
miR-30b
miR-101
miR-130b
miR-146b-5p
miR-181b
miR-335
B
THP-1 cells
THP-1 cells + 10 ng/mL IL-6
THP-1 cells
(ratio vs Neg CTRL)
5
4
3
2
1
0
**
*
**
***
***
miR-103
miR-126
miR-130b
miR-146b-5p
miR-151-5p
C
THP-1 cells + 10 µg/mL gAcrp30
THP-1 cells + 1 µg/mL gAcrp30
THP-1 cells
(ratio vs 10 µg/mL gAcrp30)
1.5
1.0
0.5
0.0
**
*
miR-30a
miR-146b-5p

Figure 6

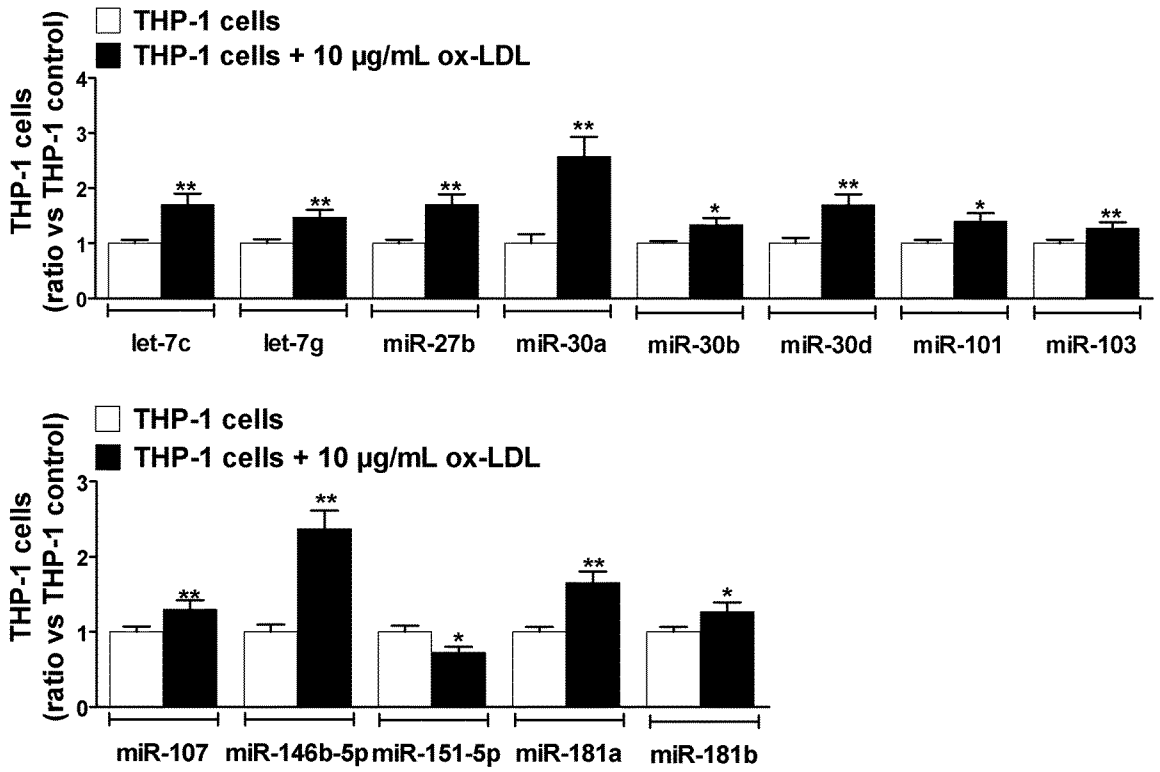


Figure 7

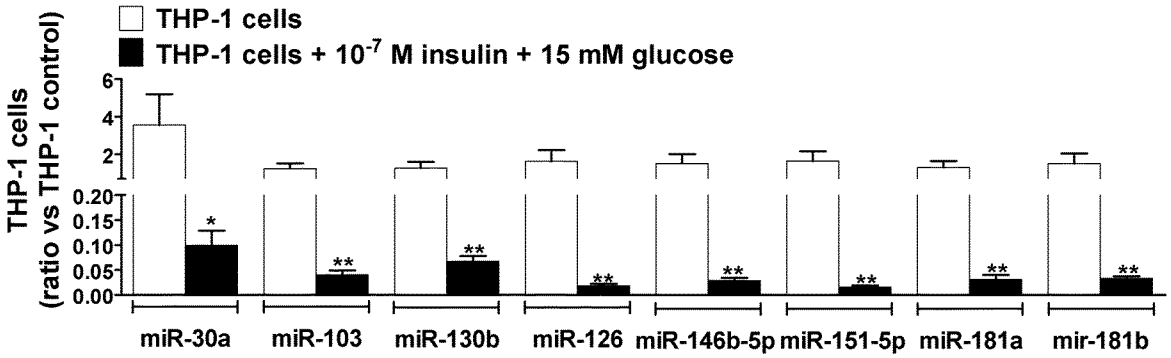


Figure 8

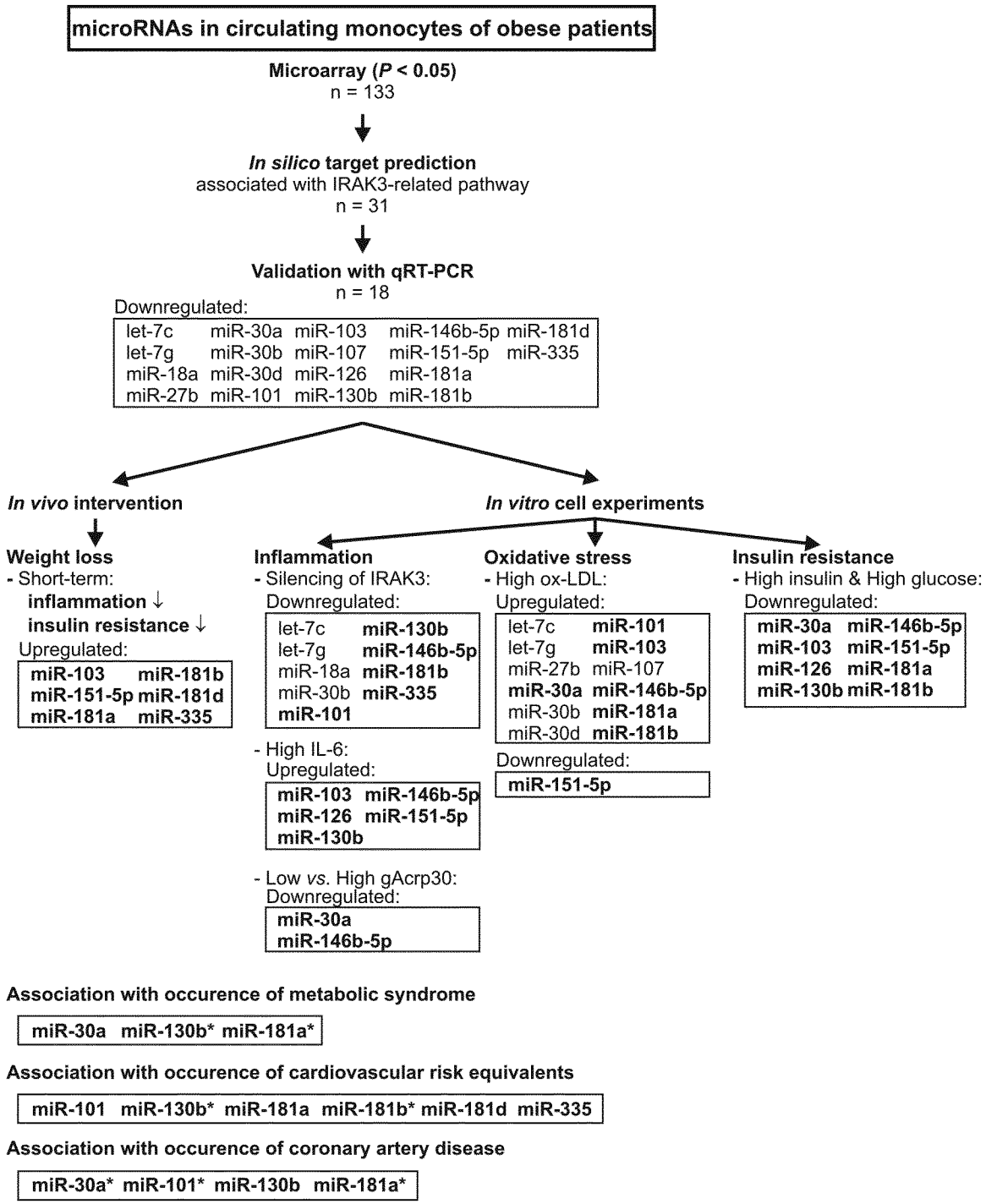

microRNAs in circulating monocytes of obese patients
Microarray (P < 0.05)
n = 133
In silico target prediction
associated with IRAK3-related pathway
n = 31
Validation with qRT-PCR
n = 18
Downregulated:
let-7c miR-30a miR-103 miR-146b-5p miR-181d
let-7g miR-30b miR-107 miR-151-5p miR-335
miR-18a miR-30d miR-126 miR-181a
miR-27b miR-101 miR-130b miR-181b
In vivo intervention
In vitro cell experiments
Weight loss
- Short-term:
inflammation ↓
insulin resistance ↓
Upregulated:
miR-103 miR-181b
miR-151-5p miR-181d
miR-181a miR-335
Inflammation
- Silencing of IRAK3:
Downregulated:
let-7c miR-130b
let-7g miR-146b-5p
miR-18a miR-181b
miR-30b miR-335
miR-101
- High IL-6:
Upregulated:
miR-103 miR-146b-5p
miR-126 miR-151-5p
miR-130b
- Low vs. High gAcrp30:
Downregulated:
miR-30a
miR-146b-5p
Oxidative stress
- High ox-LDL:
Upregulated:
let-7c miR-101
let-7g miR-103
miR-27b miR-107
miR-30a miR-146b-5p
miR-30b miR-181a
miR-30d miR-181b
Downregulated:
miR-151-5p
Insulin resistance
- High insulin & High glucose:
Downregulated:
miR-30a miR-146b-5p
miR-103 miR-151-5p
miR-126 miR-181a
miR-130b miR-181b
Association with occurence of metabolic syndrome
miR-30a miR-130b* miR-181a*
Association with occurence of cardiovascular risk equivalents
miR-101 miR-130b* miR-181a miR-181b* miR-181d miR-335
Association with occurence of coronary artery disease
miR-30a* miR-101* miR-130b miR-181a*

Figure 9

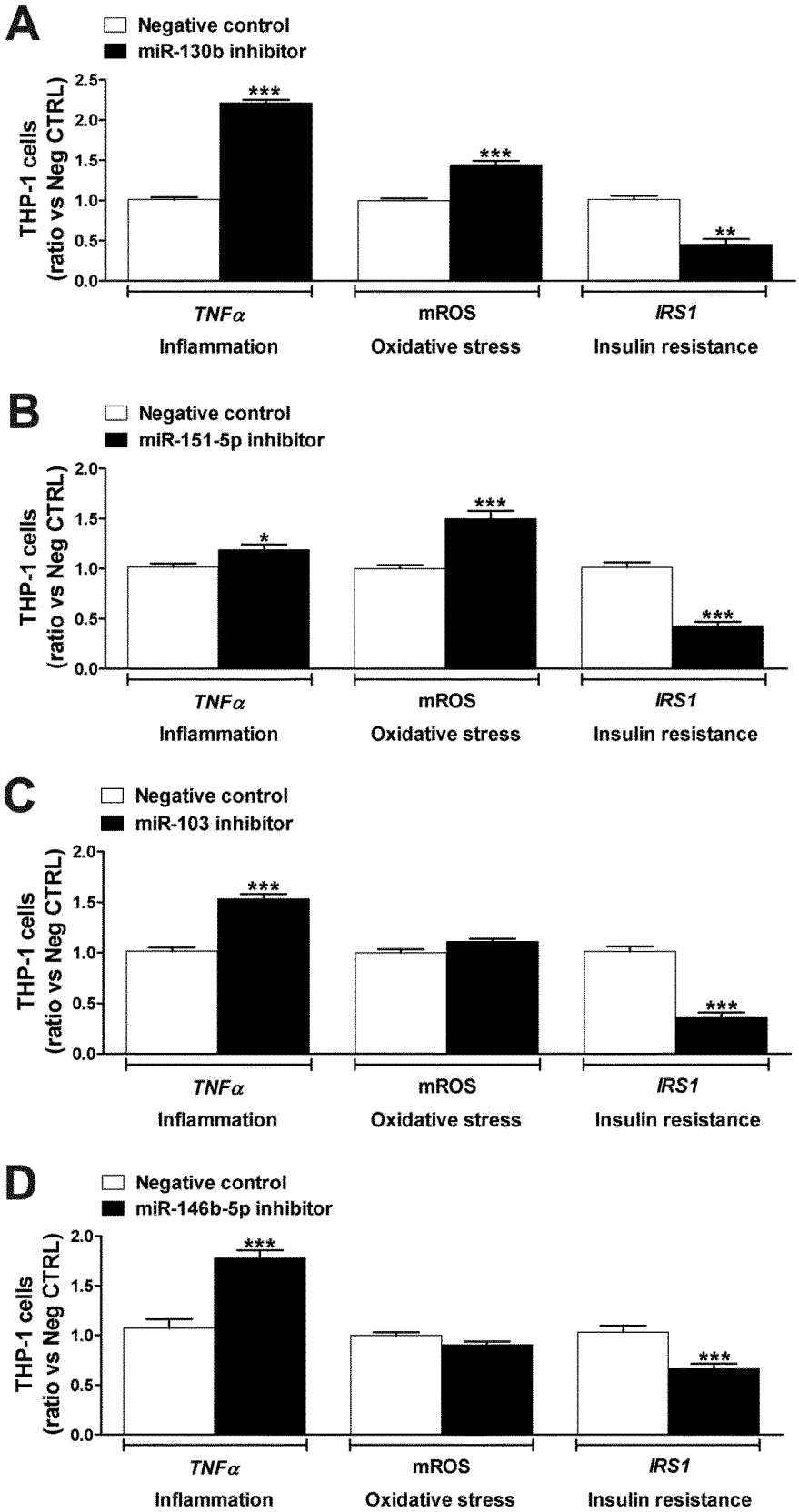

A
Negative control
miR-130b inhibitor
THP-1 cells
(ratio vs Neg CTRL)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
**
TNFα
mROS
IRS1
Inflammation
Oxidative stress
Insulin resistance
B
Negative control
miR-151-5p inhibitor
THP-1 cells
(ratio vs Neg CTRL)
2.0
1.5
1.0
0.5
0.0
*
***
***
TNFα
mROS
IRS1
Inflammation
Oxidative stress
Insulin resistance
C
Negative control
miR-103 inhibitor
THP-1 cells
(ratio vs Neg CTRL)
2.0
1.5
1.0
0.5
0.0
***
***
TNFα
mROS
IRS1
Inflammation
Oxidative stress
Insulin resistance
D
Negative control
miR-146b-5p inhibitor
THP-1 cells
(ratio vs Neg CTRL)
2.0
1.5
1.0
0.5
0.0
***
***
TNFα
mROS
IRS1
Inflammation
Oxidative stress
Insulin resistance

Figure 9 (continued)

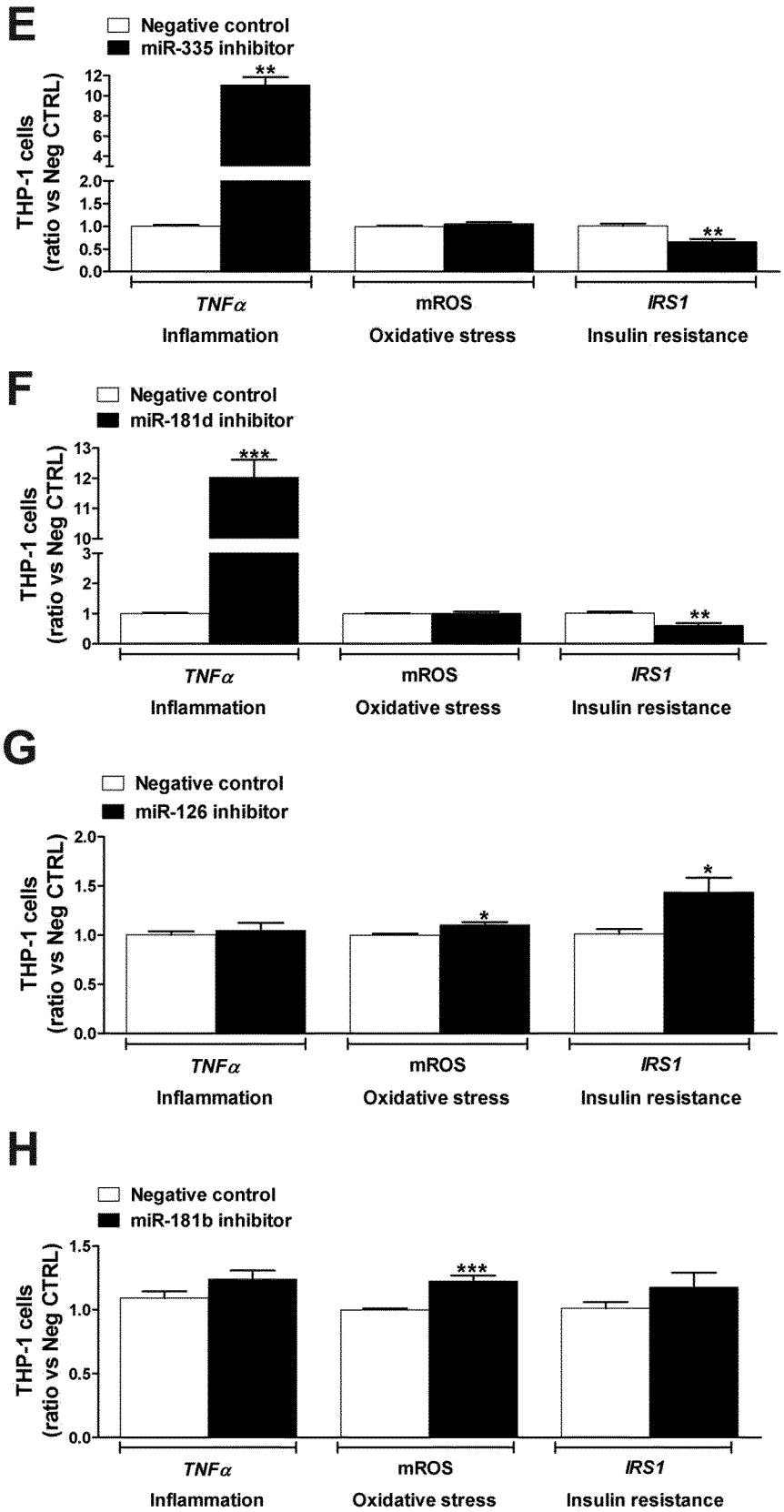


Figure 10

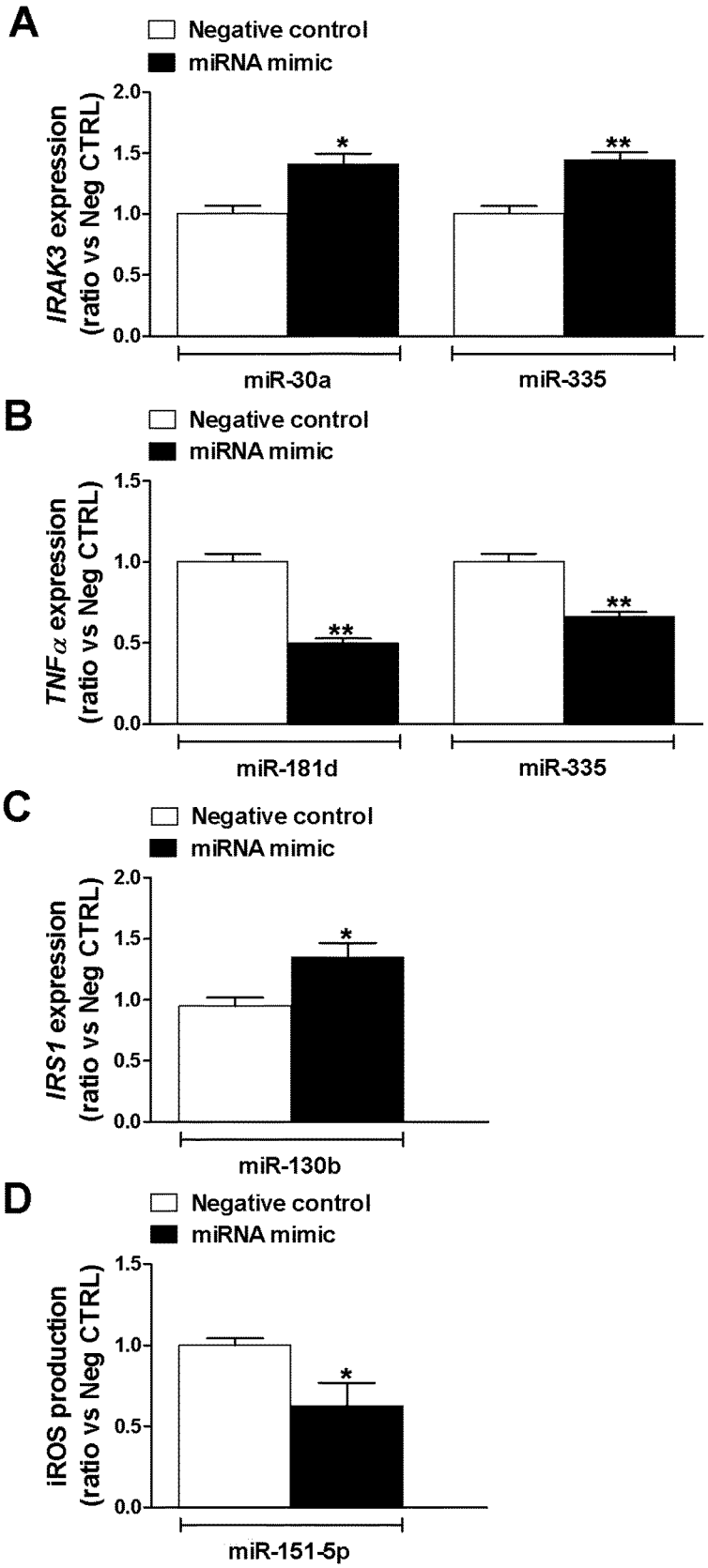
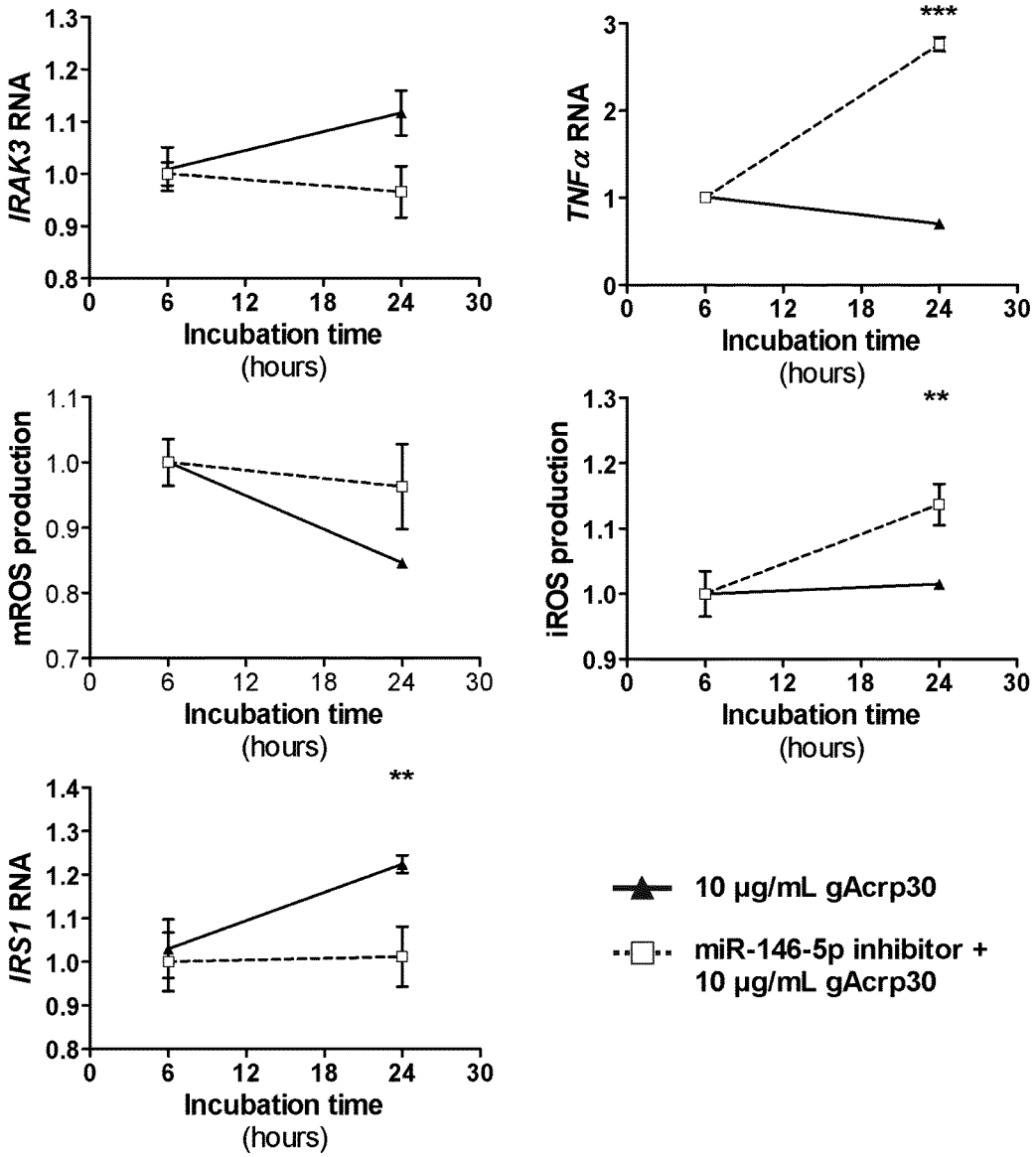

A
Negative control
miRNA mimic
IRAK3 expression
(ratio vs Neg CTRL)
2.0
1.5
1.0
0.5
0.0
*
**
miR-30a
miR-335
B
Negative control
miRNA mimic
TNFα expression
(ratio vs Neg CTRL)
1.5
1.0
0.5
0.0
**
**
miR-181d
miR-335
C
Negative control
miRNA mimic
IRS1 expression
(ratio vs Neg CTRL)
2.0
1.5
1.0
0.5
0.0
*
miR-130b
D
Negative control
miRNA mimic
iROS production
(ratio vs Neg CTRL)
1.5
1.0
0.5
0.0
*
miR-151-5p

Figure 11


IRAK3 RNA
1.3
1.2
1.1
1.0
0.9
0.8
0
6
12
18
24
30
Incubation time
(hours)
TNFα RNA
***
3
2
1
0
mROS production
1.1
1.0
0.9
0.8
0.7
iROS production
**
1.3
1.2
1.1
1.0
0.9
IRS1 RNA
**
1.4
1.3
1.2
1.1
1.0
0.9
0.8
10 µg/mL gAcrp30
miR-146-5p inhibitor +
10 µg/mL gAcrp30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/071394

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [X] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/071394

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAZZONI FLAVIA ET AL: "Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC; US, vol. 106, no. 13, 31 March 2009 (2009-03-31), pages 5282-5287, XP002541471, ISSN: 0027-8424, DOI: 10.1073/PNAS.0810909106 [retrieved on 2009-03-16] the whole document ----- -/--	1-36

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier 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
25 January 2012	10/02/2012

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

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/071394

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/088668 A2 (CEPHEID [US]; VILANOVA DAVID [FR]; PERSING DAVID H [US]; DELFOUR OLIVE) 5 August 2010 (2010-08-05) see whole doc. esp. page 96 ff.	1-36
X	CHEN TING ET AL: "MicroRNA-125a-5p partly regulates the inflammatory response, lipid uptake, and ORP9 expression in oxLDL-stimulated monocyte/macrophages.", CARDIOVASCULAR RESEARCH 1 JUL 2009 LNKD-PUBMED:19377067, vol. 83, no. 1, 1 July 2009 (2009-07-01), pages 131-139, XP002667891, ISSN: 1755-3245 the whole document	1-16
A	HENEGHAN H M ET AL: "Role of microRNAs in obesity and the metabolic syndrome", OBESITY REVIEWS, BLACKWELL SCIENCE, GB, vol. 11, no. 5, 1 May 2010 (2010-05-01), pages 354-361, XP002634305, ISSN: 1467-7881, DOI: 10.1111/J.1467-789X.2009.00659.X [retrieved on 2009-09-29] the whole document	1-36
A,P	CUI JIAN GUO ET AL: "Differential Regulation of Interleukin-1 Receptor-associated Kinase-1 (IRAK-1) and IRAK-2 by MicroRNA-146a and NF-kappa B in Stressed Human Astroglial Cells and in Alzheimer Disease", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 285, no. 50, December 2010 (2010-12), pages 38951-38960, XP002667892, the whole document	1-36

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/071394

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010088668 A2	05-08-2010	AU 2010207975 A1	18-08-2011
		CA 2751213 A1	05-08-2010
		EP 2391738 A2	07-12-2011
		US 2010227325 A1	09-09-2010
		WO 2010088668 A2	05-08-2010