Human Adipocytes and the Composition of Plasma Lipoproteins: Linking Obesity with Type 2 Diabetes and Cardiovascular Disease

Monireh Dashti Rahmatabadi

Description of the front cover figure: Representation of the structural association between lipoproteins and adipocyte-lipid droplets.

Description of the back cover figure: Representation of the endothelial structure and expression of the coagulation factors by vascular cells involved in atherothrombosis.

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Human Adipocytes and the Composition of Plasma Lipoproteins: Linking Obesity with Type 2 Diabetes and Cardiovascular Disease

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by

Monireh Dashti Rahmatabadi

Born on 25th October 1971

Tehran, Iran

ERASMUS UNIVERSITEIT ROTTERDAM

Promotor:

Prof. dr. M. P. Peppelenbosch

Inner Committee:

Prof. dr. E.G. Sijbrands

Prof. dr. Ernst J. Kuipers

Dr. C. A. Spek

Copromotor:

Dr. F. Rezaee

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Chapter 1

General Introduction

An Overview to the Function of the Adipose Tissue and Plasma Lipoproteins and Their Axis Roles in Regulation of Energy Metabolic System and Age-Related Diseases; Type 2 Diabetes and Atherosclerosis

Monireh Dashti [1,2], Maikel P. Peppelenbosch [2] Farhad Rezaee [1]

[1] Department of Cell Biology, UMCG, University of Groningen, The Netherlands

[2] Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands

Derived from invited review papers:

- Role of adipose tissue in pathophysiology of energy metabolic system disorders (***in submission process***)
- Hedgehog signaling as an antagonist of ageing and its associated diseases. ***BioEssays,Volume 34, Issue 10, October 2012***
- Nutrient signaling in adipose tissue and its consequences for metabolic disease

1. PREFACE

Maintaining energy homeostasis is one of the fundamental tasks of the body that has to be performed, and it is largely achieved through the control of blood glucose level. Thus normoglycemia is regulated through proper energy delivery to the energy-demanding tissues or storage in the adipose tissue through the lipoprotein compartment. The processes constitute a multitude of tightly regulated and highly tuned mechanisms, which is only partly understood and involved in all tissues. Nevertheless, the adipose tissue and in particular adipocytes together with the skeletal muscle and liver seems to be the most important organs for dealing with challenges to keep body energy and energy metabolic system in homeostasis. Although through most of mankind's history, lack of energy was the main challenge in current societies excess intake of calories and especially fats and sugars as well as their main manifestation, and hyperglycemia-related diseases has become a serious problem. The situation is compounded by the increase of sedentary life-styles and the use of high level of tobacco (Cabrera de et al., 2007; Oh et al., 2005). Currently, 65% of the global population lives in countries where obesity kills more people than malnutrition (WHO - Global strategy on diet, physical activity and health, 2010). The metabolic diseases include obesity-induced metabolic syndrome, lipodystrophia, and type 2 diabetes mellitus (T2DM) represent an ever increasing challenge to health care. A guiding principle of this thesis is that many aspects of these diseases can be better understood through insight into the energy metabolism of the adipocytes as well as improved knowledge of the adipocytes and their production. Furthermore, I shall argue that the lipoprotein compartment is

not only a mediator between the adipose compartment and the periphery with respect to the transport of energy-rich hydrophobic molecules, but also is an important transport modality for adipocyte-generated endrocrinological signals.

2. THE ENERGY METABOLIC SYSTEM

The energy metabolic system ensures to regulate the energy metabolism in body and is difficult to categorize. It is mainly composed of energy transporting and storing molecules (e.g. lipids and glucose), lipid carriers (lipoproteins), the endocrinological system (e.g. pancreas, adipose tissue, hypothalamus, growth hormones, thyroid, and adrenal gland hormones), the metabolically active tissues (e.g. adipose tissue, skeletal muscles, liver, and kidney) and the metabolic pathways (mainly insulin, adeosine monophosphate-activated protein kinase (AMPK), inflammatory molecules). Energy metabolism is defined as a finely tuned regulatory system to ensure the balance of the retour of energy in the form of lipids and carbohydrates in the body and is reflected by the level of the glucose in circulation. Circulatory glucose levels depend on four parameters; i: prandial glucose (food-derived) ii: the consumption of glucose in the tissues (mainly skeletal muscles and the brain), iii: the capacity of the body to store excess glucose in the adipose tissue in the form of triglycerides and iv: the endogenous glucose production through gluconeogenesis in the liver and kidney. Among the involved systems, adipose tissue and skeletal muscle are considered as the main energy reservoir and consumer units in the body respectively. Thus, adipose tissue and skeletal muscle play a main role in

determination of the quality of the metabolic system function. A disturbance in the function of these two organs can trigger the metabolic diseases including metabolic syndrome and their medical complications including coagulopathies and atherosclerosis. Here we shall argue that especially regulation of energy metabolism in adipose tissue is an underestimated but principal component regulating these processes.

2.2. General principles of the regulation of energy metabolism

Nutrient- and metabolic- status sensing is highly fundamental to the eukaryotic life; therefore the involved biochemical principles are broadly similar within eukaryotes. To adjust cell metabolism to the intracellular energy status and extracellular environment, the liver kinase B / AMPK / tuberous sclerosis complex / mammalian target of rapamycin complex 1 (LKB1/AMPK/TSC/mTORC1) pathway integrates information regarding the intracellular energy, oxygen status, the presence of growth factors and nutrient availability, which translates this into the regulation of cell growth (Figure 1) (Shah et al., 2004; van et al., 2011). Lower energy status is reflected in an increased (adenosine monophosphate/adenosine triphosphate ratio) AMP/ATP ratio, directly resulting in higher AMPK activity. Although AMPK can directly influence cellular physiology via the stimulation of glucose import and mitochondrial activity (Lim et al., 2010), its main action is to influence mTOR activity. AMPK inhibits mTOR, known as the central energy sensor of the cell (Wang et al., 2007), via TSC. mTOR activity

provides via S6 kinase a protein synthesis-permissive signal, which actively counteracts autophagia (Rabinowitz and White, 2010; Singh et al., 2009a).

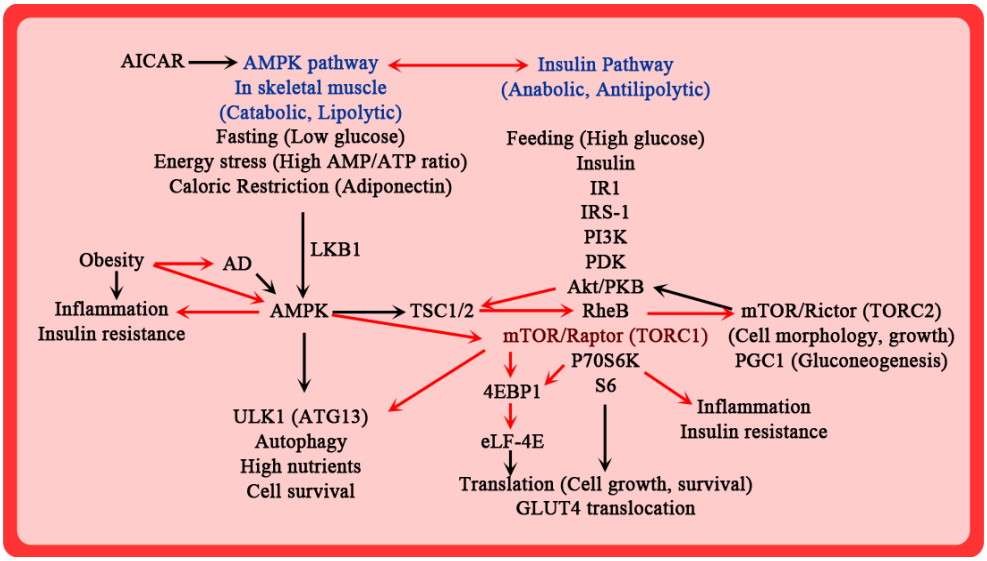

Figure 1: Illustration of the antagonist association between the insulin and AMPK pathways, two main energy metabolic systems of body.

LKB1/AMPK/TSC/mTORC1 pathway is the fundamental nutrient and metabolic status sensing in the body that help cells to adjust their metabolism to the intracellular energy status and extracellular environment. The anabolic insulin pathway and catabolic AMPK (in the skeletal muscle) pathways have a negative regulatory effect on each other and both determine the level of the energy status of the cell. AMPK pathway is activated following lower energy status, which increases the level of AMP/ATP retio in the body. Increase of the level of adiponectin following coloric restriction has a stimulatory effect on the stimulation of AMPK. AMPK decreases inflammation by enhancing fatty acid oxidation in cell. It also has a stimulatory effect on autophagy and TSC, which inhibits insulin pathway. RheB in insulin pathway via positive influence on mTOR/Rictor (TORC1) influences on the central energy sensor of the cell. TORC1 inhibits autophagy and stimulates cell growth and survival. IR: Insuli receptor, IRS: Insulin receptor substrate, AMPK: Adenosine monophosphate-activated protein kinase, PI3K: Phosphatidylinositol 3 kinase, PDK: Phosphoinositide dependent kinases, Akt/PKB: Protein kinase B, RheB: Ras homolog enriched in brain, P70S6K: P70

ribosomal S6 kinase, GLUT4: Glucose transporter type 4, EBP1: ErbB-3 binding protein, eLF-4E: Eukaryotic initiation factors-4E, ULK1: unc-51-like kinase 1, ATG13: Authophagy gene, AD: Adiponectin, AICAR: 5 aminoimidazole 4 carboxamide 1 dribofuranoside.

Human diseases such as PJS and TSC knockout animal models and *in vitro* experiments indicate that both the LKB1 kinase and the TSC1:TSC2 complex are essential proteins in regulating cell growth under conditions of metabolic stress (Jansen et al., 2009). In addition, the system is under control of the PKB/Akt pathway. Anabolic stimuli, like insulin, employ this pathway to increase mTOR activity. The effect of nutrient signaling in adipose tissue is highly cell type-dependent and thus modulation of this pathway may hold great clinical promise through this system

2.3. The link between obesity and metabolic disorders

The notion that the adipocyte compartment is an important component of the normal and aberrant energy balance and its associated disorders comes from the association that has been made between obesity and metabolic disorders. Obesity is the new major risk worldwide. An overload of energy in the form of triglycerides within lipid droplets of adipocytes is the main cause of obesity (Guilherme et al., 2008). Numerous epidemiological studies implicate the hypertrophy of adipocyte compartment to energy metabolic disorders such as insulin resistance and type 2 diabetes mellitus and their afflictions including coagulopathies, atherosclerosis and cardiovascular diseases. The nature of the problem is further compounded by the observation that both the obesity and its associated disorders seem to

display even greater prevalence over the world. The factors driving the obesity epidemic are mainly both the changes in diet with a greater dependency on sugars and fats with a concomitant decrease in fibre in combination with a reduced physical activity. Also, failure to develop a good therapy in dealing with metabolic disorders is remained to be an issue here. The development of humanity has undoubtly included many episodes of extreme starvation and as a result we are highly capable of storing excess energy in the form of lipids in the body for usage at a later time. The subcutaneous fat layers are the physiological place for this storage and would also protect us against cold. During the activity, the stored energy is released allowing better survival. However, in the whole world calorie restriction is not much of an issue anymore, therefore obesity is depressingly common. Efficient breakdown of fat may be further compounded by differences between individuals with respect to threshold levels of lipid storage and release as well as in difference in gut flora components. In turn, obesity provokes systemic chronic inflammation, which is then directly related to metabolic disorders and cardiovascular disease (Wang and Nakayama, 2010) and malignancy (Cole, 2009). Hence, this book is meant to highlight the role of obesity in initiation of inflammation (Guilherme et al., 2008; Ye and Keller, 2010) and the energy metabolic system disturbances including metabolic syndrome and atherosclerosis.

3. THE COMPONENTS OF THE FAT TISSUE IN NUTRIENT SIGNALING

Survival of a species is dependent on two main processes, which are clearly interrelated; proper maintenance of the level of energy in the body and mounting an immune response to pathogens (Wellen and Hotamisligil, 2005). Nutrient- and pathogen-sensing pathways are both evolutionary highly conserved, which are interrelated to each other at many points for instance the crucial role of mTOR gene in both nutrient pathways as well as immunity (Hotamisligil and Erbay, 2008). mTOR inhibitors are among the most available strong immunomodulators that are often used in transplantation medicine) (McMahon et al., 2011). During obesity, adipose tissue develops low grade inflammation, which is primed by adipocytes and followed by a concerted reaction of the other constituting cell types of the adipose tissue (Wellen and Hotamisligil, 2005; Meijer et al., 2011). Adipose tissue is one of the largest tissues in the body and is composed of different cells such as adipocytes, residual macrophages, mesenchymal stem cells, preadipocytes, endothelial cells, and fibroblasts (Figure 2). This diversity of cells in the adipose tissue represents its vast function and importance in different pathways including metabolic system (Dennison et al., 2007; Hattori et al., 2004) and immune system (Meijer et al., 2011) and both functionalities are related to the nutrient signaling. Adipose tissues are categorized based on the type (Brown Adipose Tissues (BAT) and White Adipose Tissues (WAT)) and location (Visceral (Vis) and Subcutaneous (SC)) in the body. These two classes are separate from each other and are important in the evaluation of the metabolic system functionality.

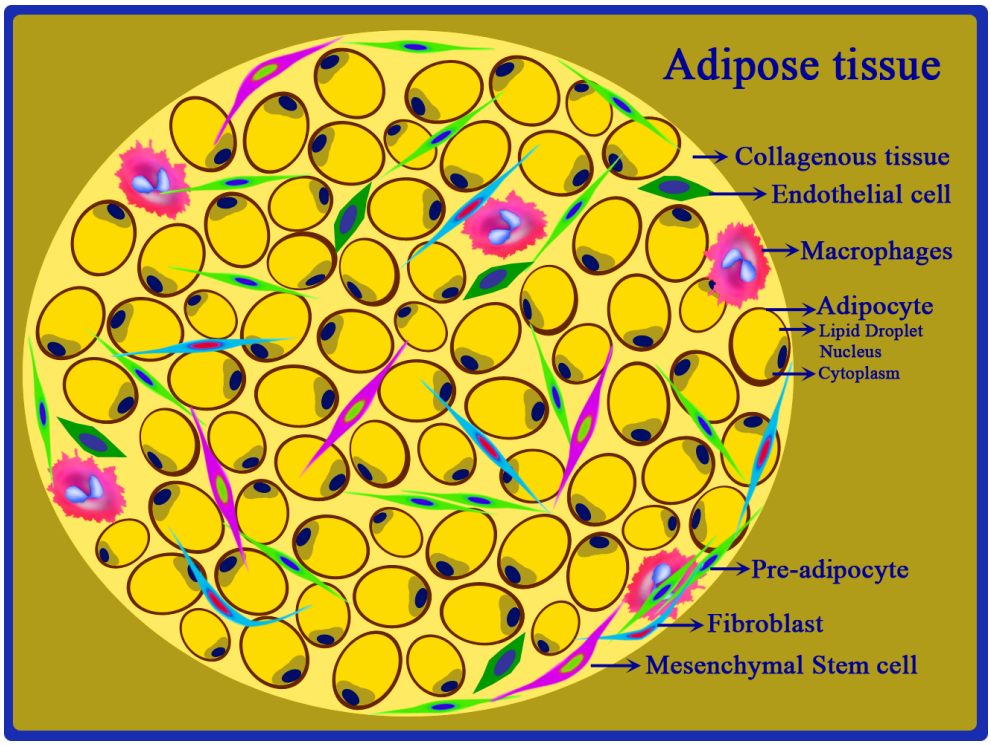

Figure 2: The structure of adipose tissue.

Adipose tissue is composed of a collagenous background together with different cell types, which are seeded in this connective tissue. Adipocytes are the highest level of cells in the adipose tissue and the main cells for the storage and release of the cytokines of this tissue. They are in close association with the residual macrophages and during obesity their interaction lead to stimulation of macrophages and initiation of chronic inflammation. Preadipocytes are the next cells and are the linker between adipocyte and macrophages as the main cells of the metabolic and immune systems. Other cells of the adipose tissue are fibroblasts, mesenchymal stem cells and endothelial cells that each one have an important role in different pathways including the metabolic, coagulation and inflammatory systems.

3.1. Brown adipose tissue adipocytes

In mammals there are two types of adipocytes with opposing functions. Brown adipose tissue-adipocytes are characterized by the small lipids droplets containing triglycerides, which are accessible for rapid hydrolysis and oxidation of fatty acids, while white adipose tissue-adipocytes have one huge lipid droplet per adipocyte for the energy storage in the form of triacylglycerol (Cinti, 2002; Nishino et al., 2008). Ultrastructurally, brown adipocytes are characterized by a high number of mitochondria packed with cristae and expressing thermogenic genes such as UCP-1 (uncoupling protein 1), which is used for fatty acid oxidation and for heat generation (thermogenesis) and warming the body (Rousset et al., 2004). Even though brown adipose tissue is the major sort of adipose tissue during the development of the fetus bodies, however during the adulthood it converts to the white adipose tissue (Guilherme et al., 2008). The percentage of the brown adipose tissue in visceral fat is higher than that in the subcutaneous fat tissue (Saely et al., 2012). The activation of human brown adipose tissue represents an opportunity to increase energy expenditure and weight loss alongside improved lipid and glucose homeostasis. Active brown adipose tissue is able to uptake a large quantities of lipid and glucose from the circulation (Becerril et al., 2010). Activity of these cells with respect to thermogenesis is largely under the control of innervated-β-adrenergic receptors on brown adipocytes. But pharmacological approaches to stimulate brown adipocyte tissue activity without central nervous system side effects might hold great promise to combat obesity-related disease (Susulic et al., 1995).

Nutrient signaling plays a pivotal role in brown adipocyte development, quite different from the situation in white adipose tissue adipocytes. Differentiation of brown adipocytes coincides with strong activation of LKB1/AMPK and canonical downregulation of mTORC1, unusually in conjunction with p38MAP kinase activation, which is a kinase normally more associated with pro-inflammatory processes. Stimulation of mTOR prevents brown adipogenesis, whereas forced activation of signaling cassette through 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)-induced AMPK activation increases UCP-1 expression and induces an accumulation of brown adipocytes in white adipose tissue (Xiang et al., 2011). Thus in contrast to classical view in which activation of mTOR promotes acquisition of cellular functionality and in which AMPK activation is associated with energy conservation, during brown adipocyte development AMPK activation is associated with the burning of calorie (storage of excessive energy) in a catabolic process (Hardie, 2004; Ronnett and Aja, 2008). It is hoped that further elucidation of the involved processes could further contribute in fight against metabolic disease.

3.2. Oxidative phosphorylation (OXPHOS)

In eukarytic cells, mitochondria are energy supplier of the cell. Within the cell, the inner membrane of mitochondria is the main site of OXPHOS. The nutrients will be oxidized to ensure the release of energy; adenosine triphosphate (ATP). During OXPHOS reaction, the electrons will be delivered from electron donor such as NADH to electron acceptor like oxygen, in a reaction called redox, releasing energy. After oxidation of

NADH in mitochondria, the energy will be avaible to generate energy for the formation of synthase. By flowing electron in electron transport chain, energy comes free to be used to move proton across inner membrane of mitochondria. With respect to OXPHOS, two pints must be noted: 1-OXPHOS is used by mitochondria to provide energy for cell, which is vital for cell and cannot be missed, and 2-From the oxidation process used in mitochondria, reactive oxygen spices (ROS) comes free, which is poison for the cell. In brown adipose tissue that mainly present in baby period and slowly replaced by white adipose tissue in adult phase, UCP plays an important role in providing of energy by mitochondria in the form of heat. UCP cause proton leak during proton transport across inner mitochondria membrane via uncoupling of OXPHOS from ATP synthesis, producing energy in the form of heat (Mitchell and Moyle, 1967).

3.3. White adipose tissue adipocytes

White adipocytes are the dominant cell type in this adipose tissue. This cell type is characterized by a very long half-life and the ability to store increasing amounts of triglycerides in its lipid droplets in mature adipocytes. However, they concomitantly loose their ability to proliferate (Hellerstein, 2003). Interestingly, the structure of lipid droplets is comparable with that of plasma lipoproteins, containing a hydrophobic core coated with a hydrophilic monolayer membrane (Cermelli et al., 2006; Farese, Jr. and Walther, 2009; Guo et al., 2009), however, there are differences with respect to phospholipids and the type of their protein composition. Many proteins are associated with the LD monolayer membrane (Ray et al., 2009).

Disregulation of the lipid metabolism in adipocytes as found in obese subjects can, to a large extent, be attributed to the increased size and storage capacity of the LD (Guilherme et al., 2008). Even in lean subject lipid droplets may occupy a high percentage of cellular volume of the adipocytes (Meijer et al., 2011).

In contrast to brown adipocyte genesis, the generation of white adipose tissue adipocytes is negatively regulated by activation of the AMPK pathway. For instance, it has been shown that various natural compounds like methyl cinnamate or mushroom extracts inhibits adipocyte differentiation via activation of the CaMKK2-AMPK pathway (Chen et al., 2012; Joo et al., 2010; Thyagarajan-Sahu et al., 2011) (rather as LKB1, which is the more common activator of AMPK). In rats, chronic stimulation of AMPK is reported to decrease adiposity through inhibition of adipogenesis (Gaidhu et al., 2011), but whether it is true for humans remains unclear as dieting (which activates AMPK in adipocytes) is not reported to reduce adipocyte number in the short or medium term (Alberdi et al., 2011). A possible explanation is the differences in leptin effects between humans and rodents. In rats, AMPK stimulation seemed to increase leptin effects on adiposity but in humans such effects of leptin are much weaker. More in general, it is expected that AMPK activation should facilitate triglyceride-loading on plasma lipoproteins in adipocytes and indeed in animals treated with AMPK inhibitors lower circulating levels of plasma triglycerides have been reported, but further research into this issue is called for.

3.4. Resident macrophages

The metabolic disorder in adipose tissue leads to an obesity state, which in turn is resulted in an inflammatory environment. Importantly, obesity and its consequent disorders change the resident macrophages to activated macrophages (also called migrated macrophages) in adipose tissue, which is a hallmark of energy metabolism disorder-induced insulin resistance (Meijer et al., 2011; Xu et al., 2003). The resident macrophages of adipose tissue or adipose tissue macrophages (ATM) have attracted substantial attention with respect to metabolic disease as they have been suggested to be the mediators of the low-grade inflammation present in fat tissue of obese people. Indeed, although conclusive evidence is still lacking and until that time other possibilities have to be kept in mind, these resident macrophages spatially seem to have a close interaction with adipocytes in the adipose tissue and this interaction is thought to stimulate cells to secrete the proinflammatory cytokines (Wellen and Hotamisligil, 2005). However, Meijer et al. suggested that the primary event in the sequence leading to chronic inflammation in adipose tissue is metabolic disorder in adipocytes, followed by production of immunological mediators by these cells, which is then exacerbated by activated adipose tissue macrophages and finally the recruitment of immune cells. During the obesity, the density of macrophages in adipose tissue increases, either due to increased chemokine expression (Guilherme et al., 2008) or maybe local proliferation or conversion of mesenchymal stem cells to macrophages (Charriere et al., 2003; Charriere et al., 2006; Cousin et al., 1999; Xu et al., 2003). Notably, the resident macrophages in adipose tissue and other tissues-containing

macrophages also have a role in tissue repair (Harford et al., 2011; Mosser and Edwards, 2008).

Macrophage infiltration increases the fat lipolysis, which leads to an increase of the circulatory free fatty acid (FFA) levels and their sedimentation in the other tissues with a plethora of health-related issues as a consequence (Guilherme et al., 2008). AMPK appears to be a key enzyme that counteracts the high-lipid load-induced inflammatory pressure in these macrophages (Yang et al., 2010). Animals genetically engineered to be defective in macrophage AMPK activity displayed a highly inflammatory phenotype in their adipose tissue macrophages. Inhibiting fatty acid oxidation (a key effector mechanism of AMPK kinase to improve the cellular energy balance) also was pro-inflammatory in these cells and, conversely pharmacological activation of AMPK counteracted inflammation (Galic et al., 2011). If adipose tissue macrophages are indeed of cardinal importance in metabolic disease, pharmacological targeting the associated pathways (e.g. using rapamycin which mimics AMPK activation in its inhibitory effect on mTOR) (Ginion et al., 2011) may prove a viable option in dealing with such diseases.

3.5. Mesenchymal stem cells, fibroblasts and preadipocytes

Adipose tissue is a rich source of mesenchymal stem cells (MSCs), a highly immunomodulatory cell type with the capacity of self-renewal, proliferation, differentiation, plasticity and intimately involved in tissue repair. The concentration of the MSCs in adipose tissue may be hundreds more than bone marrow (Franco Lambert et al., 2009; Riordan et al., 2009;

Zuk et al., 2002) and thus it is reasonable to assume that this cell type can influence the physiology of fat. Although autophagia is in general associated with protection of cells from hypoxic and hyponutrient stress (Kanamori et al., 2011), this functionality of the autophagic response may be stronger in the MSC compartment. Indeed AMPK activation has emerged a major mediator for MSC survival following hypoxia through AMPK-mediated mTOR inhibition-dependent autophagic responses (Zhang et al., 2012). Mesenchymal stem cells are able to differentiate into preadipocytes and their precursor fibroblasts (Cristancho and Lazar, 2011). The molecular details to govern the transition of fibroblasts to pre-adipocytes are slowly emerging and involve regulation through the prolyl isomerase Pin1, possibly through enhancing mTOR signaling by stabilizing its upstream activator PKB/Akt. Pre-adipocytes have multilocular lipid droplets (Nishino et al., 2008) (in contrast to mature adipocytes which contain one droplet) and are thought to be immune active cells through phagocytic and antimicrobial properties (Wellen and Hotamisligil, 2005). Profiling indicates that molecularly they are closer to macrophages than adipocytes and might be able to differentiate into the former cell type (Charriere et al., 2003). Remarkably, in obese subjects the capacity of preadipocytes to differentiate adipocytes is impaired (Isakson et al., 2009). In general, preadipocytes act as a link between the metabolism and innate immunity (Charriere et al., 2003). It must be noted that autophagy is necessary for adipogenesis and regulation of adipose mass (Singh et al., 2009b; Zhang et al., 2009). As mentioned earlier AMPK activation inhibits differentiation of pre-adipocytes to white adipocytes but facilitates the transition to the brown adipocyte type (Daval et al., 2006). In toto, AMPK activation has a protective role in these components of fat tissue, whereas mTOR activation increases sensitivity of adipose tissue lipid uptake.

3.6. Perspectives with regard to the physiological energy metabolism

When the details of nutrient pathway signaling started to emerge, it was always assumed that the way forward of employing this information for improved treatment of metabolic disease would lie in negatively regulating of this system not clear. It was reasoned that if this system enhances cellular conservation and increase metabolic efficiency, it would reduce energy requirements and thus increase adiposity. It has now become clear, however, that the situation is quite reverse. AMPK activation increases brown adipocytes, thus enhancing energy metabolism while simultaneously promoting lipolysis in white adipocytes and protecting adipose tissue resident macrophages from inflammatory responses. The combined anti-adiposity profile and anti-inflammatory profile resulting from AMPK stimulation is expected to be highly beneficial when managing metabolic disease and thus pharmacological therapy of this disease through manipulation of nutrient signaling pathways appears highly promising.

4. OTHER COMPONENTS OF THE ENERGY METABOLIC SYSTEM

4.1. Skeletal muscle

An important tissue in the metabolic system is skeletal muscle, as it is vastly distributed through the body and is the main consumer of the energy (Gross et al., 2008). Malfunction of this tissue affects the level of glucose consumption and is therefore strongly related to hyperglycemia. There is a close correlation between functioning of adipose tissue and skeletal muscle. A hypothesis is that altered release pattern of free fatty acids into the circulation from an abnormally inflamed adipose tissue provokes insulin resistance in the muscle and aggravates hyperglycemia (Guilherme et al., 2008).

4.2. Liver

Hepatocytes, in the normal situation, do not have any vast direct effect in the consumption or storage of energy in the form of lipids, but their influence in the glucose-related metabolic pathways, including glycolysis, glycogenesis (Thorens, 2008), gluconeogenesis (Koo et al., 2005) is obviously huge and the liver is location of the storage and release of the glucose in response to excess of the energy or to starvation. Hepatocytes also profoundly express genes involved in lipid metabolism (Du et al., 2007) and are capable of synthesizing triglycerides (Kabashima et al., 2003) and

loading them on lipoproteins for transport to the adipocytes or other tissues (Glaumann et al., 1975). Indeed, they also synthesize the apolipoproteins and can assemble these into lipoproteins such as VLDL and HDL (Wroblewska, 2009). Also important in this respect are the synthesis of the cholesterol, bile acids, the synthesis of the coagulation factors, absorption and lipolysis of the chylomicrons etc (Merkel et al., 2002).

Primordial multicellular organisms probably combined the functions of the adipocyte and the hepatocyte in a single cell type and (in) Drosophila still the liver together with adipose tissue and macrophages are one unit naming "fat body" organ (Balistreri et al., 2010). Maybe, the fatty liver state associated with obesity still reflects something of this ancient evolutionary heritage as this disease is characterized by fat accumulation in the liver and also fat storage in the macrophages (foam cells). The latter are also important in atherosclerosis (Horowitz, 2003; Wellen and Hotamisligil, 2005).

4.3. Macrophage compartment

Macrophages are the main cells of the innate immune system, provide a wide array of functionalities including phagocytosis, and tissue repair (Tidball, 2005). They are distributed throughout the body and are reside inside the different tissues including adipose tissue (ATM), where are involved in obesity-associated low-grade inflammation by enhancing malfunction of the tissue in lipid sequestration of the lipids and lipolysis. Chronic inflammation in the adipose tissue is thought to be the trigger of systemic inflammation and insulin resistance in the metabolic syndrome

(Guilherme et al., 2008). In addition macrophages seem to have a role in coagulation, linking this cell type to atherosclerosis (Hansson, 2005).

4.4. Kidneys

Kidney cells are one of the main components of the metabolic system and thus intimately involved atherosclerosis in addition of a plethora of other diseases. Their most important metabolic roles lie in the gluconeogenesis (Meyer et al., 2002; Meyer et al., 2003), glycogenolysis (Stumvoll et al., 1999), and urea biosynthesis, which has a link to the Krebs cycle through arginosuccinate (Haines et al., 2011). The components of the renin-angiotensin system (RAS) particularly angiotensin converting enzyme (ACE), and angiotensin II, type-1 receptor (AT1-R), are also involved in the pathogenesis of the atherosclerosis (O'Brien, 2006). Through this thesis I do not explicitly aim to study the role of this organ, but its importance does warrant study of its physiological function.

4.5. Pancreas

The pancreas secretes the main metabolic hormones of the body, insulin and glucagon. Insulin in normal situation secretes after postprandial hyperglycemia by the beta cells and stimulates glucose and amino acid absorption. Glucose absorption by the beta cells increases their ATP/ADP ratio, which leads to the closure of ATP-sensitive K+ (KATP) channels and thus to membrane depolarization. In turn depolarization causes opening of

the voltage-dependent Ca^{2+} channels (VDCC) and the subsequent Ca^{2+} influx increases free cytosolic Ca^{2+} concentration and stimulates exocytosis of preformed insulin-containing-vesicles and causes hyperinsulinemia immediately after meal to counteract the increases in blood glucose levels (Holz, 2004; Kawaguchi et al., 2006; Thorens, 2008). Insulin enhances the gene expression and synthesis of the glucose transport proteins (GLUT) and transport of the vesicles containing GLUT to the membrane to stimulate the absorption of glucose by the cells of the body, which is enhanced by AMPK (Lim et al., 2010). Following the increase of the intracellular glucose concentration, the pathways of the glucose catalysis (e.g. glycolysis) and glucose-stored pathways (glycogenesis) become stimulated and generate energy which is then available for anabolic purposes. Generally, insulin mainly activates the synthesis pathways of the glycogen, lipids, and proteins and inhibits their degradation. Protein synthesis leads to the hypertrophy of the cells and anabolic lipogenesis (Zomeno et al., 2010) stores lipid in the adipose tissue, skeletal muscle, and liver (Suh et al., 2005). Insulin also has a stimulatory effect on the secretion of the alpha cells-glucagon, which is secreted following hypoglycemia and has an opposite effect to that of insulin (Kawamori et al., 2009). Glucagon functions through cAMP-induced PKA activation and increases the lipolysis of the triglycerides by stimulation of the hormone sensitive lipase (HSL) enzyme (Plee-Gautier et al., 1996; Watt et al., 2004). It stimulates glycolysis in the muscles but reduces it in the liver to increases the glucose availability for the muscle cells through the circulation (Jiang and Zhang, 2003). It stimulates the liver phospholipase and glycogenolysis (Pilar et al., 1991), and inhibits (3-hydroxy-3-methylglutaryl-coenzyme A) HMGCoA reductase and cholesterol synthesis (Edwards et al., 1979). Current dogma is that the

pancreas is the sole source of insulin in the body, but in the present work I shall challenge this notion.

4.6. Hypothalamus

The hypothalamus and the associated pituitary gland form the central appetite and energy balance regulator through the production of a variety of endocrinological mediators (GIP, peptideYY, ghrelin, cholecystokinin, GLP-1, oxyntomodulin) (Stanley et al., 2005) thus influencing the pathways of the energy balance such as AMPK (Kola, 2008). Hypothalamic AMPK itself has a critical role in the central regulation of energy balance, but the exact functionality with respect to mediator release remains unclear (Xue and Kahn, 2006). Leptin mediates its anorectic effect via increasing the stimulation of the MC4 receptor signaling and inhibition of the central AMPK (Kahn et al., 2005). Inhibition of the hypothalamic AMPK reduces food intake, fat mass body, body weight, and hepatic glucose production (Brabant et al., 2004) as well as stimulates energy expenditure and consequently energy balance (Lim et al., 2010; Morton and Schwartz, 2011). The hypothalamus also influences on the pituitary gland and can have influence on the functionality of the metabolic-related hormones such as growth hormone, adrenal gland, thyroid gland, and androgens through the hypothalamic–pituitary–adrenal or thyroid axis or effects on the reproductive system. It also affects the autonomic nervous system (ANS), which can have influence on the metabolic system (Kalsbeek et al., 2010).

4.7. Perspectives

Many different tissues act in conjunction with the adipose compartment to regulate energy balance. Much of the communication involved is mediated via lipoproteins. In this thesis I shall further characterize composition and functionality of these molecules.

5. LIPOPROTEINS

The aqueous environment of the body poses problems with the transport of hydrophobic substances between the organs. Hence, animals have evolved plasma lipoproteins that facilitate the transport of triglycerides and cholesterol between the liver, adipose compartment and tissues (Dashti et al., 2011). Originally these structures were not thought to be of major importance for transmitting endocrinologically relevant signals, but this view is now changing. In 2006, Panakova and collegues (Panakova et al., 2005) showed that lipophorin (the Drospholia equivalent of VLDL) carried hydrophobic ligands activating Wnt and hedgehog morphogen pathways and these effects were physiologically highly relevant in the model organism, whereas in past years we have shown that the morphogen is carried by lipoproteins in humans as well (Bijlsma et al., 2006a; Bijlsma et al., 2006b; Queiroz et al., 2010). Interestingly, we recently reported that vitamin D resides in LDL and that this is relevant for modulating signaling in atherosclerotic plaques (Bijlsma et al., 2006c; Queiroz et al., 2012). Apart from hydrophobic morphogenes, also other hydrophobic signaling molecules may reside in lipoproteins. Many hormones are highly

hydrophobic and evidence is available that lipoproteins may be involved in their efficient transport through the body. In $ApoE^{-/-}$ mice for instance, both endocannabinoid (Bartelt et al., 2011) and glucocorticoid (Raber et al., 2000) signaling is disturbed and is strongly associated with inflammation, insulin resistance and hepatic steatosis, suggesting endocannabinoid carrying by lipoproteins is a relevant process. Thus the lipoprotein compartment may have an underestimated role in the human endocrinology.

5.1. Lipoprotein constituents

Lipoproteins are biochemical transporters that are composed of proteins and lipids such as triacylglycerol (TAG), free cholesterol, cholesterol esters, and phospholipids like phosphatidylcholines (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and sphingomyelin (SM)) (Figure 3) (Dashti et al., 2011).

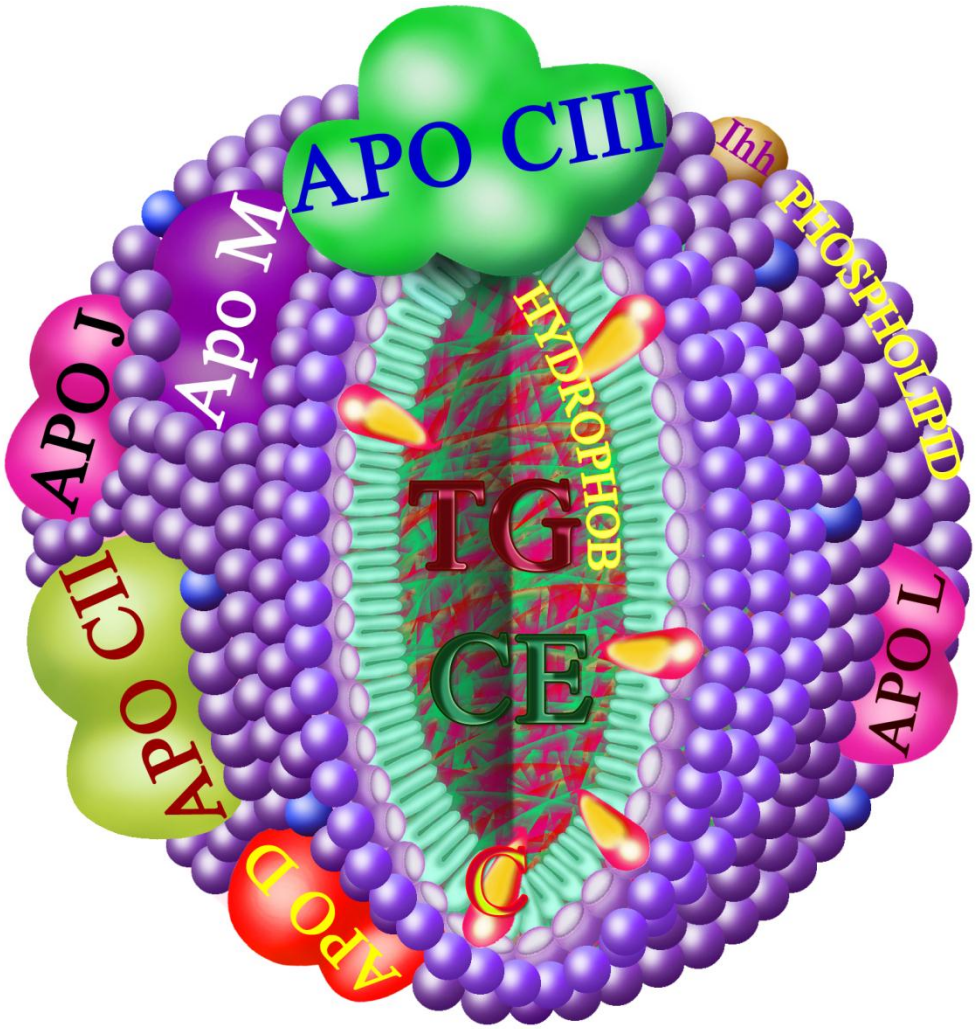

Figure 3. The structure of a Lipoprotein.

Plasma lipoproteins composed of a hydrophobic core containing triglycerides (TG), cholesterol (C) and cholesterol ester (CE) surrended by a phospholipid layer, which is highly hydrophilic. The major constituted proteins of lipoprotein particles are called apolipoproteins such as apolipoprotein M, apolipoprotein J (also called clusterin) etcetera..

Different compositions of proteins and lipids in these assemblies produce different kind of lipoproteins that the main ones based on their diameter and

density, which represent the percentage of their proteins and triglycerides are high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoproteins (IDL), very low-density lipoprotein (VLDL), and chylomicrons (CM) respectably (Mahley et al., 1984). HDL is the smallest and has more density with the highest percentage of proteins. Their structure consists of an amphipathic monolayer of lipids, which is composed of the assembly of the hydrophilic headgroups of the phospholipids and free (nonesterified) cholesterol together with the (apo)proteins that face to the aqueous phase and cover the intra hydrophobic (non-polar) part of their structure which are triglycerides and cholesterol esters (Hoofnagle and Heinecke, 2009). The polarity of the surface lipoproteins avoids their aggregation and let them to be solubilized in the circulation (Tzen et al., 1992). The main function of lipoproteins in the body is transport of the lipids through the circulation from the source of lipids such as intestine and liver to the other tissues that require these fats as their energy suppliers or use as structural materials such as membrane. The particle proteins via their interactions with each other and also with the other proteins including enzymes, and the cell surface proteins determine whether the lipids should be absorbed from or exported to particular tissues. The overall metabolism and structure of the lipoproteins is determined with apolipoproteins and also the interactions with the peripheral receptor molecules.

5.2. Lipoproteins and disease

LDL through the distribution of cholesterol from the liver to the tissue and especially the endothelium poses pathological challenges relating to atherosclerotic plaque formation (Bijlsma et al., 2006a). Conversely, HDL is involved in the transport of lipids from tissues and back to the liver or excrete from the body; a process called reverse cholesterol transport (RCT) (Mulay et al., 2012). Thus, HDL has a protective effect in the body. Chylomicrons and VLDL are involved in the transport of triglycerides, whereby chylomicrons transport the newly absorbed triglycerides from intestine to the skeletal muscle for consumption, adipose tissue for storage or to the liver to be used for synthesis of VLDL and secreted to the circulation for transport to the adipose tissue. Another nomenclature, which is used for explanation of the lipoprotein metabolism pathway, based on the origin of their lipid, is exogenous and endogenous lipoproteins pathways in which the lipid components of the lipoproteins are synthesized by intestine from the dietary lipids or liver respectably (Rivellese et al., 2004). In the exogenous pathway, the intestinal lipid (triglycerides, phospholipids, cholesterol), are absorbed by the epithelium and assembled with apo B48 to make the nascent chylomicron (nCM) and secreted to the lymphatic vessels (via its apoproteins) and release directly (bypass liver) to the circulation via the subclavian vein. In the blood, HDL delivers its apolipoproteins (C II and E) to the nCM to make a mature chylomicron (mCM). These exchangeable apolipoproteins protect the TG-rich particles (CM and VLDL) from non-specified interaction with the plasma and lead to their correct configuration for the action of lipoprotein lipase (LPL) enzyme. In the skeletal muscle and adipose tissue, the mature chylomicron via activation of the endothelial LPL

and its cofactor apolipoprotein C II ensures the hydrolysis of triglyceride to the glycerol and fatty acids to be consumed or stored by the tissues and in particular adipose tissue. The hydrolyzed chylomicron, known as remnant chylomicron (rCM), is transferred to the liver through the circulation, which in turn rCM is end up to the endocytosis mediated by the interaction of apolipoprotein E with the rCM receptors in the liver (Adiels et al., 2008; Austin et al., 1998; Mahley et al., 1984; Merkel et al., 2002; Sarwar et al., 2007). Ultimately, rCM are degraded in lysosome, which is resulted in the release of fatty acids and glycerol (Frayn, 1998). In the endogenous pathway, liver is the main source of VLDL lipid constituent. The assembly of intracellular triglyceride and cholesterol in the liver are maintained by the apolipoprotein B100 and delivered to the circulation by lipid transporters (Frayn, 1998; Rivellese et al., 2004). The same as chylomicrons, HDL provides apoE and apoCII to VLDL (Murdoch and Breckenridge, 1995; Murdoch and Breckenridge, 1996). VLDL is also considered as triglyceride-rich lipoprotein particle. LPL ensures the release of fatty acids and glycerol via hydrolyzation of triglycerides. VLDL plays, thus, an important role in the delivery of free fatty acid (FFA) to the adipose tissue to store energy as inactive fuel in the form of triglycerides in the lipid droplets (LD) within adipocytes or supply active energy for skeletal muscles and other tissues via FFA delivery (Frayn, 1998). Many studies have shown that hypertriglyceridemia (with VLDL as TG carrier) the same as hypercholesterolemia (with LDL as cholesterol transporter) is directly linked to the risk of cardiovascular morbidity and mortality (Ezenwaka and Davis, 2000). IDL as interplayer between VLDL and LDL is formed by the hydrolization of remnant VLDL (Mahley et al., 1984) and the released energy components in the form of glycerol and fatty acids will be used by the skeletal muscles and adipocytes.

As in chylomicrons, interaction of the rVLDL with their cognate receptors of the liver are ended to the hydrolyzation to constitute the rIDL or LDL lipoproteins. LDL has a similar fate as rCM. The release cholesterol in the endothelial cells is phagocytized by the macrophages and form the foam cells (macrophages loaded with lipids), which is one of the main component of the atherosclerosis (Hansson, 2005).

Atherosclerosis is an inflammatory disease that is triggered by oxidation of LDL cholesterol (LDL-C), which in turn the ox-LDLs are trapped in the extracellular matrix of the sub-endothelial space. The ox-LDL is absorbed by the tissue macrophages to form the foam cells and promote inflammatory gene expression (e.g. NFkB pathway) that initiate the inflammatory response. Inflammation primes the formation of fatty streak and in turn the arterial calcification (Ross, 1993; Ross, 1999). This is starting for the aggregation of the coagulation cells that express different coagulation factors for initiation and maintenance a coagulation state for a short period and tissue repair that follows with stimulation of the fibrinolysis pathway for ending and removing this process (Cesarman-Maus and Hajjar, 2005)

5.3. Lipoprotein lipase (LPL)

These pancreatic, hepatic, and endothelial lipases are soluble enzymes whose main function lies in the hydrolysis of the triglycerides of the circulatory lipoprotein particles (mainly the triglyceride-rich chylomicrons and VLDL) and convertting the triglycerides to non-esterified fatty acids (NEFA) and 2-monoacylglycerol (Chatterjee and Sparks, 2011; Funaki, 2009; Guilherme et al., 2008). The apolipoproteins on lipoprotein particle

surface are often cofactors (especially apoC2) for the function of the LPL although apoC3 is an inhibitor of LPL activity (Shachter, 2001; Yu and Ginsberg, 2005). Apart from its enzymatic function, LPL also acts as a linker between lipoproteins and cell surface receptors/proteoglycans and thus facilitates the uptake of lipoproteins by the tissues (Yu and Ginsberg, 2005). In the literature, malfunctioning of LPL has been associated with a plethora of pathological conditions including atherosclerosis, obesity, diabetes, chylomicronaemia, Alzheimer disease, and cachexia (Mead et al., 2002; Takasu et al., 2012). The enzyme is produced by the parenchymal cells of the liver, but also in the adipocyte and a variety of other cell types including macrophages (Mead et al., 2002). Much is known about the factors guiding its expression, which involves a complex interaction between sterol regulatory element binding protein (SREBP), cytokines, lipopolysaccharide (LPS) (Mead et al., 2002), peroxisome proliferator-activated receptor (PPAR)-α , cAMP response element binding protein (CREBP) and activator protein-1-like factors (Mead et al., 2002; Sartippour and Renier, 2000; Staels et al., 1996). In addition, expression of LPL is also controlled by nutritional status, blood glucose levels, fatty acids, and the levels of hormones as insulin, catecholamine and thyroid, growth hormone, estrogen, prolactin, parathyroid hormone, retinoic acid and vitamin D3 (Mead et al., 2002). In addition, the level of LPL production especially in adipose tissue and skeletal muscle determine whether dietary lipids will mainly be stored in the fat tissue or used for energy consumption respectively. Thus, the control of LPL production is important for understanding obesity and pathological weight loss (Kageyama et al., 2003; Pedersen et al., 1999). Food intake increases LPL activity in adipose tissue, whereas exercise leads to a higher level of enzyme activity in the skeletal muscle (Frayn, 1998). Of note, expression of LPL by adipose tissue and

skeletal muscle is often considered to constitute an anti-atherosclerotic influence, while the expression of LPL by the endothelial cells and macrophages has a pro-atherosclerotic effect. Insulin stimulates LPL activity in the adipose tissue, whereas insulin resistance is associated with a proinflammatory state of the adipose tissue in which production of the cytokines such as TNFα and IL-6 reduce the expression of LPL in the adipose tissue (Frayn, 1998). In turn this provokes hypertriglyceridemia and susceptibility to the coronary artery disease. Conversely induced-inhibition of adipose tissue-associated LPL is also the pathophysiology of the cachexia in the cancers (Sakayama et al., 2008).

Thus it is expected that anti-geriatic factors, if they exist, should also influence LPL activity. In this thesis I want to explore if such factors might exist and try to link these to adipose tissue physiology and the lipoprotein compartment. Through this thesis I shall especially explore the role of the hedgehog morphogen in this context, albeit largely theoretically if not experimentally.

5.4. Hedgehog signaling pathway linked with age-related diseases; Atherosclerosis

Hedgehog signaling pathway is one of the evolutionary conserved pathways that have a role in regulation of embryonic development (Casso et al., 2011), stem cell network signaling (Katoh, 2007) and energy metabolic system (Cousin et al., 2007). Therefore, it has effect on the age-related diseases including diabetes (Thomas et al., 2000) and atherosclerosis (Bijlsma et al., 2006a; Doherty et al., 2003) through the common cell types

mainly fibroblasts (Bijlsma and Spek, 2010; Choi et al., 2010; Lavine et al., 2006), monocytes (Dunaeva et al., 2010), and mesenchymal stem cells (MSCs) (Fontaine et al., 2008).

Adipose tissue and adipocytes as the axis of the energy metabolic system regulation is regulated by the function of the hedgehog signaling pathway (Cousin et al., 2007). The anti-adipogenic and osteogenic capacity of the hedgehog signaling through the MSCs link the adipose tissue to the bone and explain the etiology of osteoporosis in the senescent people (Cousin et al., 2007; James et al., 2010). In this thesis I shall explore the notion that Hedgehog constitutes an anti-geriatric signal, also with respect to atherosclerosis (Figure 4) (Dwyer et al., 2007; Bijlsma et al., 2006a; Morrow et al., 2009). The therapeutically benefits of Hedgehog in vascular disease are particularly evident from a studies involving Shh myocardial gene therapy in counteracting experimental chronic myocardial ischemia (Bijlsma et al., 2006a; Bijlsma and Spek, 2010; Kusano et al., 2005), whereas also experimental peripheral ischemia benefits from ectopic expression of Shh (Luo et al., 2009). The effects here are not as much that the atherosclerotic process per se inhibited but that Hedgehog signaling mediates shunting angiogenesis in addition to effects on apoptosis as described above for neurons. Conversely, genetically or pharmacologically interfering with Hedgehog signaling compromises endothelial functionality and thus prepones age-related diminished function of the vasculature (Kanda et al., 2003; Luo et al., 2009), whereas ischaemic insult by itself provokes substantial production of Hedgehog (Bijlsma et al., 2006a; Kusano et al., 2005). These effects of Hedgehog improving endothelial function are multifold, but especially increased secondary production of angiogenic factors, increased survival signaling of endothelial cells through phosphatidylinositol-3-OH-kinase and recruitment of endothelial precursor

cells have been well-documented. In toto, the evidence that Hedgehog signaling counteracts age-related changes in the endothelial compartment is compelling and thus Hedgehog production represents an important anti-geriatric signal here, although the effects do not as much prevent the atherosclerotic process but help in limiting the damage inflicted.

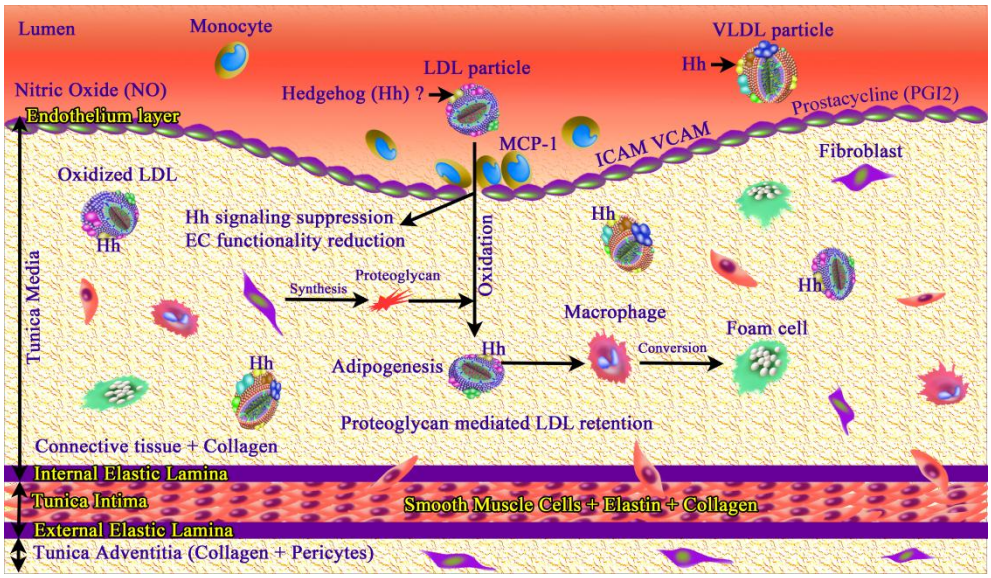

Figure 4: The role of Hedgehog and the endothelial function.

In the circulation, VLDL is considered as carrier of Ihh, although LDL is also suggested as hedgehog transporter but there is no evidence for this notion. A normal arteriole is composed of endothelium, and the basement membrane, which is composed of a collagenous connective tissue layer, consisting of collagen fibres, smooth muscle cells (SMCs) and elastic fibers that provide support to the vessels. The internal and external elastic laminaes (IEL and EEL) line the two sides of the SMC layer and the EEL separates the SMCs from a last layer, which is called adventitial layer. In a healthy vessel, the intact internal elastic lamina, which are lies directly peripheral from the endothelial cells. Under the intact state, hedgehog signalling suggested to be involved in the improvement of endothelial function. A suppression of hedgehog signalling may lead to less integrity of endothelial cells, which in turn make it vulnerable to damage by OX-LDL. The process of plaque formation and inflammation starts by local deposition and trapping of LDL molecules. LDL may

become subject of oxidation and the resulting oxidized-LDL (ox-LDL), is efficiently taken up by macrophages, which are not well capable of dealing with lipid load involved and form foam cells. During the subsequent atherosclerotic reaction, the IEL layer is degraded by macrophage-cathepsin K allowing the SMCs to migrate from media to the nascent plaque. Pericyte and myofibroblasts in atherosclerotic plaque are subject to aberrant differentiation events. In plaque, a suppressed hedgehog signalling may promote adipogenesis locally. **VCAM:** Vascular cell adhesion molecule-1, **ICAM-1:** Intercellular adhesion molecule-1, **MCP-1:** monocyte chemoattractant protein-1.

6. ADIPOSE TISSUE: FUNCTIONS, PROPERTIES, AND ITS ROLE IN THE METABOLIC DISORDERS

Despite its negative connotations in these times of the obesity epidemic, fats play a fundamental role in our body. Adipose tissue is intimately involved in functions such as temperature isolation, structural support of organs, endocrinology, immunity and obviously the storage excess energy in the form of triglyceride. Lipid droplet-sequestration of excessive energy is one of the characteristic functionalities of the adipose tissue and it is enhanced via the pattern of the lipid droplets-associated proteins such as FSP27 (Farese, Jr. and Walther, 2009; Guilherme et al., 2008).

FSP27 enhances the unilocularization of the separated growing lipid droplets mainly in the subcutaneous adipose tissue (Nishino et al., 2008; Puri and Czech, 2008). The uniocular lipid droplets have a better capacity of the sequestration and storage of lipids than the multicular droplets because of their lower surface contact with the lipolytic enzymes. In the adipose tissue and in particular adipocytes, the expression of FSP27 protein is 100 times higher as the other cell types like for instance the hepatocytes (Puri

and Czech, 2008). The overload of the free fatty acids in the circulation and the peripheral tissues such as skeletal muscle and liver and the absence of a functional lipid droplets in these tissues lead to lipid metabolites-induced insulin resistance and consequently organ dysfunction and hyperglycemia (Farese, Jr. and Walther, 2009; Hirabara et al., 2010). One assumption is that the dysfunction of lipid droplets results from an overload of triglycerides that provoke accumulation of long chain fatty acids (LCFA) in the cytoplasm of adipocytes and consequently incapacity of the mitochondria to process them (Funaki, 2009; Guilherme et al., 2008; Kennedy et al., 2009; Watt and Spriet, 2010). In addition, the peroxisomes may not be able to provide adequate anti-oxidative resistance to cope with the reactive oxygen species (ROS)-stress following lipid overload (Delille et al., 2009; Ye and Keller, 2010). The released free fatty acids are shuttled back into the circulation (FFAs pass through the membrane and enter the circulation via FFA transporters) compounding problems elsewhere in the body (Hotamisligil, 2006; Hummasti and Hotamisligil, 2010; Zhao and Akerman 2006). Figure 5 shows the structure of a lipid droplet.

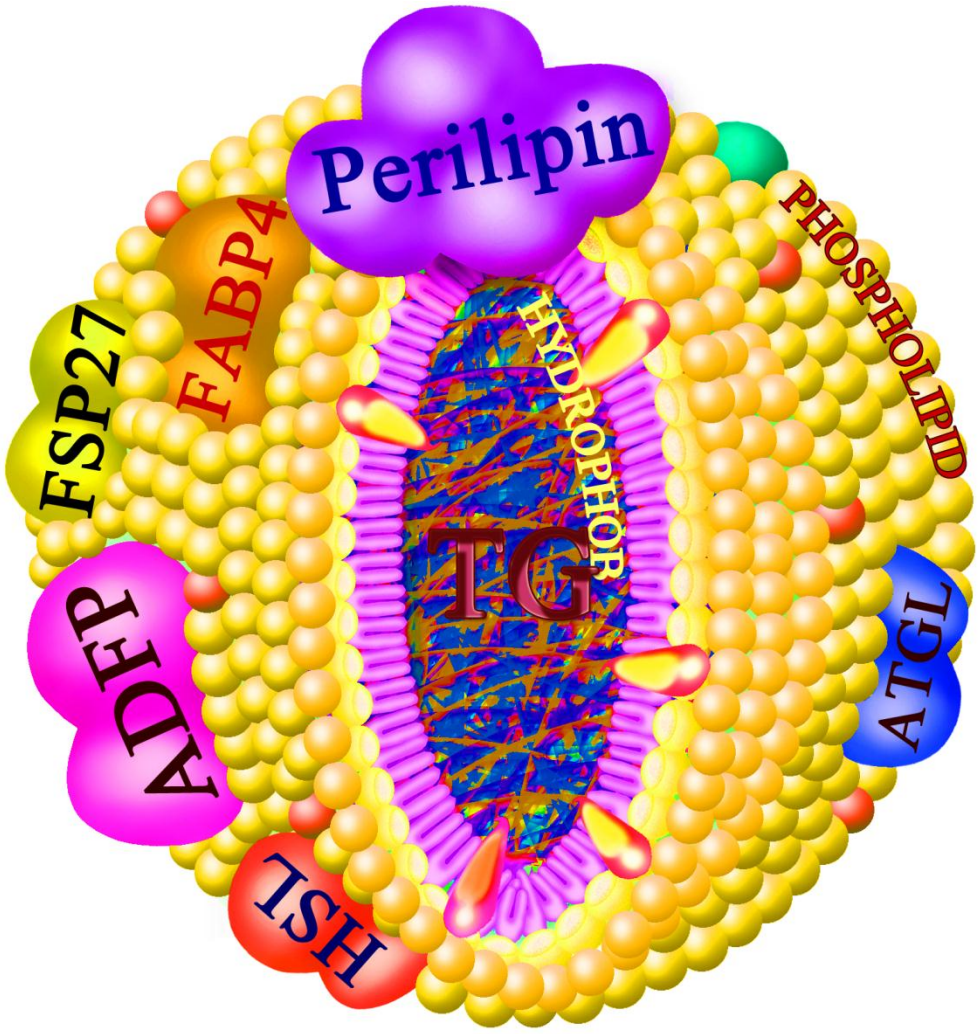

Figure 5. The structure of a Lipid droplet.

The lipid droplet consisted of a hydrophobic core containing triglycerides (TG), Cholesterol (C) and cholesterol ester (CE) coated by a hydrophilic phospholipid layer. The major constituted proteins of lipoprotein particles are Perilipin, Adipose differentiation-related protein (ADRP = ADFP = adipophilin)), Fatty acid binding protein-4 (FABP-4), adipose triglyceride lipase (ATGL) and Fat specific protein-27 (FSP-27).

6.1. Endocrinological function of the adipocyte compartment

From the endocrinology point of view, adipocytes are the source of a number of adipokines, which affect the function of central nervous system (CNS) and metabolic tissues with a major role in maintenance of the systematic energy status of the body (Morton and Schwartz, 2011). During the obesity, hypertrophy and hyperplasia are hallmark of malfunction of adipose tissue, which is reflected in protein catalogue of adipose tissue-secreted proteins (Balistreri et al., 2010). Therefore, the secretion of the physiological amount of the adipokines (Guilherme et al., 2008; Hotamisligil, 2006; Yu and Ginsberg, 2005) is linked to the proper function of the metabolic system. Pro-inflammatory cytokines, which in general are main drivers of the innate immune response, are produced by adipose tissue at levels comparable to that to monocytes, thus the adipocyte compartment is an important source of inflammatory hormones (Meijer et al., 2011). It is easy then to envision that obesity provokes a chronic low grade inflammation, in turn important for the initiation of energy metabolic disorders. Intriguingly, comparative morphology suggests a close relationship of adipose tissue to the liver and to a lesser extent also the pancreas, suggesting that liver- and pancreas-derived endocrinological factors may be produced by adipose tissue as well. Indeed through this thesis we will show novel functionality of the adipocyte in the secretion of insulin (an important pancreas-derived endocrinological signal) and coagulation factors (which according to conventional wisdom are produced solely in the liver).

6.1.1. Hemostasis

The coagulation/fibrinolysis system is essential for wound healing after vascular injury. According to the canonical view of hemostasis, the synthesis of coagulation factors is exclusively restricted to the liver, platelets and the endothelium, but in this thesis we will challenge this notion.

The coagulation and fibrinolysis pathways are two main constituents of the hemostasic process. The capacity of the coagulation cascade to repair vascular injury provoked by loss of blood vessel integrity is crucially dependent on the presence of coagulation factors. This dependence is emphasized by the serious health problems observed in patients suffering from the congenital absence of such factors and modulators (e.g., thrombomodulin (TM) or protein C), which normally ensure rapid and appropriate fibrin clot formation. Also, there is a tightly controlled balance between clot formation and fibrin degradation tuned to the vascular injury and wound healing process, which finally lead to removal of clot from circulation. The malfunction of each of these two pathways, regarding the failure or deficiency, or malfunction of any factors of these two cascades lead to varying degree of either uncontrolled bleeding (hemorrhaging) or clotting (thrombosis). Thus studies as to the source of coagulation factor production are highly relevant.

6.1.2. Hemostasis and the vascular system

The blood vessel is composed of different layers. The first inner layer of the vascular system is made out of a single layer of endothelial cells that

protects the basement membrane and has a negative charge that repels the circulating proteins and platelets (Amoroso et al., 2001). In an intact state of vascular endothelium, blood circulates within the vessel and the hemostatic components stay in the circulation in an inactive form. Endothelium, which is coated by glycocalyx, except for controlling the permeability of the vessels and the rate of the blood flow, is able to produce and release the products that have a stimulatory or inhibitory effect on the platelets, coagulation, and fibrinolysis pathways (Amoroso et al., 2001; Reitsma et al., 2007). In an intact endothelium, prostacyclin and nitric oxide (NO) have a negative role in platelet aggregation (Yao et al., 1992). Presence of the membrane-associated molecules, thrombomodulin (TM) and heparin-like molecule, on the endothelial surface membrane via inactivation of specific coagulation factors are able to block clot forming (Irokawa et al., 1997). After a vascular injury, tissue factor (TF) released from the damaged endothelial layer activates the extrinsic coagulation pathway (also called contact pathway) and forms a complex with circulating factor VIIa (FVIIa), resulting in the activation of factor X (FX) (Mackman, 2009). In addition, factor VIII (FVIII) is an important cofactor that plays an unambiguous role in the activation of FX in the intrinsic tenase complex (FIXa, FVIII and FX) (Fay, 2004; Saenko and Scandella, 1997; Webster et al., 1976). It must be noted that the prothrombinase complex, a target of both coagulation pathways, plays a major role in the formation of the fibrin clot, and that the coagulation factor FX and its cofactor, factor V (FV), are key components of the complex. Monocytes and platelets are two main cell types, which arrive at first at the site of vascular injury. Primary hemostasis is the first step in initiation of the coagulation. In the primary hemostasis the vasculature system and platelets (thrombocytes) have a crucial role. Platelets' function happened in four phases e.g. 1- platelet activation 2-

platelet adhesion 3- platelet aggregation and 4- platelet secretion. These stages are triggered following diversion of the normal environment of platelets. After endothelial damage, hemostasis is started with immediate vasoconstriction of the arterioles in order to both minimize the blood flow to the origin and prevent blood loss. This is a fast and short event, which the function is taken over by other parameters to complete wound healing process (Troy, 1988).

After vascular repair, tissue plasminogen activator (tPA) activates plasminogen-plasmin system, which ensures the lysis of clot and removal from circulation. In the sub-endothelium part, the basement membrane composed of a collagenous connective tissue layer, which is consist of collagen fibers as well as smooth muscle cells (SMCs) and elastic fibers that provide support to blood vessels. The internal and external elastic laminaes (IEL and EEL) lining the two sides of the SMCs layer and the EEL separate the SMCs from the last layer referred to adventitial layer. In the normal state, the intact internal elastic lamina, which is lined under the endothelial cells, prevents the migration of the SMCs into the sub-endothelial connective tissue part. But in an atherosclerotic state, this layer is damaged; the SMCs pass through the penetrated IEL layer and migrate towards fibrous cap (Packard and Libby, 2008).

6.1.3. The inflammatory system and the coagulation system; Atherosclerosis

Atherothrombosis is a complex of thrombus and atherosclerotic plaque and a major cause of morbidity and death worldwide. In addition, atherosclerosis is an inflammatory disease, which started at the very young age and takes decades to be symptomatic (Ross, 1993; Ross, 1999).

Atherosclerosis and atherothrombosis are thus aged-related diseases. We already discussed the role of hedgehog to exert antigeriatic influence.

6.2. Metabolic disorders

With respect to metabolic diseases, in this thesis I want to limit myself to those that are related to the regulation of the circulatory glucose levels with common clinical and outcome parameters such as T2D, metabolic syndrome, and lipodystrophia. In this part of introduction I aim to provide further background on the definition, manifestation, pathophysiology of these diseases.

6.2.1. Determinants of the circulatory glucose level

Circulatory glucose levels depend on four parameters: prandial glucose uptake, consumption of glucose by the tissues, the capacity of the body to store excess glucose in the adipose tissue, and the endogenous glucose production through gluconeogenesis in the liver and kidney (Thorens, 2008). Blood glucose levels are controlled through insulin, an anabolic endocrinological signal that enhances the glucose uptake by the cell. Among the tissues, especially the skeletal muscles and the adipose tissues are the main consumer and storage place of blood glucose (Figure 6). Successful blood glucose control depends on concerted action between several tissues, hormones together with the operation of the autonomic nervous system (Huopio et al., 2002; Lins et al., 1986; Morton and Schwartz, 2011).

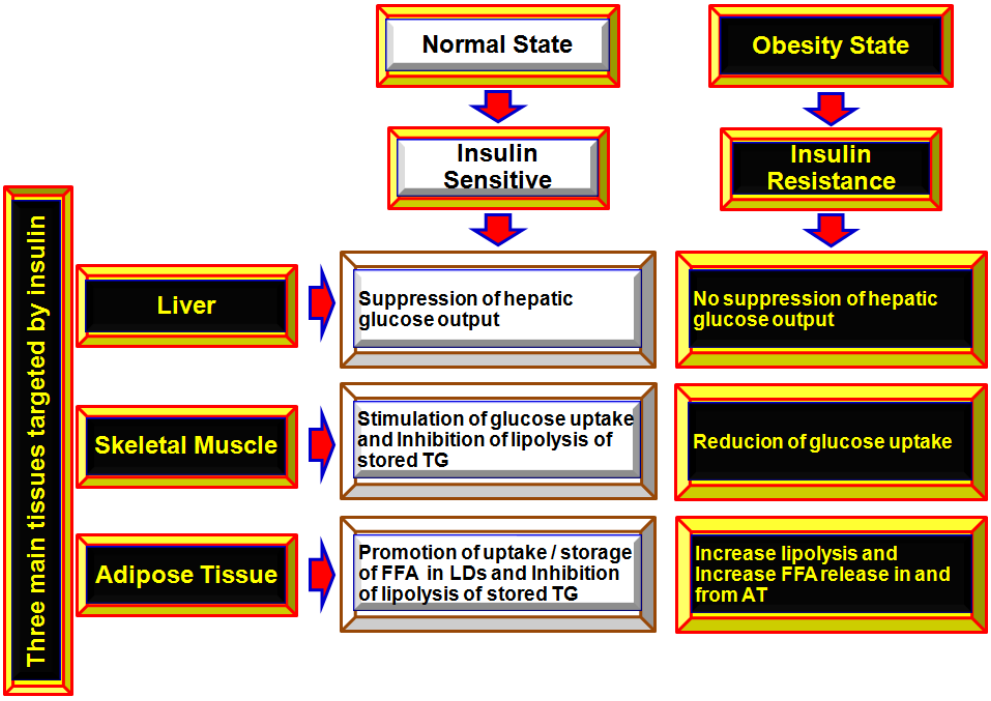

Figure 6. The role of insulin in three main tissues (Liver, Skeletal Muscle and Adipose Tissue).

6.2.2. Diabetes

The definition of the diabetes is mainly based on the level of the circulating glucose. Diabetes is defined as a disease in which the body is not able to regulate the amount of the glucose in the circulation; a state called hyperglycemia. The etiology of hyperglycemia is subsequently defined as a disturbance of the insulin pathway either due to a defect in insulin secretion following β cell destruction as in T1D, or to the insulin resistance as in T2D. Therefore, diabetes is a disease, where there is either hyperglycemia / hypoinsulinemia in T1D or hyperglycemia / hyperinsulinemia in T2D. However, this sort of interpretation regarding the ethiology of

hyperglycemia is not complete because the lipid metabolism in the metabolic system is ignored. Adipose tissue has a crucial role in the pathogenesis of the diabetes, and in this thesis we aim to show that this role is further exemplified by the insulin secretion by the adipocytes that directly influences on the level of the circulatory glucose. The clinical characteristics and differences between the type 1 and type 2 diabetes are as follows: In T1D, patients are largely young, display fatigue and regardless of their hyperplasia (orexigenia) are thin (Levy-Marchal et al., 1992). These symptoms suggest the existence of an active intracellular metabolic system and that perversely convinces this point that the cells, in spite of the hyperglycemia, are starved. In this disease, the disturbance of insulin production affects all organs including kidney, eye, feet, nervous system, adipose tissue, and skeletal muscles and the functionality of all anabolic pathways is disrupted. The main therapeutic option in T1D patients is insulin injection, but also islet transplantation is becoming more feasible.

The T2D patients are elder, obese and do not feel hungry or tired. Disturbance of the insulin pathway is mainly restricted to the metabolically active organs. They exhibit insulin resistance, ostensibly originating from chronic inflammation in adipose tissue (Balistreri et al., 2010; Nishimura et al., 2009). However, other mechanisms might also be involved, and through this thesis I hope to make contributions here.

In T2D, adipose tissue malfunction in lipid sequestration (Gletsu-Miller et al., 2009; Sattler, 2003) leads to fatty acid sedimentation in the peripheral catabolically tissues such as the skeletal muscles and the liver. The systemic insulin resistance, via reduced GLUT activity, disturbs their cellular glucose absorption. The hyperglycemia enhances the activity of the β cells to secrete insulin (Ramlo-Halsted and Edelman, 1999) to compensate the high blood glucose level. However, in advanced T2D; the pancreatic β cells because of

either insulin resistance or hyperactivity become damaged and are not able to secret enough insulin to cope with hyperglycemia. Nevertheless in these patients a measurement of circulatory insulin level is in general high, possibly indicating alternative sources for insulin. Even though insulin therapy is one of the therapeutic protocols to augment and rescue the function of the β cells, the best therapy for T2D, however, is weight loss (Guilherme et al., 2008; Kahn et al., 2005). Other possibilities are bariatric surgery (Gletsu-Miller et al., 2009) or treatment of T2D patients with thiazolidinones (Yki-Jarvinen, 2004). Further insight into the source of the high insulin levels will certainly bring this field forward.

6.2.3. Lipodystrophy and metabolic syndrome (MS)

Lipodystrophy and metabolic syndrome are a group of the energy metabolic disorders whose pathophysiological sources back to the adipose tissue. They are categorized based on their level of adipose tissue mass into either lipoatrophia (lack or absence of adipose tissue) (Figueiredo Filho et al., 2004) or lipohypertrophia or obesity linked-metabolic syndrome in which there is abnormal fat distribution from the subcutaneous to the visceral part (Hamdy et al., 2006, Wajchenberg, 2000). One of the symptoms is premature aged aspect of the face. This group of diseases present with hyperglycemia, dyslipidemia, hepatic steatosis, and cardiovascular complications (Figueiredo Filho et al., 2004; Herrero et al., 2010; Hotamisligil, 2006; Tsiodras and Mantzoros, 2006).

Their main common etiology is lack of a metabolically active adipose tissue that leads to the impairment of adipokine secretion and lipid sequestration in

adipose tissue, which deviate sedimentation of the fatty acids to the non-adipose metabolically active tissues in the form of hepatic and myocellular steatosis (Lewis et al., 2002). Overload of the intracellular energy levels in the catabolic, non-adipose tissues increases the susceptibility of ER stress and production of the lipid intermediates such as ceramid and diacylglycerol (DAG) (Watt and Spriet, 2010), which lead to insulin pathway disturbance and consequently stimulation of the inflammatory pathways (Yu and Ginsberg, 2005). Insulin resistance stimulates extra synthesis of insulin by the β cells, which in turn leads to the outputting of these cells and ultimately the occurrence of hyperinsulinemia in the initiation stage. However in the advanced stage the lipid droplets enlargement of the adipocytes tend to the hyperinsulinemia, which together with insulin resistance of the β cells suppress the insulin production. Insulin resistance also negatively affects the insulin-induced gene expression and transport of GLUTs to the membrane for glucose absorption and consequently enhances the cells for using the intracellular lipids instead of glucose which lead to hyperglycemia (Guilherme et al., 2008; Nguyen et al., 2005). In these diseases, their leptin level, as a mass-related adipokine, and their cellular leptin sensitivity is converse, whereby in the lipoathrophia the leptin level is low and cells are sensitive to it, which is used from this property in leptin replacement therapy (Oral et al., 2002). However in the lipohypertrophia, the leptin level is high and there is leptin resistance that is similar to the insulin resistance (Kahn et al., 2005). The level of the circulatory insulin in both groups is high that in the lipohypertrophia state would be due to the mass of the adipose tissue and enlargement of the adipocyte-lipid droplets-induced, while in the lipoatrophy state it is still unknown. Leptin has also an effect on the sympathetic nervous system, which could be the reason of the hypertension and cardiovascular diseases in the obese individuals (Eikelis et

al., 2003). These diseases are accompanied by ageing-like loss of adipose tissue from the subcutaneous face, osteoporosis, and atherosclerosis that would be due to the hedgehog signaling and its influence on the energy metabolic system. This review is meant to serve as basis for future research.

7. THE SCOPE OF THIS THESIS

As becomes clear from the former, the adipose tissue is an underestimated player in the immunopathophysiology of the human body, but full appreciation of its function is lacking due to both the absence of comprehensive studies of the biomedical literature as well as a void with respect to experimental data. Through the current thesis I shall try to address these concerns. To this end, I shall first deal with hedgehog proteins, a usual lipidated potent morphogen. It will appear that in conjunction with the adipose compartment this hormone might constitute the first anti-geriatric signal in the mammalian body and it should prove exiting to explore the possible uses of the signal involved for combating age-related diseases, in particular vascular pathology. As vascular pathology is closely linked with adipose compartment through lipoprotein loading and production, I continue to provide the first comprehensive description of both VLDL (chapter 3 and 4) and LDL (chapter 4) constituents. It will appear that hedgehog is specifically located on VLDL, which is in contrast with previous notions attributing the blood-derived hedgehog signal to the LDL compartment, and in this sense I feel my results significantly alter the classical view of the relation between hedgehog, the lipoprotein compartment and the adipocyte compartment. Hedgehog as a vascularizing signal, however, works in

conjunction with the coagulation system. Previous work has suggested that the coagulation factors drive important way regenerative responses after injury, hedgehog fine-tuning the exact neovascularization status. The relevance of the findings involved is further illustrated by other unconventional production of signals through the adipocyte compartment, in case insulin and findings involved in chapter 5 as well as their relevance for type 2 diabetes. Then I became interested in the expression domain of important coagulation factors and strikingly my results challenge the conventional view that such synthesis is limited to the liver. The results involved are described in chapter 6. A summarizing discussion highlighting the important novel endocrine functionality of the adipocytes in mammalian physiology concludes this dissertation and is given in chapter 7.

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Chapter 2

Hedgehog Signaling as an Antagonist of Ageing and Its Associated Diseases

Monireh Dashti [1,2], Maikel P. Peppelenbosch [2] Farhad Rezaee [1]

[1] Department of Cell Biology, UMCG, University of Groningen, The Netherlands

[2] Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands

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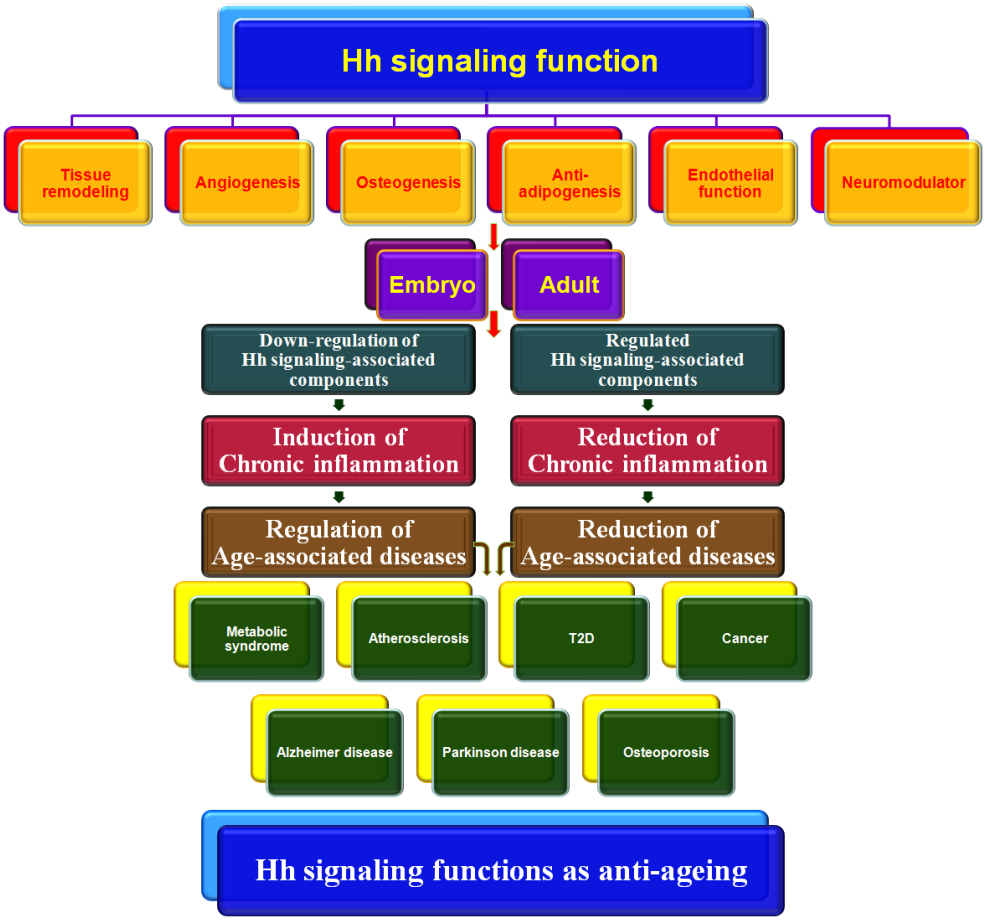
GRAPHIC ABSTRACT AND FIGURE

Hedgehog is a morphogenic signal during embryogenesis and adult life with many vital biological functions such as neuromodulators and anti-adipogenesis. Down-regulation of Hedgehog signaling is associated with ageing-related diseases like Alzheimer/Parkinson and T2D diseases and up-regulation of this signaling reduces risk of these diseases. Thus, Hedgehog signalling is antagonist of ageing.



ABSTRACT

Hedgehog is an important morphogenic signal directing pattern formation during embryogenesis but its activity remains present through adult life as well. It is now becoming increasingly clear that during the reproductive phase of life and beyond it continues to direct cell renewal (which is essential to combat the chronic environmental stress to which the body is constantly exposed) and counteracts vascular, osteolytic and sometimes oncological insult to the body. Conversely down regulation of Hedgehog signaling is associated with age-related diseases like type 2 diabetes, neurodegeneration, atherosclerosis and osteoporosis. Hence, in this essay we shall argue that Hedgehog signaling is not only important at the start of life but constitutes an important anti-geriatric influence and that enhanced understanding of its properties may contribute to developing rational strategies for healthy ageing and reduction of age-related diseases.

Introduction

The mechanisms that orchestrate the development of a multicellular organism from a single cell have fascinated people for centuries. We are still a long way from truly understanding the robust programs that allow building of or our specialized organs that regulate digestion, respiration, and hemodynamics guard us from invasion by other unsolicited organisms. However, in the past three decades, many of the genes that play a role in patterning our bodies have been identified (Behr et al., 2010; Bragdon et al., 2011; Nusse, 2003) and we have learned much about the mechanisms involved in development by characterizing the nature of these patterning genes and the way they work together (Cao and Chen, 2005; Iwasaki et al., 1993; Kumar et al., 2012; Reddi and Reddi, 2009). A major component of the developmental systems creating the body during gestation is Hedgehog. In this essay we shall introduce the Hedgehog pathway and argue that this morphogenetic system is not only of principal importance at the start of life, but that its action prevents the deterioration of the body during adult life (Benson et al., 2004; Kanda et al., 2003; Katoh, 2007; Reddi and Reddi, 2009), while its shut down is associated with senescence (Dwyer et al., 2007) of the body and makes it vulnerable to diseases of elderly. Murine models for increased Hedgehog signaling are available, for instance through the heterozygous knock-out for Patched (see below), but have until now mainly been explored to pinpoint the role of enhanced Hedgehog signaling in cancer. As Hedgehog may the first example of anti-geriatric signals in the body, it should prove highly interesting to investigate this animal in models of age-related disease as well.

GENERAL PRINCIPLES OF HEDGEHOG SIGNAL TRANSDUCTION

Hedgehog signaling is one of the highly conserved pathways in metazoan organisms, although when, for instance, Drosophila is compared to Homo sapiens variations in signaling details are also apparent (Wilson and Chuang, 2010). As stated, it is one of the key pathways directing morphogenesis during morphogenesis and this property seems critically dependent on the ability of cells to show Hedgehog concentration-dependent responses that result in alternative cell fates (Harfe et al., 2004; Wilson and Chuang, 2010). How concentration gradients of Hedgehog are precisely established remains not completely resolved. Hedgehog morphogens are a family of lipidated ligands which in vertebrates include sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh), but which all signal through the same receptors and same signal transduction cascades. After synthesis as pro-morphogens, Hedgehogs undergo autocatalytic proteolytic processing; acquire a covalent addition of cholesterol to the C terminal domain and a cysteine palmitoylation at the N terminal domain (King et al., 2008), all resulting in a highly hydrophobic protein. The subsequent release of the hedgehog molecule for intercellular signaling is an active process involving binding of the Dispatched translocator to the cholesterol moiety (Burke et al., 1999). The function of Hedgehog as a long range morphogen is evidently not hampered by its highly hydrophobic character and thus mechanisms ensuring its solubilisation must be operative (Gallet et al., 2006; Panakova et al., 2005). Various modes have been proposed to account for its transport through the aqueous environment. These include the formation of the micelle-like multimeric complexes (King

et al., 2008) in which the hydrophobic parts of the molecules are centralized via their lipophilic tails, transport by lipophorins (Drosophila) or lipoproteins (mammals) and attachment to the surface of a large membrane sheathed vesicles together with retinoic acid (Table 1) (Breitling, 2007; Panakova et al., 2005). The absolute and relative importance, however, of these alternative mechanisms remains unclear and long-range transport of Hedgehog remains a large outstanding unanswered question.

Homologies in Drosophila / Mouse / Human		
Drosophila	**Mouse**	**Human**
Microtubule	Primary cilium	Primary cilium
Ptc	Ptch 1	PTCH 1
Ihog, Boi	Cdo/ Boc	CDO/ BOC
Disp	Disp1	DISP 1
Ci- 155	Gli 3 A FL	GLI 3 A FL
Ci- 75	Gli 2/3 R	GLI 2/3 R
HIB	Spop	SPOP
Cos2	Kif 7	KIF 7

Table 1: Homologies in Drosophila, Mouse and Human

Hedgehog engages target cells in a complex fashion. Its transmembrane (TM) receptors include the 12 times lipid bilayer transferring receptor Patched (Ptc in Drosophila and Ptch1 in mammals), the Interference hedgehog (Ihog) co-receptor and Smoothened, a G protein-coupled (Smo) receptor (Murone et al., 1999). Unligated Ptc inhibits signaling by repressing Smo, but Hedgehog binding to Ptc leads to Ptc internalisation and release of Smo-dependent further signal transduction (Figure 1). Ihog, which has been best characterised in Drosophila (Camp et al., 2010), is composed of an immunoglobulin (Ig)-like domain and a Fibronectin type 3

(FN-III)-like domain (Alberts et al., 2008). The Ihog receptor binds to the hedgehog proteins and enhances alleviation of Ptc-dependent repression of Smo receptor, maybe in conjunction with Boi/Boc (Brother of Ihog in Drosophila, brother of Cdo in mammals) (Yao et al., 2006). Ptc inhibits Smo via production of a 3-hydroxy steroid and by controlling Smo trafficking (Breitling, 2007). In mammals, cells use the primary cilium for signal activation. In the absence of the hedgehog ligand, the inactive Smo receptor is located outside the cilium and inhibited by the active inhibitory receptor Ptch. After ligand binding and following Ptch degradation, the active Smo receptor translocates to the cilium in a process involving the Intraflagellar transport (IFT) protein in conjunction with Kinesin II (Kif3 family) and β-arrestin-2 (Arrb2) (Figure 1). Further signaling occurs through a structure denominated the called hedgehog signaling complex (HSC), also apparently located at the primary cilium in mammals or the microtubule skeleton in general in Drosophila. The main targets for Hedgehog signaling are the Ci (Drosophila) / Gli (mammals) transcription factors, which constitute the main principal modulator(s) of the hedgehog-driven transcription. Ci (Gli) protein(s) act either as activators of transcription (the full-length Ci-155 in Drosophila and Gli2/3A in mammals) or as repressors of transcription (the cleaved Ci-75 in Drosophila or Gli2/3R in mammals), hedgehog signal transduction converting transcriptional repression to activation by inhibiting proteolysis-favouring Ci/Gli phosphorylation (Figure 1). The resulting transcriptional changes are critically important for pattern formation during embryogenesis, but as we shall argue in this essay remain of cardinal importance during adult life as well and mediate anti-geriatric gene transcription (Wilson and Chuang, 2010).

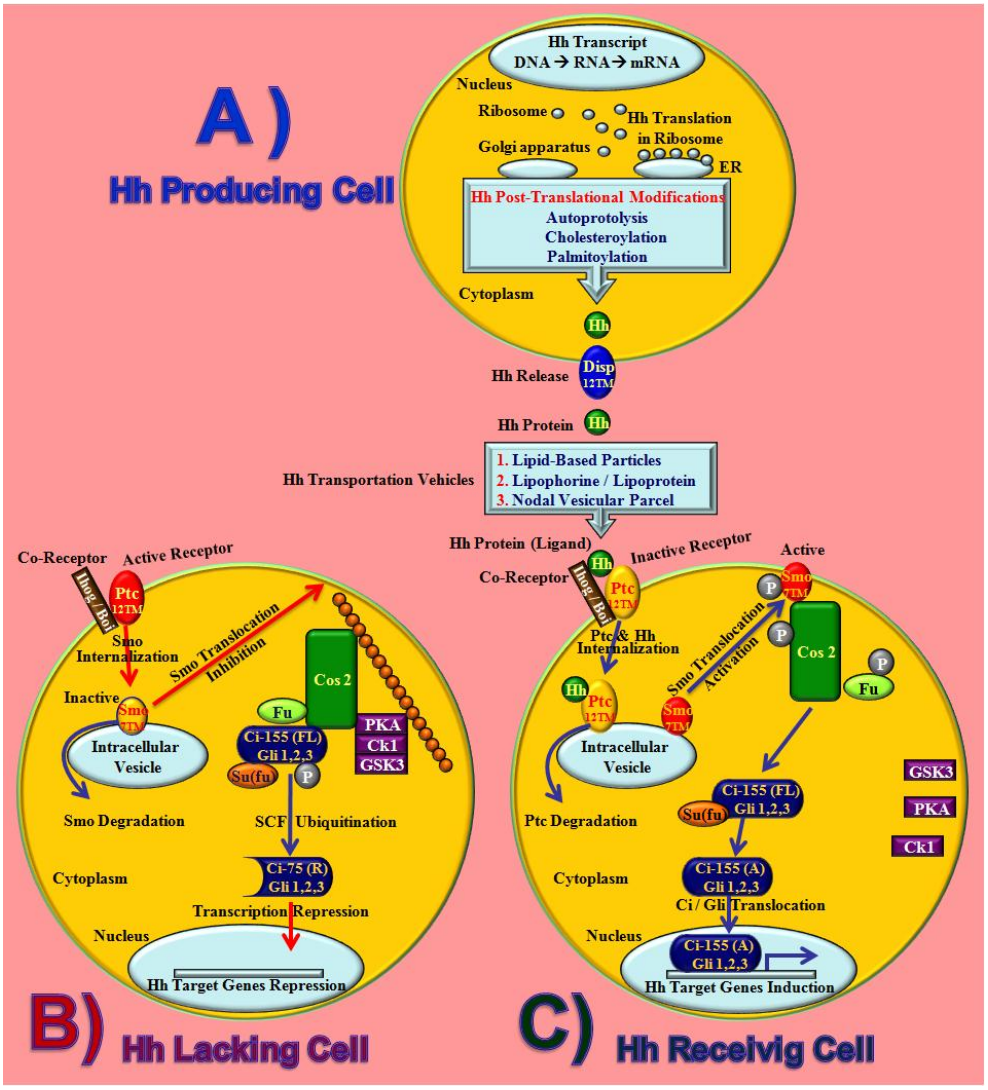


Figure 1: The Hedgehog signaling pathway. Panel A: Synthesis, secretion and transport of the hedgehog protein. Hedgehog production: After transcription of Hh in the nucleus and translation Hh on the ER membrane, Hh is processed both in the ER and the Golgi apparatus. This processing includes the autoproteolytic cleavage of the C-terminal domain, Hh palmitoylation and the cholesterol addition. The palmitoylation of Hh is necessary for efficient hedgehog signaling and the cholesterol addition is needed for the Hh oligomerization, but result in a highly hydrophobic protein. Hence Hh release has to be an

active process and this is mediated via the Dispatched (Disp) receptor. The thus released hydrophobicbic Hh proteins can be then transported in three ways towards their target tissues: 1- Lipid-based particles, 2- carry lipophorins of plasma lipoproteins and 3- via so-called nodal vesicular parcels. The relative importance of these modes or whether other transport modalities exist remains unclear but represents an important question in the field. Hh: Hedgehog; Disp: Dispatched (encodes a twelve transmembrane domain protein (D12) protein containing Sterol sensing domain (SSD). **Panel B: The Hedgehog signaling system in the absence of Hh.** When Hh is not present the Hh receptor Ptc is constitutively active and inactivates Smo, possibly via its sequestration and degradation by the intracellular endosome and inhibition of Smo translocation to the plasma membrane surface in a mechanism which possibly involves the Ptc-mediated translocation of a 3-β-hydroxysterol over the membrane. In absence of Hh, Cos2, Fu, Ci-155 are complexed to SuFu and microtubule skeleton. A number of kinases including PKA, CK1, and GSK3 kinases are recruited by Cos2 to this complex and phosphorylate Ci. This phosphorylation targets Ci for ubiquitination and partial degradation via SCF complex. A 75 kb cleaved fragment form of Ci (Ci-75) or Gli, however, translocate to the nucleus where it act as a transcriptional repressor for Hh target genes. **Panel C: Hh reception:** Hh ligand forms a complex with the Ptc receptor as well as with co-receptors like iHog or Boi. This complex is then internalized, thus removing the inhibitory action of patched of Smo, possibly mediated by a translocation of Smo from intracellular vesicles to the plasma membrane and hyperphosphorylation of Smo. Smo subsequently recruits Fu and Cos 2 to the membrane, where these proteins become phosphorylated. In turn this results in the detachment of Fu from Cos 2, relieving Ci from otherwise constitutive phosphorylation, preventing partial proteolysis and accumulation of the intact 155 kb fragment of Ci transcription factor or Gli (1-3), which upon translocation to the nucleus activates transcription of the Hh target genes. Hh: Hedgehog; P: Phosphate group; Ub: Ubiquitination; A: Activator; R: Repressor; FL: full-length; Red arrows: Inhibitory effect; Smo7TM: 7-pass transmembrane spanning Smoothened receptor, Ptc12TM: 12-pass transmembrane, Sterol-sensing domain (SSD), Red receptors refers to activated receptors (e.g. Smo at membrane surface); Yellow receptors refers to Inactivated receptors (e.g. Ptc) Ihog: Interference hedgehog co-receptor;

Boi: brother of Ihog, Ci: Cubitus interruptus; Ci-155 A: 155 kb intact active form of Ci; Ci-75 R: 75 kb cleaved form of Ci; zinc-finger transcription factor; Cos2: Costal2; atypical kinesin-like protein; Fu: Fused; a putative serine/threonine kinase; Su(fu): Suppressor of fused (PEST domain protein); PKA: Protein kinase A; CKI: Casein kinase I; GSK3: Glycogen synthase kinase.

POSSIBLE FUNCTIONALITY OF HEDGEHOG IN THE AGEING PROCESS

Generally speaking, following development and a juvenile phase animals enter the reproductive phase. Following exhaustion of the reproductive potential, however, a long phase and a gradual decline may follow before the organism dies. Especially for humans is the evolutionary forces that drive and counteract ageing (Kiss et al., 2009). Generally, it involves a reduced capacity to regenerate tissues (including the immune system) comprising their functionality, a reduced capacity to combat genotoxic damage resulting in a sharp increase in cancer incidence following the end of the reproductive and offspring-rearing phase, and the accumulation of sclerotic and fibrotic lesions in the body, especially the vasculature. In humans this phenomenon is associated with a wide range of pathologies, including senile dementia, atherosclerotic disease, and osteoporosis (Kirkwood, 1996). Earlier concepts of ageing were based on the idea that the body invests a certain amount of energy in mounting defences against especially oxidative stress, depending on the life span needed for efficient reproduction but that over time damage accumulates and following passage of certain tipping point senescence quickly gathers pace. New data involving genetically modified mice to exhibit reduced anti-oxidative

defences and studies in exceptionally long-lived animals have questioned this notion (Speakman and Selman, 2011). Although ageing is an extremely complicated process, it now appears that reduced capacity to cope with external stress and a reduced potential to mount regenerative answers to insult, drives the geriatric process. In turn, this reduced functional and regenerative capacity is the consequence of reduced size and agility of the various stem cell compartments in the body. For instance, increasing evidence shows that central molecular players in Alzheimer's disease influence the generation of new neurons, and noteworthy alterations in neurogenesis take place earlier than the onset of hallmark lesions or loss (Lazarov and Marr, 2010). The rapid pace of neurodegeneration and dementia is attributed to a reduced activity in neuronal stem cell compartments resulting in inadequate neuronal and glial replenishment following injury, whereas evidence has been presented that in benign senile dementia similar mechanisms play a role (Lazarov et al., 2010). Also in, for instance, the immune system, a reduced capacity of the hematopoietic stem cell compartment to generate especially lymphoid cell types may be involved in the increased propensity to succumb to infectious disease (Beerman et al., 2010). If, however, reduced functionality in the somatic stem cell compartments is responsible for ageing, the factors that govern the behaviour of this compartment might well be important to control the pace of ageing. It would thus be interesting to execute experiments in which experimental rodents are subjected to treatment with Smo agonists and subsequently investigated for the size of the various stem cell compartments. As Hedgehog is a major regulator of stem cell function, this would suggest that the morphogen could guide ageing through influencing the stem cell compartment. The function of hedgehog as anti-ageing-related diseases is depicted in figure 2.

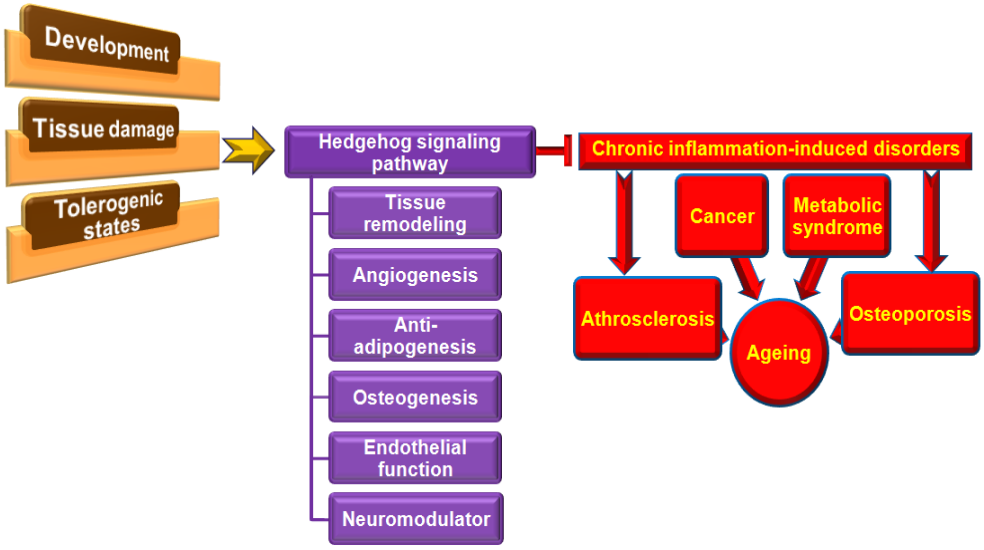


Figure 2. A schematic representation of the hedgehog signaling stimulators, inhibiting/reducing the chronic inflammation-induced pathophysiological disorders: a linking between induction of hedgehog signaling and reduction of the different age-related diseases.

HEDGEHOG SIGNALING AND THE STEM CELL NICHE

Maintenance of the body requires continues replenishing of the organs with fresh effector cells, which are typically produced from a small stem cell pool in the specific organs. Ageing and the associated diminished functionality of organs is often attributed to diminished size and action in the stem cells, compromising the capacity of the organs to withstand mechanic and xenobiotic insult. Many stem cell niches require Hedgehog signaling (Katoh, 2007; Rubin and de Sauvage, 2006; Tang et al., 2006) especially in tissue derived from the ectodermal lineage (e.g. in the central

nervous system or in the skin) (Sasai and De Robertis, 1997) but there is also good evidence that Hedgehog is required in the hematopoietic stem cell niche and others (Bijlsma et al., 2006a). The role of Hedgehog as an important factor for maintaining stem cells is illustrated by the importance attributed to this signaling system for maintaining cancer stem cells and inhibitors of Hedgehog signaling are being proposed as tools to test the cancer stem cell (Merchant and Matsui, 2010). Among the mechanisms employed by Hedgehog signaling to maintain the stem cell phenotype is suppression of p16 (INK4a). A fragment of GLI2 transcription factor directly binds and inhibits the p16 promoter and stem cell senescence is associated with the loss of GLI2 (Bishop et al., 2010). Indeed p16 is a well-established mediator of cellular senescence (Rayess et al., 2012) and its expression rises with age in many tissues, as does the accumulation of dysfunctional senescent stem cells (Campisi, 2005), providing a direct link as to how Hedgehog signaling can counteract ageing through the stem cell compartment. Thus Hedgehog can counteract senescence by maintaining the stem cell phenotype but conversely this property of Hedgehog also probably contributes to its role in promoting the process (Altaba et al., 2002). It is important to note that other components of morphogenetic code (Hogan, 1996) often mainly act as antagonists of stem cell functions. Members of the TGFβ/BMP family of morphogens, for instance, mainly act as antagonists of stemness, as evident from the expression of BMP antagonists in many stem niches to protect the stem cell from the action of these morphogens (Hardwick et al., 2008) or the requirement for BMP antagonists like noggin in the medium of stem cell-derived organoid cultures (Sato et al., 2011). Also FGF family members tend often to favour differentiation over stem cell expansion throughout the body (Turner and Grose, 2010). In this sense BMP and FGF-like morphogenes are more pro-geriatric as anti-geriatric

substances. Wnt ligands and ligands stimulating Notch signaling are often potent in promoting stem cell maintenance and expansion, but differ from Hedgehogs that they are essentially short-range paracrine factors. Thus Hedgehog seems unique in its capacity to confer long-range signaling stimulatory activity on the stem cell compartment.

HEDGEHOG SIGNALING MAINTAINS VERSATILITY IN THE SKELETON

A good example is that how Hedgehog via acting on stem cell functionality may be found in the bone. With advancing age, the conditions of the bones deteriorate and especially in elderly women osteoporosis is a major health problem. During skeletogenesis, Shh and Ihh provide positional information and initiate or maintain cellular differentiation programs regulating the formation of cartilage and bone and are of particular importance for osteoblast generation (Das et al., 2011; Koga et al., 2008). During adult life bone is lost, and has been attributed to a shift in the balance osteogenic (bone forming) and adipogenic (fat forming) mesenchymal stem cell (MSC) differentiation (Abiola et al., 2009; Cousin et al., 2007; Fontaine et al., 2008; Ng et al., 2008). In rodent adipocytes, hedgehog signaling pathway (mainly Shh) interferes with adipocyte differentiation through an unknown mechanism. Hedgehog favours the osteogenic differentiation over adipogenic differentiation by acting upstream of the Peroxisome proliferator-activated receptor gamma (PPARγ), which in turn mediates the anti-adipogenic transcription factors GATA2 and 3 (Cousin et al., 2007; Hu et al., 2005; Kim et al., 2007). In addition, in bone hedgehog signaling

directly interferes with the transcription of CCAAT/enhancer binding protein (CEBP)α and Sterol Regulatory Element Binding Protein (SREBP)-1c, which mediate adipogenesis, while conversely osteogenic transcription factors are induced. In diabetic patients, high glucose interferes with the Shh signaling and Shh induced bone regeneration, explaining the association between the hyperglycaemia and osteoporosis (Guan et al., 2009), thus highlighting the important role of continued Hedgehog signaling during life for maintaining skeletal strength and evidence has been provided that decreasing levels of Shh in the bone with advanced age correlate with impaired osteogenesis and is thus critical for osteoporosis in the elderly (Dwyer et al., 2007). In this sense the continued action of Hedgehog on skeletal bone stem cell compartments forms a good example how the action of this hormone can counteract the consequences of ageing.

HEDGEHOG SIGNALING CONTRIBUTES TO THE PROTECTION OF THE BRAIN AGAINST NEURON LOSS

Another example as to how Hedgehog signaling can counteract ageing is found in the nervous system. Increasing age is accompanied by a loss of mental faculties that is attributed to the loss of neuronal cells and associated reduced plasticity in cerebral functioning. Neurons in general are robust cells, but as the central nervous system requires constant replenishing with neurons from neuronal stem cell compartments, as it is for instance evident from Alzheimer's disease where defects in the stem cell compartment are important factors in the progressive neuron loss, but active hedgehog

signaling is generally important for maintaining neurons in adults (Han et al., 2008). In apparent concordance, also in Alzheimer's disease Hedgehog has been proposed as molecule counteracting the disease process through increased stem cell activity (Paganelli et al., 2001) and persistent Hedgehog signaling is necessary for neuronal stem cells to acquire their identity during development (Ihrie et al., 2011). Thus the evidence that continued Hedgehog signaling through the stem cell compartment plays a cardinal role in enabling the brain to regenerate neurons throughout life seems compelling.

Importantly, apart from its role in maintaining neuronal stem cell populations, it seems that capacity of neurons to activate transcriptional programs associated with increased robustness against chemical, nutritional or ischemic stress is sensitive to external cues and that Hedgehog signaling plays an important role in this state (Dai et al., 2011). In primary cultures of cortical neurons oxidative insult results in Shh production. As inhibition of Hedgehog production exacerbates apoptosis and neurotoxicity, whereas artificial hyperactivation reduces neuronal cell death it appears that Shh is an important endogenous protective hormone for the cortical neuronal compartment (Dai et al., 2011). Similar results were also reported for spinal neurons (Ng et al., 2008). Mechanistically, Shh responses to improve survival of neurons seems kaleidoscopic and complex as increased activation of survival pathways, induction of expression of the anti-apoptotic mitochondrial protein Bcl-2 with concomitant decreased expression of its pro-apoptotic relative Bax, enhanced expression of trophic factors like BNDF and VEGF and direct inhibition of cell stress-sensing pathways have all been reported, probably highlighting that increased survival of post-mitotic cells is a bona-fide effect of Hedgehog signals. As

ageing involves diminished size of such post-mitotic compartments, this again represents an important anti-geriatric signal.

Neurodegenerative disease can be highly serious health complication in cerebral animals as humans are. Thus although treatment with pharmacological Smoothened agonists will undoubtedly be associated with increased risk for malignant transformation, the possible gains in brain function through enhanced stem cell function and decreased propensity to further neuron loss may still be considered a valid therapeutic risk in the future.

SKIN

A final example as to how loss of Hedgehog may cause ageing-related phenomena is found in the skin. Reduced skin elasticity is one of most obvious effects of advancing age. Hedgehog is an important morphogen here, maintaining the basally-located stem cell compartment, as evident for instance from the frequent development of basal cell carcinoma in patients having only one functional allele of Patched (Teglund and Toftgard, 2010). Furthermore, decrease capacity for skin repair is associated with reduced activation of Hedgehog signaling in the bulge cells, an important stem cell in the skin. Finally, in a model for aged skin, juvenile markers can be induced by lentiviral-mediated overexpression of Gli1 (Rittie et al., 2009). Thus in multiple places in the body bona fide evidence for a role Hedgehog signaling in combating ageing through modulation of the stem cell compartment is available.

HEDGEHOG COUNTERACTS STRESS-RELATED LESIONS

From the former it is clear that Hedgehog directs tissue rejuvenation through trophic actions on the stem compartment throughout the body and may be the only long-range signaling molecule to do so. Functionality in providing a juvenating signal in the body is further supported by the role Hedgehogs play in counteracting the effects of chronic insult to the body. During life, the body constantly suffers fibrotic and atherosclerotic lesions. Although such lesions can already be detected in young individuals, beyond the reproductive phase the number of especially atherosclerotic lesions suddenly increases, due to an apparent incapacity to control and counteract growth of such lesions by compensatory responses. There is evidence that Hedgehog provides an important signals to the tissues that such compensatory responses should be mounted and thus that Hedgehog function as an anti-geriatric signal does not only lie in the dynamics of stem cell function but in how the body deals with the metabolic demands conferred by ageing (Reddi and Reddi, 2009; Tseng et al., 2008).

HEDGEHOG SIGNALING LIMITS ATHEROSCLEROTIC DISEASE

Atherosclerosis is one main manifestations of advanced age and a major cause of the associated aetiology. It has a close association with the other disorders of the energy metabolic diseases including metabolic syndrome

and type 2 diabetes. Different pathways are involved in their pathophysiology, including the immune system and coagulation system. Lipoproteins have different effects in this process. While LDL and VLDL have a stimulatory role in the atherosclerosis, HDL has a protective effect on the blood vessel, which counterintuitive in view that both are important carriers of cholesterol (Queiroz et al., 2010). In this context it is important to note that sterol metabolism is closely related to hedgehog signaling (Breitling, 2007; Colles et al., 2001; Dwyer et al., 2007; Gallet et al., 2006; Kim et al., 2007; Omoigui, 2007). Hedgehog is sterolated and Hedgehog derivatives mediate the inhibitory function of Ptch receptor on Smo receptor in the absence of the hedgehog molecule (Bijlsma et al., 2006c). Thus, sterol disorders affect hedgehog pathway and development either by improper sterolation of the hedgehog protein or by a reduced responsiveness of the cells to the hedgehog proteins. In the circulation, the transport of the highly hydrophobic hedgehog molecules in Drosophila occurs via lipophorin and in mammals VLDL is loaded with Hedgehog in the adipocyte compartment. There has also been extensive speculation on the notion that also LDL carries Hedgehog, but hard data is lacking (Bijlsma et al., 2006b; Callejo et al., 2008; Panakova et al., 2005). The therapeutically benefits of Hedgehog in vascular disease are particularly evident from a studies involving Shh myocardial gene therapy in counteracting experimental chronic myocardial ischemia (Bijlsma et al., 2006a; Bijlsma and Spek, 2010; Kusano et al., 2005), whereas also experimental peripheral ischemia benefits from ectopic expression of Shh (Luo et al., 2009). The effects here are not as much that the atherosclerotic process per se inhibited but that Hedgehog signaling mediates shunting angiogenesis in addition to effects on apoptosis as described above for neurons. Conversely, genetically or pharmacologically interfering with Hedgehog signaling compromises endothelial functionality

and thus prepones age-related diminished function of the vasculature (Kanda et al., 2003; Luo et al., 2009), whereas ischaemic insult by itself provokes substantial production of Hedgehog (Bijlsma et al., 2006a; Kusano et al., 2005). These effects of Hedgehog improving endothelial function are multifold, but especially increased secondary production of angiogenic factors, increased survival signaling of endothelial cells through phosphatidylinositol-3-OH-kinase and recruitment of endothelial precursor cells have been well-documented. In toto, the evidence that Hedgehog signaling counteracts age-related changes in the endothelial compartment is compelling and thus Hedgehog production represents an important anti-geriatric signal here, although the effects do not as much prevent the atherosclerotic process but help in limiting the damage inflicted.

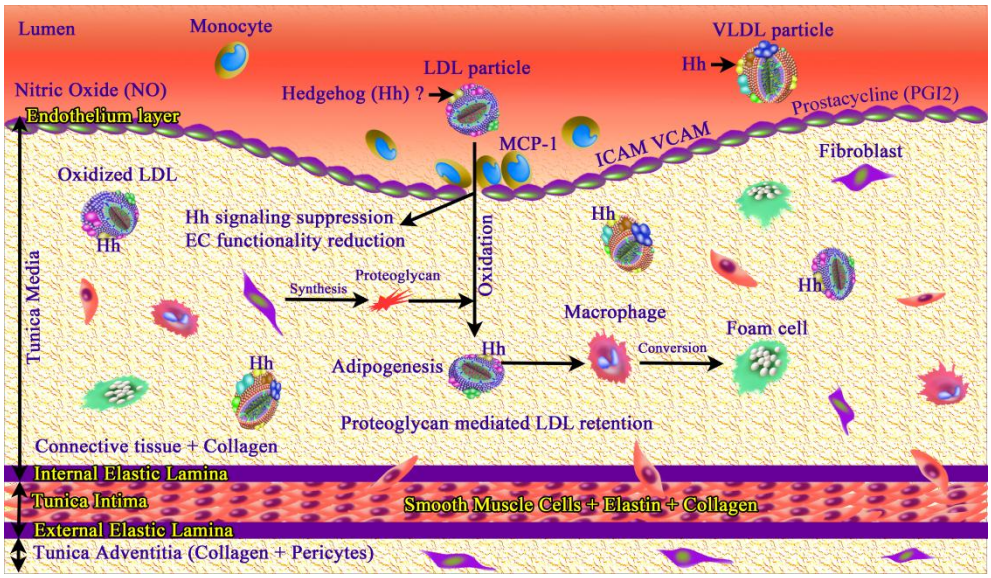


Figure 3: The role of Hedgehog and the endothelial function. In the circulation, VLDL is considered as carrier of Ihh, although LDL is also suggested as hedgehog transporter but there is no evidence for this notion. A normal arteriole is composed of endothelium, and the basement membrane, which is composed of a collagenous connective tissue layer,

consisting of collagen fibres, smooth muscle cells (SMCs) and elastic fibers that provide support to the vessels. The internal and external elastic laminaes (IEL and EEL) line the two sides of the SMC layer and the EEL separates the SMCs from a last layer, which is called adventitial layer. In a healthy vessel, the intact internal elastic lamina, which are lies directly peripheral from the endothelial cells. Under the intact state, hedgehog signaling suggested to be involved in the improvement of endothelial function. A suppression of hedgehog signaling may lead to less integrity of endothelial cells, which in turn make it vulnerable to damage by OX-LDL. The process of plaque formation and inflammation starts by local deposition and trapping of LDL molecules. LDL may become subject of oxidation and the resulting oxidized-LDL (ox-LDL), is efficiently taken up by macrophages, which are not well capable of dealing with lipid load involved and form foam cells. During the subsequent atherosclerotic reaction, the IEL layer is degraded by macrophage-cathepsin K allowing the SMCs to migrate from media to the nascent plaque. Pericyte and myofibroblasts in atherosclerotic plaque are subject to aberrant differentiation events. In plaque, a suppressed hedgehog signaling may promote adipogenesis locally. VCAM: Vascular cell adhesion molecule-1, ICAM-1: Intercellular adhesion molecule-1, MCP-1: monocyte chemoattractant protein-1.

CONCLUSION

Hedgehog signaling is one of the most highly evolutionary conserved pathways in metazoan body with a pivotal importance during embryogenesis, but with abundant activity afterwards. Importantly, though, an accumulating body of evidence suggests that its remaining functionality has not only an important role tissues repair, but is also involved the maintenance of the tissues and preventing their senescence. In this

Hedgehog my de facto act as an anti-geriatric signal and its reduced expression associated with advanced age may trigger ageing.

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Chapter 3

A Phospholipidomic Analysis of All Defined Human Plasma Lipoproteins

Monireh Dashti [1, 2], Willem Kulik [3], Frans Hoek [4], Enno C. Veerman [5], Maikel P. Peppelenbosch [1, 2] Farhad Rezaee [1,6]

[1] Department of Cell Biology, UMCG, University of Groningen, The Netherlands

[2] Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands

[3] Laboratory Genetic Metabolic Diseases

[4] Department of Clinical Chemistry, Academic MC, Amsterdam, The Netherlands

[5] Dep. Dental Basic Sciences (Oral Biochemistry), ACTA, VUMC, The Netherlands

[6] Dep. Experimental and MolecularMedicine, Academic MC, Amsterdam, The Netherlands

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ABSTRACT

Since plasma lipoproteins contain both protein and phospholipid components, either may be involved in processes such as atherosclerosis. In this study the identification of plasma lipoprotein-associated phospholipids, which is essential for understanding these processes at the molecular level, are performed.

LC-ESI/MS, LC-ESI-MS/MS and High Performance Thin Layer Chromatography (HPTLC) analysis of different lipoprotein fractions collected from pooled plasma revealed the presence of phosphatidylethanolamine (PE), phosphatidylinositol (PI), and sphingomyeline (SM) only on lipoproteins and phosphatidylcholine (PC), lyso-PC on both lipoproteins and plasma lipoprotein free fraction (PLFF). Cardiolipin (CL), phosphatidylglycerol (PG) and phosphatidylserine (PS) were observed neither in the lipoprotein fractions nor in PLFF. All three approaches led to the same results regarding phospholipids occurrence in plasma lipoproteins and PLFF. A high abundancy of PE and SM was observed in very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions respectively.

This study provides for the first time the knowledge about the phospholipid composition of all defined plasma lipoproteins.

INTRODUCTION

Despite major advances in the aetiology, prevention and treatment of cardiovascular disease, and in particular atherosclerosis, it remains the leading cause of mortality and morbidity in Western countries. Atherosclerosis results from the interaction between genetic and environmental factors modulating the functions of various cell types and inflammatory molecules within the arterial wall (Berliner et al., 1995; Ross, 1993). Extensive epidemiological studies have shown that lipoproteins play a major role in atherosclerosis of which LDL cholesterol levels are directly related to the risk of coronary artery disease and high-density lipoprotein (HDL) cholesterol levels are inversely related to this risk (Castelli et al., 1986). Despite lipid cholesterol modification therapies such as LDL-lowering treatments, cardiovascular events still occur. In addition, compared to plasma cholesterol measurements, much less attention has been given to the relationship between phospholipids and atherosclerosis.

Lipoproteins are different in phospholipid composition and (apolipo) protein contents (Atkinson and Small, 1986; Davidson et al., 1994; Mahley et al., 1984). Moreover, the apolipoproteins play an important role in lipoprotein stability, binding, and catabolism (Cushley and Okon, 2002). In addition, plasma phospholipids transfer protein (PLTP) mediates the transfer of phospholipids from VLDL and LDL to HDL, and is able to modulate HDL size and composition and may also be involved in HDL cellular-mediated efflux of phospholipids and cholesterol (Tall et al., 1985; Tollefson et al., 1988; Tu et al., 1993; Wolfbauer et al., 1999). The presence of abnormal phospholipids was also shown in patients with essential hypertension

(Bagdade et al., 1995). In addition, it was recently shown that the phospholipid composition of HDL plays an important role in scavenger receptor type B class I (SR-BI) and ABC trasporter A1 (ABCA1)-mediated cholesterol efflux (Yancey et al., 2000; Yancey et al., 2004). Yancey *et al.* (Yancey et al., 2000) also demonstrated that Phosphatidylcholine (PC) enrichment of HDL increase the efflux, whereas sphingomyelin decrease the influx of HDL cholesterol. Other studies suggest that the phospholipid composition of HDL could be a key determinant in a variety of other processes, including the inhibition of adhesion molecules (Ashby et al., 2001; Baker et al., 1999; Baker et al., 2000; Subbaiah and Pritchard, 1989), the efflux of cholesterol from Fu5AH cells to human serum (Fournier et al., 1997) and in its anti-inflammatory and anti-oxidant properties (Piperi et al., 2004; Xia et al., 1999).

It is also recently suggested that hemodialysis therapy in patients with chronic renal failure play an important role in dyslipidemic profile of these patients by modification of lipid and phospholipids composition concentrations (Pruzanski et al., 2000). Furthermore, it has been recently reported that the concentrations of phospholipid composition are clearly different in acute phase HDL compared to normal HDL (Van Lenten et al., 1995).

The phospholipid content is one of the factors, which have been suggested to contribute to the closer lateral molecular packing in LDL (Ibdah et al., 1989). The phospholipid composition in surface of lipoprotein particles influences the binding of apo A-I and that is the reason why apo A-I is absent in LDL particles *in vivo* (Ibdah et al., 1989). The impairment of PC biosynthesis led to the inhibition of VLDL secretion (Verkade et al., 1993), resulting in the changing of nascent VLDL phospholipids contents (Fast and Vance, 1995). In addition**,** VLDL stimulates the contact pathway of blood

coagulation via the exposure of phosphatidylethanolamine (Klein et al., 2001). Navab *et al.* (Navab et al., 2003) have recently shown that oral phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) in the drinking water of apoE-deficient mice results in an increase of plasma HDL cholesterol levels, an improvement in HDL function, and a marked reduction in atherosclerosis**.** Moreover, numerous studies have shown that phospholipid administration leads to the reduction of atherosclerotic development *in vivo* (Burgess et al., 2003; Burgess et al., 2005; Stamler et al., 2000).

Based on above-mentioned evidence, plasma lipoproteins contain both protein and phospholipid components, either of which may be involved in the atherosclerosis, inflammation, lipid metabolism, and haemostatic processes. Thus, the plasma lipoprotein functions can be manipulated by protein or phospholipids compositions.

After genomics and proteomics, many analytical techniques have been developed and used to elucidate the human lipidome. A lipidomics approach may be a reliable way to analyze the lipid composition in biological fluids, such as human plasma lipoproteins. A reliable analysis of phospholipid lipid molecules, "lipidomics", in the context of genomics and proteomics is an essential step to understanding cellular physiology and pathology and consequently, lipid biology has become a major research target of the post-genomic revolution and systems biology.

In our previous study, we have characterized proteins associated with HDL (Rezaee et al., 2006). In this study, liquid chromatography-electrospray ionization/mass spectrometry (LC-ESI-MS) and tandem MS were performed to identify the phospholipids composition of the major lipoprotein classes. ESI allows for large, non-volatile molecules to be analyzed directly from the liquid phase. The analysis of main phospholipids

composition of lipoproteins is needed to gain additional insight into *in vivo* functions of lipoprotein-phospholipids content both in normal and pathological states.

MATERIALS AND METHODS

Human blood was drawn from overnight-fasted normal healthy volunteers after informed consent, by venipuncture into tubes containing sodium-citrate as anticoagulant. Subsequently, plasma was obtained by centrifugation at 1,000 x g for 15 minutes at 4 ºC and used freshly or stored at -80 °C for further analysis. All experiments were performed in accordance with relevant guidelines and regulations and had the approval of the local Medical ethical committee (Academic Medical Center Amsterdam/ Univerisity Medical Center Groningen).

Lipoprotein isolation by salt-gradient ultracentrifugation

One-step ultracentrifugation was used according to Kleinfeld *et al.*[33] with some modifications (omission of sucrose in the salt gradient, and use of a 103,000 x g instead of a 200,000 x g centrifugation step) to obtain the major lipoprotein fractions (VLDL, IDL, LDL, and HDL) and HDL-2 and VHDL as well as Plasma Lipoprotein Free Fraction (PLFF). In this study, all plasma lipoprotein fractions were analyzed except Lipoprotein small a (LPa) and that was due to very small amount of extracted lipid fraction. The collected lipoprotein fractions were concentrated by concentrator

(Eppendorf) and were freshly used in the experiments and the rest stored for 10 days at 4°C. Only for the isolation of chylomicrons (CM) we applied other approach.

CM isolation

12 ml non-fasted plasma was spun in a SW41.14 rotor for 1 hour at 29000 rpm in a Beckman ultracentrifuge. The top 3 ml was collected and spun under the same conditions. The top approximately 2.5 ml was again eluted and spun under the same circumstances. Finally, the top 2 ml collected fraction (rich in CM) was subjected to one-step ultracentrifugation according to Kleinfeld *et al.* (Queiroz et al., 2010) with some modifications. This strategy was taken to remove as much VLDL possible from the chylomicrons fraction. All ultra, HPGC and mass spectrometry experiments involved CM fraction were separately performed en analyzed.

High performance gel-permeation chromatography (HPGC), cholesterol analysis and protein analysis

The cholesterol profiles of the major lipoprotein types were determined applying HPGC (Kleinveld et al., 1996; Levels et al., 2001; Queiroz et al., 2010; Rezaee et al., 2006; Weerheim et al., 2002). 60 µl of lipoprotein fractions collected by ultracentrifugation was introduced to gel-permeation chromatography using a Superose 6 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) (Kleinveld et al., 1996; Levels et al., 2001; Queiroz et al.,

2010; Rezaee et al., 2006). Chromatographic profiles of commercially available plasma lipid standards (SKZL, Nijmegen, The Netherlands) served as a reference. To obtain a purified ultra IDL fraction, Ultra IDL fraction was repeatedly exposed to HPGC. The protein analysis was used for the ultra collected IDL fraction. All conditions for the protein analysis of the ultra collected IDL fraction are the same as above-mentioned procedure meant for the cholesterol analysis.

Nephelometry

Apo A-I and apo B content in the lipoprotein fractions were measured by immunoprecipitation using nephelometry in a BN ProSpec nephelometer protein analyser (Dade Behring, Marburg, Germany) according to the manufacturer's instruction. The reagents for the quantitative determination of apo A-I and apo B were purchased by Dade Behring (Dade Behring, Marburg, Germany).

Western blot analysis

50-100 µg of protein present in the lipoprotein and plasma lipoprotein free fractions were denatured and separated by 7.5 % (w/v) SDS-PAGE and transferred from the gel to low fluorescent polyvinylidene difluoride (LPF-PVDF) membranes (GE Healthcare Biosciences). The blots were then blocked at 4 °C for overnight. After blocking, blots were incubated with a monoclonal antibody against apo B (178444, Calbiochem, Darmstadt,

Germany). Apolipoprotein B protein was purchased by sigma (Gmbh, Germany). Bound antibodies were detected with a CyDye-coupled goat anti-mouse antibody according to the manufacturer's instructions for ECL Plex Western Blotting System (GE Healthcare Biosciences). PageRuler prestained protein ladder (Fermentas, Germany) were run in parallel. The protein concentration of the lipoprotein fractions was determined by absorbance at 280nm (an OD of 1 equals 1 mg/mL).

Lipoprotein and plasma lipoprotein free fraction associated Phospholipid extraction

The phospholipids from 200 µl of each plasma lipoprotein and lipoprotein free fractions (three independent Lipoprotein isolation by salt-gradient ultracentrifugation) were extracted with 4 ml of ice-cold solution of chloroform: methanol (1:1 v/v). The suspension was incubated for 30 min at 4 °C. Subsequently, the mixture was centrifuged at 4,000 rpm for 15 min at 4 °C. The supernatant was collected and the pellets were treated with 3 ml chloroform: methanol (2:1 v/v) followed by incubation and centrifugation. The collected supernatants were pooled, pelletted under nitrogen gas, solubilized in chloroform: methanol: water (50:45:5 v/v) and stored at -80 °C. This phospholipid extraction approach is a single-phase extraction (monophasic) (Houtkooper et al., 2009). Based on this monophasic approach, we assume that we did not loose any phospholipids in our plasma lipoprotein fractions.

Liquid Chromatography- ElectroSpray Ionization-MS (LC-ESI-MS)

LC

The LC system consisted of an HP1100 series binary gradient pump, a vacuum degasser, a column temperature controller (all from Hewlett Packard, Wilmington, DE, USA) and a Gilson 231 XL autosampler (Gilson, Villiers-le-Bel, France). 20 µL of sample was loaded onto an analytical LiChrosphere Si 60 column (250 x 2 mm, 5µm particle size, Merck, Darmstadt, Germany) and the column temperature was maintained at 22 °C. Samples were eluted with a flow rate of 0.3 mL/min and a linear gradient between solution A (CH_3OH/H_2O, 90:10 v/v, 0.1% NH_4OH) and solution B ($CHCl_3/CH_3OH$, 98:2 v/v, 0.01% NH_4OH). The gradient was programmed: from T=0 min, 20% A and 80% B towards t = 5 min, 100% A; t = 5-8 min 100% A; t = 8-8.1min change to 100% B; t = 8.1-14 min 100% B. t = 14-14.1 min: return back to the starting composition; a flow of 0.5 ml/min was used to equilibrate the column. All gradient steps were linear, and the total analysis time, including the equilibration, was 18 min.

A splitter between the LC column and MS allowed introduction of eluant with a flow of 30 µl/min. An electrically operated valve selected the introduction of eluant in the MS between t = 3 and 14 min.

ElectroSpray Ionization - Mass Spectrometry (ESI-MS)

A Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, UK), was used in the positive and negative electrospray ionization (ESI)

mode. Nitrogen was used as nebulizing gas and argon was used as collision gas at a pressure of 2.5 x 10^{-3} mBar (collision energy 20-40 eV). The capillary voltage used was 3KV. The source temperature was set at 80 °C and optimal cone voltage was 45 V. Full scans were acquired between (+/-) m/z 400 and (+/-) m/z 1000 in 3s/scan. Specific phospholipid identities were confirmed by the characteristic constant neutral loss or parent scans in the corresponding retention time windows (for PE). All LC-ESI/MS experiments were performed with two independent lipoprotein isolation by salt-gradient ultracentrifugation.

Liquid Chromatography –ESI-tandem Mass Spectrometry (LC-ESI-MS/ MS)

LC

Chromatographic separation was achieved on a Surveyor modular LC System (Thermo Finnigan Corporation, San Jose, CA, USA) consisting of a thermostated autosampler, a low-flow quaternary MS pump and a 2.1×250 mm silica column, 5µm (particle size) (Merck, Darmstadt, Germany).
Samples were eluted with a flow rate of 0.3 ml/min and a linear gradient between solution A (CH_3OH/H_2O, 85:15 v/v, 0.1% NH_4OH) and solution B ($CHCl_3/CH_3OH$, 97:3 v/v, 0.01% NH_4OH). The gradient was programmed: from T=0 min, 20% A and 80% B towards T=10 min, 100% A; T=10-12 min 100% A; T=12-12.1min change to 100% B; T=12.1-17 min 100% B. T=17-17.1 min: return back to the starting composition. A splitter between the LC column and the mass spectrometer was used; 75 µl/min was introduced into the mass spectrometer.

ESI-MS/MS analysis

MS/MS analyses were performed on a TSQ Quantum AM (Thermo Finnigan Corporation, San Jose, CA, USA) operated alternating in the negative- and positive ion electrospray ionization (ESI) mode in consecutive runs. The SID was set at 10 V; spray voltage was 3600 V and the capillary temperature was 300 °C. In the MS/MS experiments Ar was used as collision gas at a pressure of 0.5 mtorr; collision energy ranged between 20-40 eV for the different optimized transitions. In the negative mode mass spectra were obtained by continuous scanning between m/z 400 - m/z 1000 (2s/scan). In the positive mode characteristic constant neutral loss (CNL) or parent (P) scans were used to selectively detect specific phospholipids in their corresponding retention time windows: CNL (141) for PE, CNL (172) for PG, (260) for PI, CNL (185) for PS, CNL and P (184) for PC, LPC, SM (Taguchi et al., 2005). CNL (172) for PG and (260) for PI was described previously by Sommer *et al.* (Sommer et al., 2006) and Cole *et al.* (Cole and Enke, 1991).

Cardiolipins are easily identified based upon their retention time and typical clusters of doubly charged ions as described previously by Houtkooper *et al.* (Houtkooper et al., 2009) and Han *et al.* (Han et al., 2006) and we applied this approach to determine CL in plasma lipoprotein samples.

High Performance Thin Layer Chromatography (HPTLC)

HPTLC was performed as described previously (Weerheim et al., 2002). Very short description: TLC was applied on HPTLC plates, which is

purchased from Merck (Darmstadt, Germany). The impurities of plates were removed by methanol: ethyl acetate treatment (6:4) in a Camag horizontal developing chamber. The plates were activated for 10 min at 130°C. Samples and one mixture of phospholipids standards were applied to one HPTLC-plate. Subsequently, the plate was developed in a Camag horizontal developing chamber.

The HPTLC-plate was dried under an air stream (40°C) on a heating plate (DESAGA, Heidelberg, Germany) for 10 min at 40°C. Finally, phospholipids were separated as described previously (Weerheim et al., 2002).

RESULTS

Characterization of lipoproteins

High performance gel-permeation chromatography (HPGC)

To investigate whether one-step ultracentrifugation provided lipoprotein fractions of acceptable purity, the collected lipoprotein fractions were subjected to HPGC (figure 1).

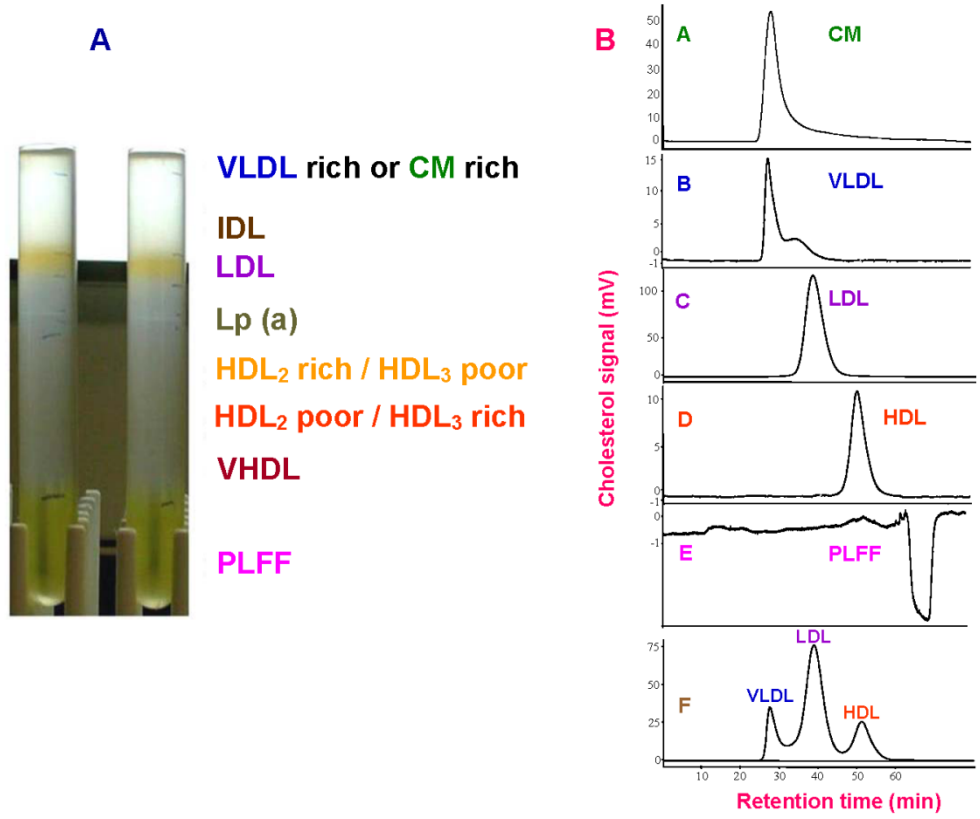

Figure 1 A-B. Panel **A** shows the major human plasma lipoprotein fractions after one-step salt gradient ultracentrifugation. Panel **B** shows Cholesterol profiles of major plasma lipoprotein fractions separated by High Performance Gel-Permeation Chromatography (HPGC). Lipoprotein fractions obtained by one-step ultracentrifugation were subjected to size-exclusion chromatography as described in Material and Methods. Panel A: CM fraction, Panel B: VLDL fraction, panel C: LDL fraction, panel D: HDL fraction, panel E: Plasma lipoproteins free fraction and panel F: standard cholesterol.

The HDL fraction obtained by ultracentrifugation resulted in a single peak with a retention time of approximately 51 min (figure 1D). HPGC elution of a commercial HDL lipoprotein standard revealed identical elution patterns, which confirmed that the fraction contained only HDL (figure 1F).

VLDL and LDL fractions isolated by the same method yielded peaks eluting at approximately 28 min and 40 min, respectively (figure 1B and C). Same retention times were found when commercial LDL and VLDL lipoprotein standards were analyzed by HPGC, confirming the identity of the peaks (figure 1F). However, the intermediate-density lipoprotein (IDL) peak was also detected in the VLDL fraction. No peaks were detected in the plasma lipoprotein free fraction (figure 1E). The IDL fraction obtained by a combination of ultracentrifugation and HPGC yielded a peak eluting at approximately 35 min (figure 2). Although the IDL fraction contains LDL, the fraction was very highly enriched in IDL. The subjection of CM fraction to HPGC resulted in a single peak with a retention time of 28 min which is similar to elution peak of VLDL (figure 1A). Although both CM and VLDL fractions yielded a peak eluting at 28 min, the IDL peak was not observed in CM fraction.

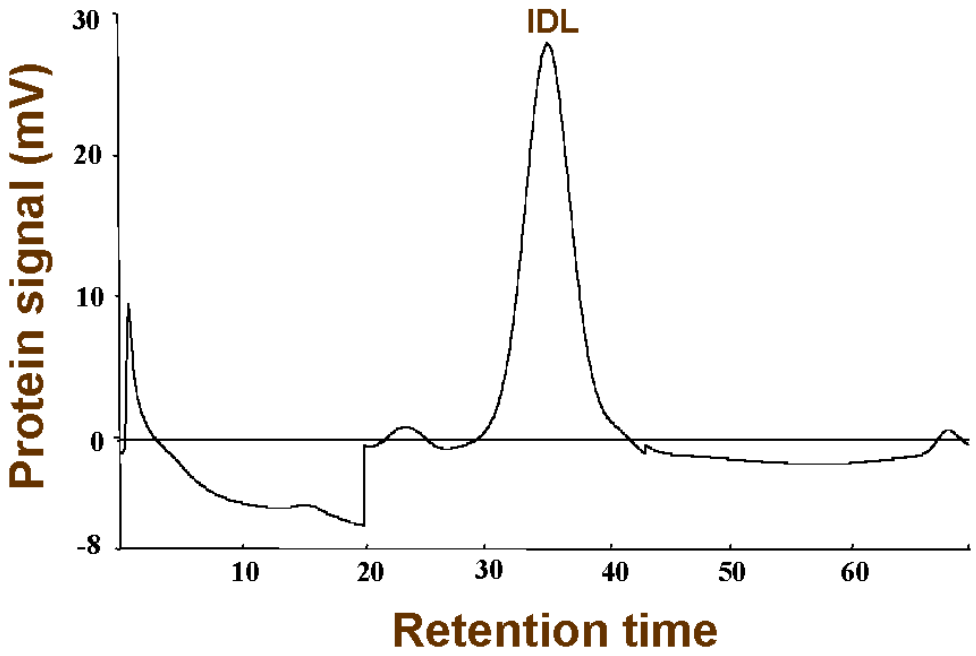

Figure 2. Protein profile of IDL fraction.

The ultra collected IDL fraction were several times exposed to HPGC to purify the IDL, therefore we used protein profile (to re-use elution for HPGC exposure) instead of cholesterol profile as described in Material and Methods. However, the cholesterol profile of ultra collected IDL fraction (before purification with HPGC) revealed approximately the same elution peak.

Nephlometry and Western blot analysis of apolipoproteins

Nephelometry was performed to further investigate the results obtained by HPGC concerning the purity of the isolated lipoprotein fractions collected by ultracentrifugation. Nephelometric analysis revealed the presence of apo A-I in the HDL fraction (approximately 1.1 g/l), but not in the VLDL, IDL and LDL or plasma lipoprotein free fractions. In addition, no cross-contamination of apo B was observed in the HDL or PLFF fractions while it was found to be abundant in the CM (0.52 g/l), VLDL (approximately 0.7 g/l), IDL (0.7 g/l) and LDL (1.2 g/l) fractions. As displayed in figure 3, Western blotting with anti-apo B antibodies confirmed the results obtained by HPGC and nephlometry.

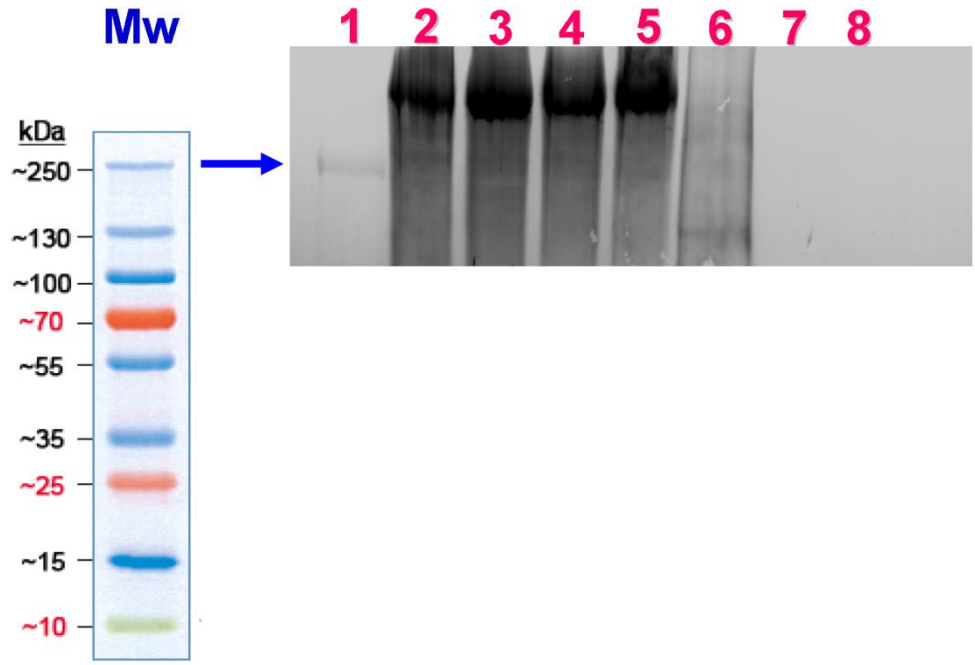

Figure 3. Western blot analysis of purified plasma lipoproteins.

Plasma lipoprotein- associated proteins (20-100 µg) were separated by 6 % (w/v) SDS-PAGE under denatured conditions, blotted for 20 h and probed with monoclonal anti-apo B antibodies as described in Materials and Methods. The plasma lipoproteins used were CM (lane 2, 80 ug), VLDL (lane 3, 80 ug), IDL (lane 4, 80 ug), LDL (lane 5, 80 ug), Apo B protein (lane 6, 20 ug), and HDL (lane 7, 100 ug). The lane 1 is protein marker and lane 8 is plasma lipoprotein free fraction (PLFF, 100 ug). The molecular weight standards (M_w) are in kilo Daltons (kDa) and indicated on the left of the figure. The approximately 500 kDa band is indicated by arrow on the left of the figure.

As depicted in figure 3, anti-apo B antibodies showed the presence of an intense band of about 550 kDa corresponding to full length apo B100 in VLDL, IDL and LDL fractions, but not in HDL and PLFF fractions. This indicated that the one-step ultracentrifugation protocol resulted in a purification of the major lipoprotein fractions (and a combination of ultra

and HPGC for IDL purification) without contamination with other lipoproteins.

Identification of plasma lipoprotein phospholipids composition

Liquid Chromatography-ElectroSpray Ionization-Mass Spectrometry (LC-ESI-MS)

LC-ESI-MS analysis in the negative and positive mode led to the identification of different phospholipids associated with lipoproteins. All samples were run twice in positive ion and negative mode, allowing the detection of the predominant positive and/or negative ions of each class of phospholipids. Although we did not observe CL, PG and PS in all lipoprotein fractions as well as PLFF (table 1, supplement (http://www.nature.com/scientificreports)) the PI (figure 1 A-D, supplement (http://www.nature.com/scientificreports)) and SM were only detected in the plasma lipoprotein fractions (figure 2 A-D, supplement (http://www.nature.com/scientificreports)). The PC, Lyso-PC and PE were also detected in all fractions (table 1, supplement (http://www.nature.com/scientificreports)). However, PE was not measured in PLFF fraction, using LC-ESI-MS approach (see Materials and Methods; ESI-MS paragraph). Also, CM was not analyzed by LC-ESI-MS strategy.

LC- ESI- tandem MS (LC-ESI-MS/MS)

LC-ESI-MS/MS was applied to further examine the observed phospholipild profile in the major lipoprotein fractions and PLFF obtained by LC-ESI-MS analysis.

LC-ESI-MS/MS strategy revealed the presence of PE (figure 4 A-E), PI (figure 5 A-E) and SM (figure 6 A-E) in all lipoprotein fractions but not in PLFF. The occurrence of PC and Lyso-PC were shown in all lipoprotein fractions and PLFF (figure 7 A-E and figure 8 A-E respectively), using LC-ESI-MS/MS. Although traces of SM were found in PLFF fraction, it could have resulted from very small traces of VHDL fraction and not vice versa. Furthermore, the CL, PG, and PS were also not detected in all fractions, applying this strategy. Although CL, PG, and PS were not observed in all fractions, lipoprotein fractions and PLFF obtained by LC-ESI-MS/MS strategy corresponded with the LC-ESI-MS profiles of the same fractions. The global intensity of each analyzed phospholipid in the different fractions was shown in table 1.

PL	1	2	3	4	5	6	7	8	9	MW (Da)
L-PC	116	17	5	53	110	63	0	20	51	525
SM	719	173	72	1258	891	5	71	247	65	747
PC	3803	1080	180	6022	230772	61	236	3144	561	791
PI	68	22	6	97	298	0	0	38	14	867
PE	164	64	6	189	633	0	0	96	26	748

Table 1. Relative quantitative analysis of major phospholipids in 8 plasma lipoprotein fractions as well as in novel plasma lipoprotein free fraction as plasma control based on the concentration of phospholipids used in standard mixture and expressed in nmol/ml.

Plasma lipoprotein phospholipids composition and PLFF fraction were extracted and applied to one-dimensional, horizontal HPTLC as described under Materials and Methods and legend of figure 9. Samples were numbered from 1 to 9. Samples were: (1-Chylomicrons, 2-VLDL, 3-IDL, 4-LDL, 5-HDL_2, 6-PLFF (Control), 7-Lp(a), 8-HDL_3 and 9-VHDL. The concentration was expressed as nmol/ml. Molecular weight is abbreviated as MW and expressed as kilodalton (kDa).

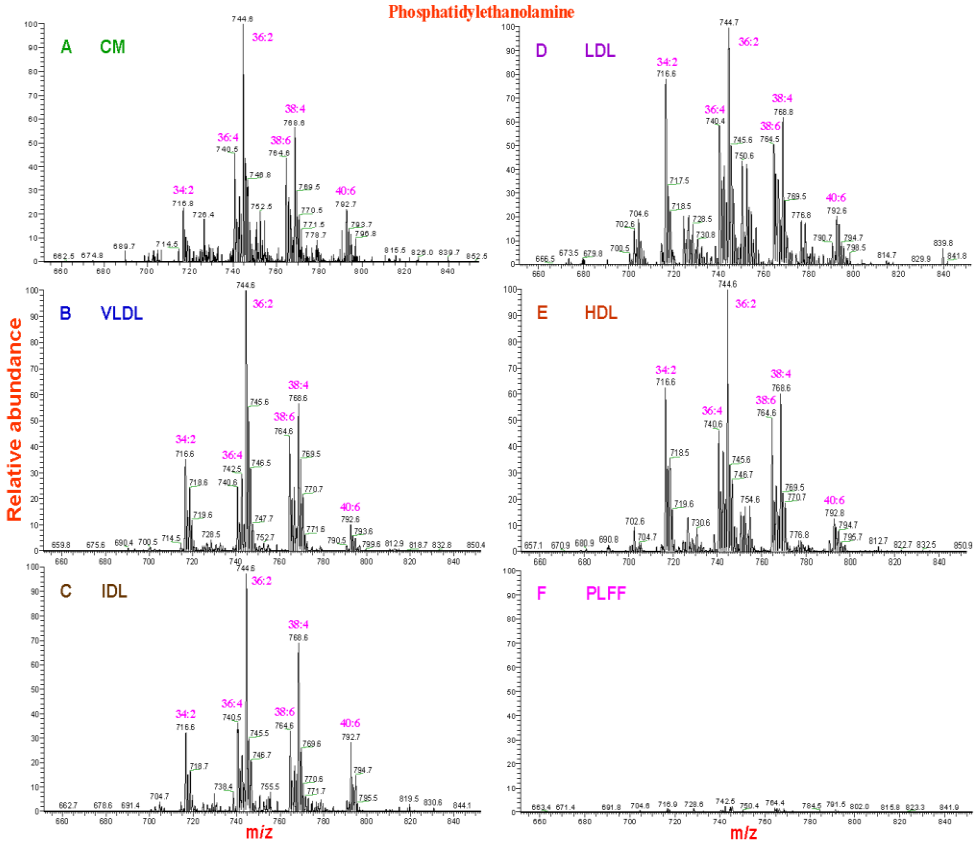

Figure 4. High-performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of plasma lipoproteins and plasma lipoprotein free fraction.

We did not observe any adducts ions like Na or K. positive scan [M + H]+. Characteristic part of the parent-scan for phosphatidylethanolamine (PE) in the corresponding retention time window.

'36:2' specifies the composition of the protonated molecular ion as a species with 36 C-atoms in total in the acyl-chains with 2 unsaturated bonds in total; other assignments are analogous. A) CM fraction, B) VLDL fraction, C) IDL fraction, D) LDL fraction, E) HDL fraction and F) PLFF fraction.

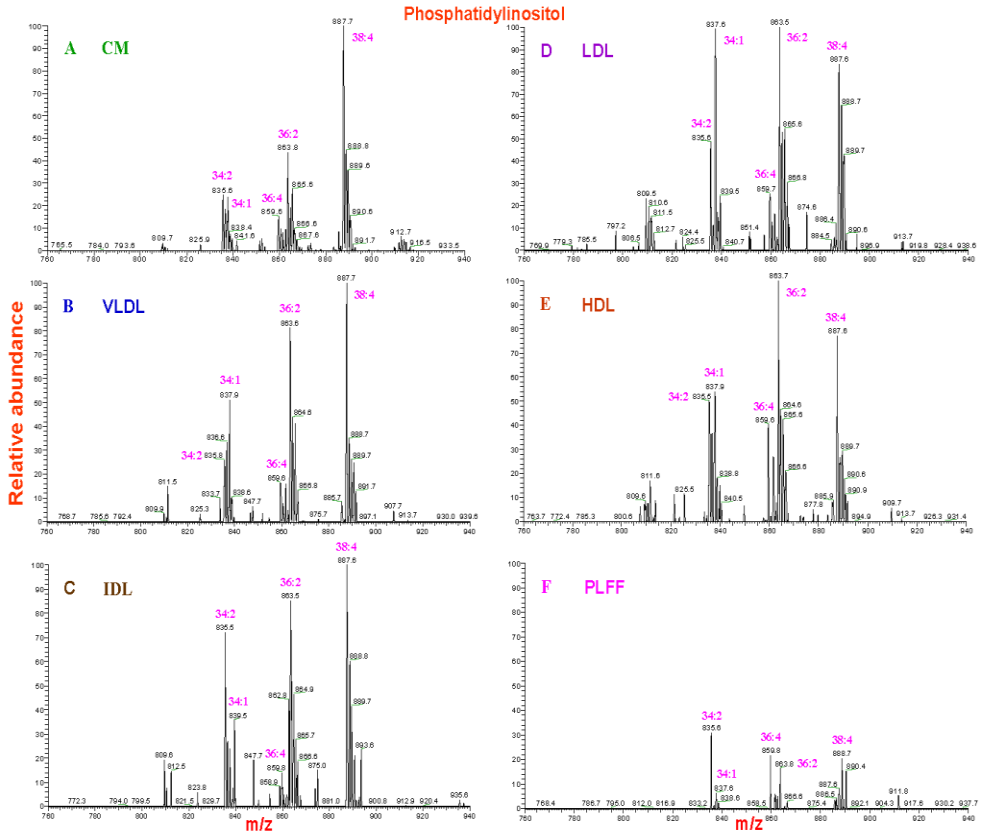

Figure 5. High-performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of plasma lipoproteins and plasma lipoprotein free fraction.

We did not observe any adducts ions like Na or K. positive scan [M + H]+.Characteristic part of the parent-scan for phosphatidylinositol (PI) in the corresponding retention time window.

'36:2' specifies the composition of the protonated molecular ion as a species with 36 C-atoms in total in the acyl-chains with 2 unsaturated bonds in total; other assignments are

analogous. A) CM fraction, B) VLDL fraction, C) IDL fraction, D) LDL fraction, E) HDL fraction and F) PLFF fraction.

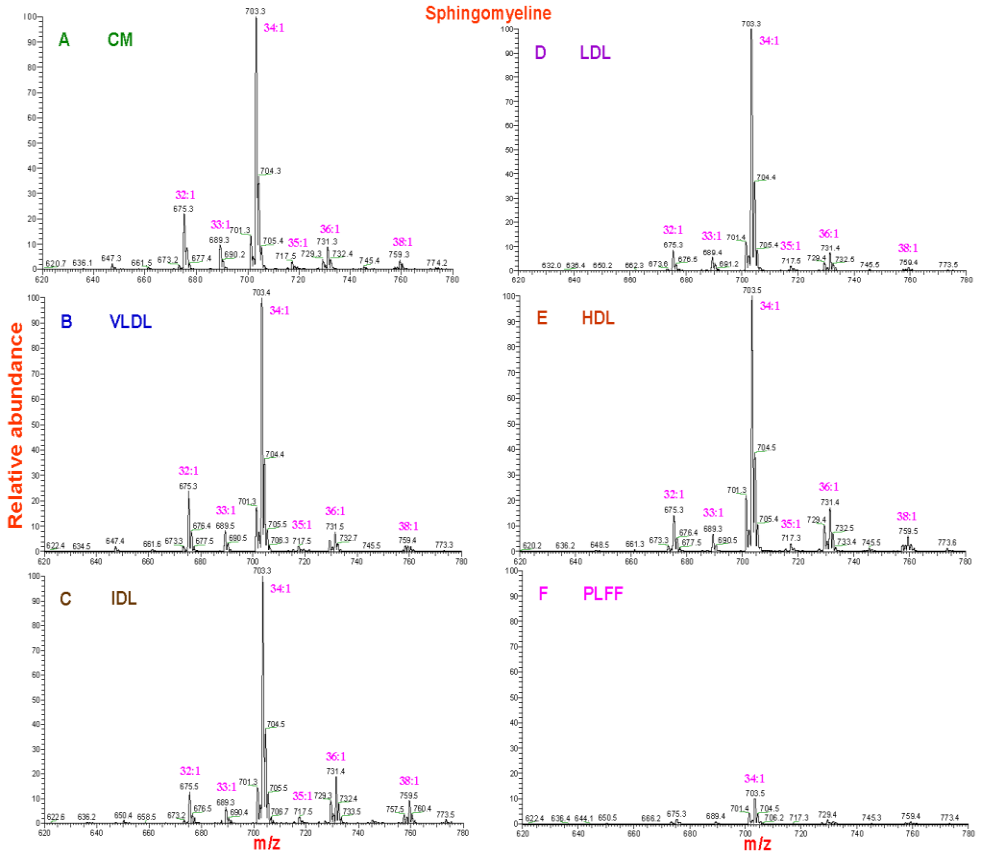

Figure 6. High-performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of plasma lipoproteins and plasma lipoprotein free fraction.

We did not observe any adducts ions like Na or K. positive scan [M + H]+.Characteristic part of the parent-scan for sphingomyeline (SM) in the corresponding retention time window.

'34:1' specifies the composition of the protonated molecular ion as a species with 34 C-atoms in total in the acylchain and alkyl chain of the sphingosine moiety with 1 unsaturated bond in total; other assignments are analogous. A) CM fraction, B) VLDL fraction, C) IDL fraction, D) LDL fraction, E) HDL fraction and F) PLFF fraction.

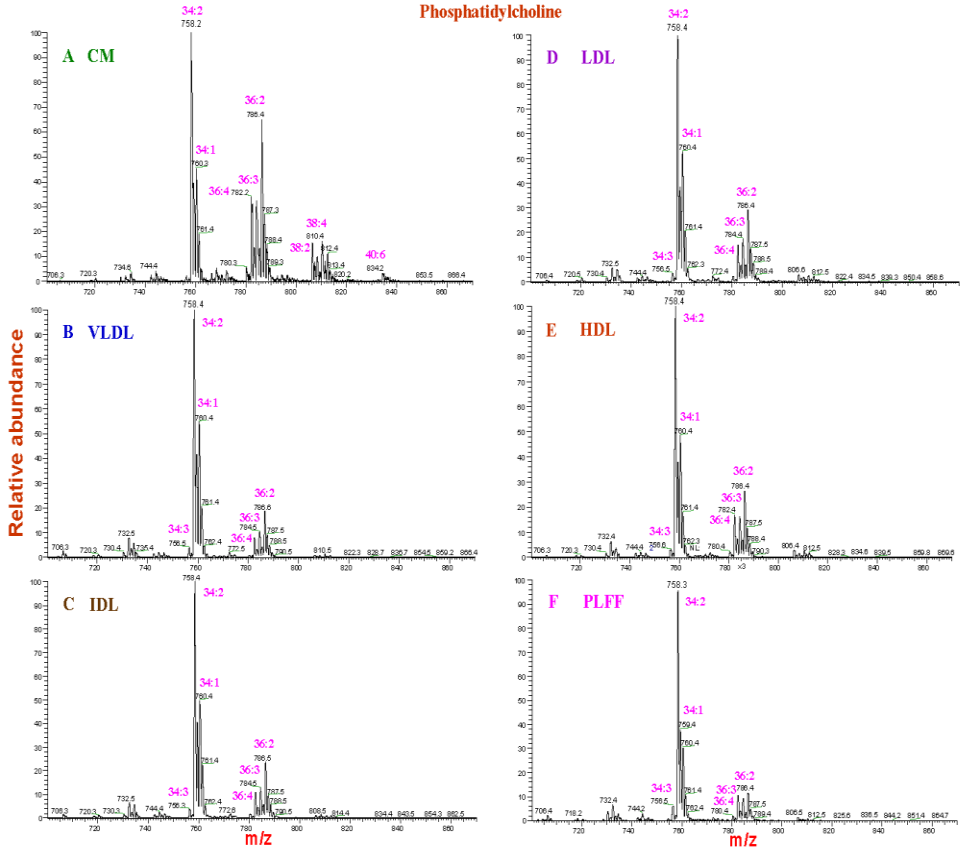

Figure 7. High-performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of plasma lipoproteins and plasma lipoprotein free fraction.

We did not observe any adducts ions like Na or K. positive scan [M + H]+. Characteristic part of the parent-scan for phosphatidylcholine (PC) in the corresponding retention time window.

‘34:2’ specifies the composition of the protonated molecular ion as a species with 34 C-atoms in total in the acyl-chains with 2 unsaturated bonds in total; other assignments are analogous. A) CM fraction, B) VLDL fraction, C) IDL fraction, D) LDL fraction, E) HDL fraction and F) PLFF fraction.

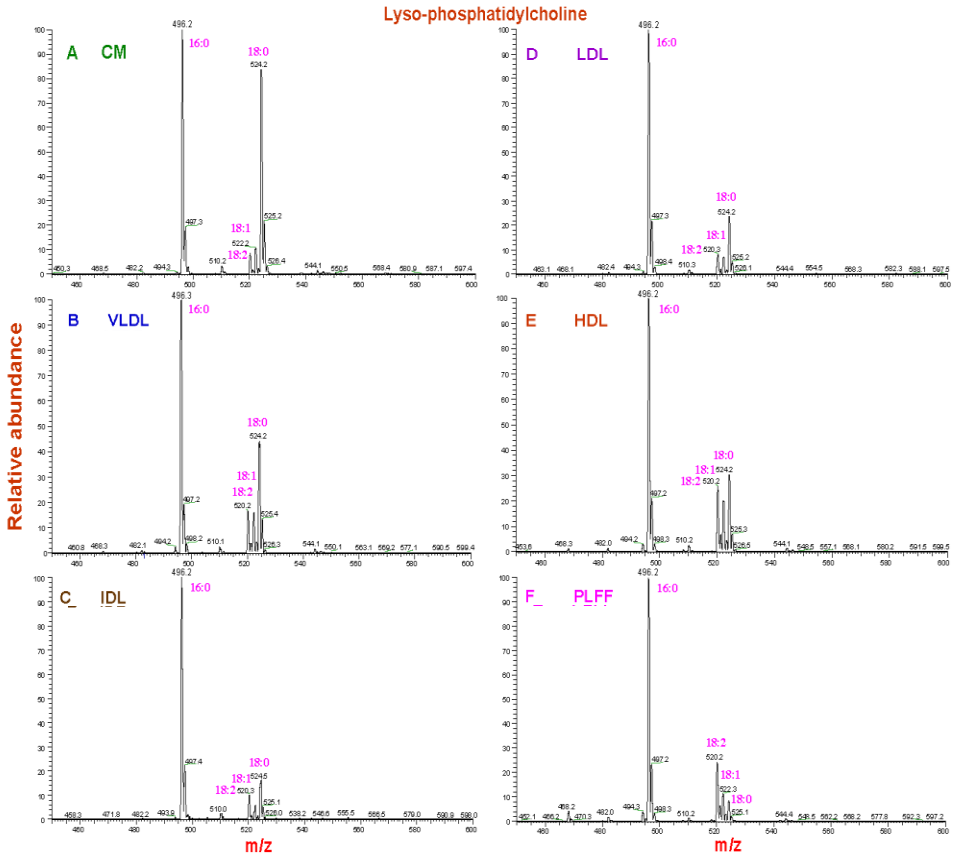

Figure 8. High-performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of plasma lipoproteins and plasma lipoprotein free fraction.

We did not observe any adducts ions like Na or K. positive scan [M + H]+.Characteristic part of the parent-scan for lyso-phosphatidylcholine (LPC) in the corresponding retention time window.

‘18:0’ specifies the composition of the protonated molecular ion as a species with 18 C-atoms in the acyl-chain with 0 unsaturated bonds in total; other assignments are analogous. A) CM fraction, B) VLDL fraction, C) IDL fraction, D) LDL fraction, E) HDL fraction and F) PLFF fraction.

High Performance Thin Layer Chromatography (HPTLC)

HPTLC was performed to further confirm the results obtained by MS and tandem MS. The relative abundance of plasma lipoprotein-associated phospholipids as determined by LC-ESI-MS and LC- ESI- tandem MS method agrees very well with those results obtained by HPTLC approach (figure 9). HPTLC method also confirmed the presence of PE, PI and SM only in lipoprotein fractions and not in PLFF fraction (figure 9). Moreover, as depicted in figure 9 CL, PG, and PS were not detected in all fractions applying HPTLC and corresponded to the results obtained either by LC-ESI-MS or LC- ESI-MS/MS (tandem MS). Similar results were also obtained by HPTLC as compared to the other two above-mentioned strategies regarding PC and Lyso-PC (MS data of HDL-2 and VHDL not shown). Although HPTLC confirmed the results obtained by two other strategies, the relative quantity of the major phospholipids was calculated and shown in table 2. As displayed in table 2, the relative concentration of PC in HDL2 was tremendously higher than the rest of plasma lipoproteins.

PLFF	PHOSPHOLIPIDS							
	PE	PG	PS	PI	PC	SM	LP-C	CL
VLDL	+++++	nd	nd	++++	++++	+++	+	nd
IDL	+	nd	nd	++	+++	++	+++++	nd
LDL	+	nd	nd	+++	++++	+++++	++	nd
HDL_2	+	nd	nd	+++	++++	++	+	nd
HDL_3	++	nd	nd	+++++	+++++	+++	++	nd
VHDL	+	nd	nd	++++	+++	++	+++	nd
PLFF	nd	nd	nd	nd	+	+	++++	nd

Table 2. Global relative abundance of each phospholipid based on the intensity of the most intense peak.

Relative abundance of phospholipids in the different plasma lipoprotein fractions were indicated by +, Very small traces of a detected phospholipids are indicated by small +. The abbreviations are: Plasma lipoprotein fraction (PLPF), Plasma lipoprotein free fraction (PLFF) and not detected (nd). The results for CM fraction was obtained in a separate tandem MS run, hence relative intensity of CM were not included in this table. A similar pattern was observed for the phospholipids composition of CM and other lipoprotein fractions, although CM fraction was separately analyzed. Please see the list of abbreviation in supplement (http://www.nature.com/scientificreports)

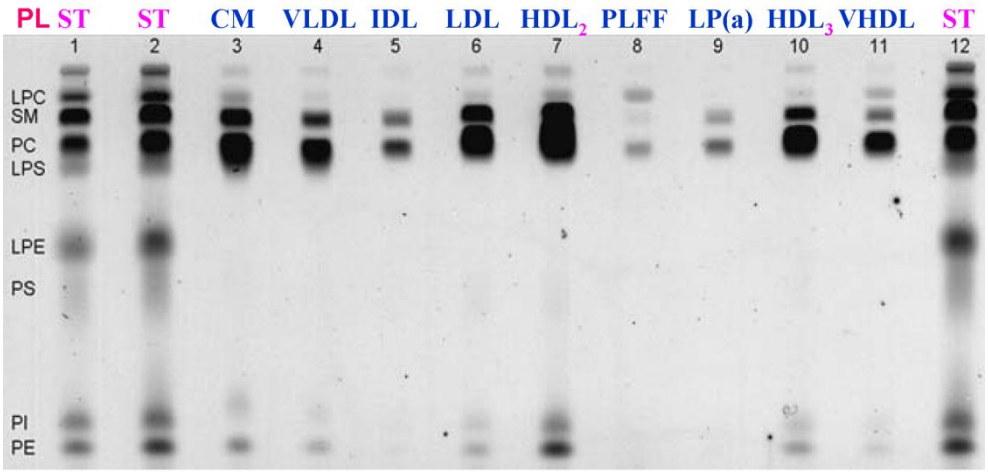

Figure 9. Separation of phospholipids from plasma lipoprotein fractions and plasma lipoprotein free fraction by HPTLC.

Plasma lipoprotein phospholipid composition and PLFF fraction were extracted and applied to one-dimensional, horizontal HPTLC as described under Materials and Methods. Representative separations are shown of a mixture of purified (ST=standard) phospholipids (lane 1 (10 µl), 2 and 12 (20 µ)). The following concentration of phospholipids (PL) were used in standard mixture and expressed in ng/µl (Lyso-PC 51, SM 101.8, PC 100, PI 50, PE 49.7, Lyso-PS 67.2, Lyso-PE 53 and PS 50.2). The following volumes of lipoprotein

phospholipids extracts were utilized in HPTLC (Chylomicrons (lane 3, 3 µl), VLDL (lane 4, 4 µl), IDL (lane 5, 5 µl), LDL (lane 6, 2 µl), HDL_2 (lane 7, 2 µl), PLFF (lane 8, 5 µl), Lp(a) (lane 9, 2 µl), HDL_3 (lane 10, 4 µl), and VHDL (lane 11, 5 µl). The different phospholipids composition of standard mixture is indicated on the left and lane number on the top of figure.

DISCUSSION

To shed light in the role of lipoproteins in patho-physiologic processes it is essential to elucidate the make-up of the lipoprotein particles, i.e. its phospholipid and protein composition. Since recent clinical studies have stressed the atheroprotective and proathero role of HDL and LDL respectively (Nofer et al., 2001; Sachinidis et al., 1999), further studies of these particles have become even more crucial. In this study, as first step, we applied a LC-ESI/MS and LC-ESI-MS/MS strategy to lipoprotein fractions to identify lipoprotein-associated phospholipids. Due to its high sensitivity, selectivity and specificity, LC-ESI-MS/MS is without parallel the most sophisticated technique for assessing the lipid composition of biological samples such as the phospholipid content of plasma lipoproteins. Lipoproteins were isolated from human pooled plasmas and the quality of purity of collected lipoproteins (VLDL, IDL, LDL and HDLs) was determined using three independent techniques: HPGC, nephelometry and Western blotting (Rezaee et al., 2006). Furthermore, it is basically impossible to separate chylomicrones from VLDL to collect the pure VLDL or CM due to the continuum of VLDL and CM sizes by ultracentrifugation and HPGC. Although CM and VLDL fraction could be contaminated with each other, the first fraction was rich in CM and the second one rich in VLDL. However, we did not observe any specific differences between the

fraction rich in CM and VLDL fraction with respect to phospholipid composition.

Nephelometric analysis exhibited that apo A-I (the major constituent of HDL) was only observed in the HDL fractions, whereas apo B (the major constituent of CM, VLDL, IDL and LDL) was not. Furthermore, cholesterol analysis, protein analysis and elution behaviour of ultracentrifugation isolated VLDL, IDL (extra purified steps by HPGC), LDL, HDL and PLFF confirmed the purity of the fractions. Finally, Western blot analysis revealed the absence of apo B in the isolated HDL fractions and the presence of apo B in the isolated CM, VLDL, IDL and LDL fractions. These results confirm the utility of our strategy to collect lipoprotein fractions from plasma that was not contaminated with other lipoproteins, although CM and VLDL fractions could be contaminated with each other.

LC-ESI/MS analysis resulted in the identification of sphingomyelin, PE, PI, PC and Lyso-PC in all lipoprotein fractions. However, we did not observe PG, PS and CL in plasma lipoprotein and lipoprotein free fractions. This could be due to the presence of only very low amounts of these phospholipids (below the detection limit of the LC-ESI-MS technique) in plasma lipoproteins and lipoprotein free fractions under normal conditions in plasma. The very low abundances of these phospholipids were also mentioned by Deguchi *et a.l.* (Deguchi et al., 2000). Also, PG constitutes 2% of the total plasma phospholipids as shown by Schwars *et al.* (Schwarz et al., 1977) and was confirmed by Uran *et al.* (Uran et al., 2001). Since plasma divided in 9 fractions in this study, the concentration of PG (2%) will be also divided in these 9 fractions. That could be the reason why PG was not detected in our samples.

LC-ESI-MS/MS was used as a complementary technique to improve selectivity and consequently the signal/noise ratio of the preselected

compound classes; overall it lowers limit of detections (LOD). By using the MS/MS mode (classes of) compounds can easily be identified, filtering out the interference of all those compounds that do not meet the MS/MS conditions. LC-ESI-MS/MS approach led to the identification of the same phospholipids as we identified in the different fractions when we used LC-ESI-MS, Indicating that LC-ESI-MS/MS confirms the results obtained by LC-ESI-MS.

It is intriguing to note that PE, PI and SM were only observed in plasma lipoproteins and not in PLFF. This finding confirmed the specificity of our utility to identify lipoprotein-associated phospholipids. Furthermore, on searching the literature, we were unable to find any study in which the plasma lipoprotein free fractions were used as control for plasma lipoproteins. In addition, HPTLC was used as an alternative method to support and confirm the results obtained by MS and tandem MS approach regarding phospholipids composition of the major plasma lipoproteins. Three independent strategies led to the identification of a similar lipoprotein-associated phospholipids pattern, indicating a high confidence in the obtained results. Interestingly, the molecular profile appeared to be conserved between the different lipoproteins even though the concentrations of the lipid classes show slight differences except for PC species 38:2, 38:4 and 40:6 in CM fraction. These three PC species were only detected in CM fraction and were complete absent in other fractions.

Although LC-ESI-MS and LC-ESI-MS/MS were applied as qualitative approach, HPTLC was used as semi-quantitative in this study. MS and tandem MS were used as qualitative approach, because of three points: 1- the use of different internal standard for each plasma lipoprotein fraction, 2; the use of different internal standard for each main phopholipid and lipid species and 3; the combination of step 1 and 2 increase the complexity of

samples and in turn the use of many different internal standards hampered to obtain correct quantification. This complexity was also described by Uran *et al*. (Uran et al., 2001). However, Uran *et al*. have shown a limit detection of 0.1-5 ng for blood-associated phospholipids. For this reason, HPTLC was used as semi-quantitative approach in this study and not MS approach

Numerous studies show that low HDL-C can be considered as an independent risk factor of atherosclerosis even when LDL-C is low (Castelli, 1988). In addition, it is also well studied that increased HDL-C reduces the risk for cardiovascular disease (Castelli, 1988; Gordon et al., 1977). Although there are many available strategies to increase the HDL-C, the present available therapies with the current drugs have a limited effect because of low efficacy and severe side effects of these drugs (Burgess et al., 2005) [29]**.** An alternative therapy for the increase of the HDL-C levels is PI therapy via diet as shown recently by Burgess *et al.* and the others (Burgess et al., 2003; Burgess et al., 2005; Stamler et al., 2000). Burgess *et al.* have shown that PI stimulates cholesterol reverse transport via an increased efflux from peripheral tissues to HDL and in turn the transport of cholesterol from HDL to the liver, bile, and faeces for clearance or removing (Burgess et al., 2003; Burgess et al., 2005; Stamler et al., 2000). They have also demonstrated that when PI is taken orally during two weeks therapy leads on the one side the increase of apo-AI concentrations and on the other side the decrease of plasma triglyceride levels. Our results revealed that PI is only present on plasma lipoproteins, and this may increases the specificity of therapy with PI via diet to affect the lipoprotein composition levels. Although the mechanism study of PI effect on lipoprotein is beyond our study, the PI therapy via diet is very simple and performable without major and severe side effects.

Another novel finding in this study is also the presence of SM only on lipoproteins. Although SM plays a major role in the constitution of all lipoproteins, the physiological function of SM in plasma is not until recently clear. Papasani *et al.* (Papasani et al., 1999) have shown that SM inhibits the oxidation of lipoproteins. Furthermore, Rey *et al.* (Rye et al., 1996) have shown in reconstituted high-density lipoproteins studies that SM influences the structure of discoidal and spherical rHDL as well as it inhibits the LCAT reaction in discoidal rHDL. It has been recently suggested that presence of SM in the lipoprotein surface involved in lipoprotein lipase-mediated lipolysis, and that cholesterol enrichment in the lipoprotein surface does not affect lipolysis (Arimoto et al., 1998; Saito et al., 1997). Moreover, it has been reported that SM can be used as marker for the clearance of remnant-like particles (Schlitt et al., 2005). Also, Jiang *et al.* have suggested that plasma SM concentration is a risk factor for atherosclerotic process (Jiang et al., 2000). Although the function of SM in lipoproteins remains to be fully clarified, the SM could be also considered as an alternative therapy and pharmacological intervention to affect lipoprotein levels. To our knowledge, the observed effects of PI (Burgess et al., 2003; Burgess et al., 2005; Stamler et al., 2000) and SM (Arimoto et al., 1998; Gordon et al., 1977; Rye et al., 1996; Saito et al., 1997; Schlitt et al., 2005; Subbaiah et al., 1999) in plasma could be only possible via lipoproteins, since PI and SM were only present on lipoproteins (with high intensity of SM in LDL fraction) and not detected in plasma lipoprotein free fractions.

Next to other studies (Fournier et al., 1997; Klein et al., 2001; Vaysse et al., 1985), our approach also revealed the presence of PE on lipoproteins. Recently, Agren *et al.*(Agren et al., 2005) demonstrated that there is a selective secretion of PE and sphingomyelin molecular species during VLDL secretion when they compare the molecular species phospholipids

content of VLDL with liver phospholipids composition. Furthermore, it has been recently shown that Prothrombinase enhancement through quantitative and qualitative changes appear to affect very low-density lipoprotein in complex with C-reactive protein, and the qualitative changes appeared to be resulted from a deficiency of PE in VLDL from disseminated intravascular coagulation patients with the biphasic waveform (Dennis et al., 2004). In addition, Phosphatidylethanolamine N-methyltransferase appears to be an essential component in the secretion of apoB100-containing VLDLs (Noga et al., 2002). Since PE is only present in lipoproteins, (with highest abundancy in VLDL fraction) its physiological role could be only possible via lipoproteins.

LC-ESI/MS analysis further revealed the presence of PC and Lyso-PC on lipoproteins. Parthasarathy *et al.* have reported that 40% of the PC in LDL is converted to lyso-PC during oxidized modification. Also, the lyso-PC concentration is shown to be increased in atherosclerotic plaques in mice fed an atherogenic diet (Parthasarathy et al., 1985; Portman and Alexander, 1969). Furthermore, Lyso-PC induces human coronary artery Smooth muscle cells migration (Kohno et al., 1998). In addition, Lyso-PC increased in atherogenic lipoproteins and atherosclerotic lesions, and has been shown to stimulate transcription of a variety of endothelial genes relevant to atherogenesis (Ueno et al., 1999).

In summary, this study resulted in the identification of 5 phospholipids associated with lipoproteins of which PE, PI and SM were only present in lipoproteins. The role of these identified lipoprotein-associated phospholipids in atherothrombosis awaits further investigation and may provide new insights into the mechanism(s) of atherothrombotic diseases and the future pharmacological intervention.

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Chapter 4

An In-depth Proteomic Analysis of Human Plasma Very Low-Density Lipoprotein (VLDL) and Low-Density Lipoprotein (LDL)

Monireh Dashti [1, 2], Mahdi Motazacker [3], Han Levels [3], Marcel de Vries [1], Maikel P. Peppelenbosch [2], Farhad Rezaee [1]

[1] Department of Cell Biology, UMCG, University of Groningen, The Netherlands

[2] Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands

[3] Department of Vascular Medicine, Academic MC, Amsterdam, The Netherlands

(Under review)

ABSTRACT

Apart from transporting lipids through the body, the human plasma lipoproteins VLDL and LDL are also thought to serve as a modality for intraorganismal protein transfer, shipping proteins with important roles in inflammation and thrombosis from the site of synthesis to effector locations. To better understand the role of VLDL and LDL in the transport of proteins, we applied a combination of ORBITRAP-LTQ-XL (nLC-MS/MS) with both in-SDS-PAGE gel and in-solution tryptic digestion of pure and defined VLDL and LDL fractions. We identified the presence of 95 VLDL and 51 LDL associated proteins including all known apolipoproteins and lipid transport proteins, and intriguingly a set of coagulation, complement system and anti- microbial proteins. Prothrombin, protein S, fibrinogen γ, PLTP, CETP, CD14 and LBP were present on VLDL but not on LDL. Prenylcysteine oxidase 1, dermcidin, Cathelicidin antimicrobial peptide, TFPI-1 and Fibrinogen α chain were associated with both VLDL and LDL. Collectively, this study provides a wealth of knowledge on the protein constituents of the human plasma lipoprotein system and provides strong support for the notion that protein shuttling through this system is involved in the regulation of biological processes such as coagulation, atherosclerosis, defense against bacteria and energy metabolism.

Introduction

Mammals have developed an elaborate system to transport lipids, in particular triglycerides and cholesterol through the body. The bulk of this transport is performed by a highly sophisticated plasma lipoprotein network composed of many lipoproteins of which very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are the main constituents. Various lipoproteins are distinguished based on size, composition and density, and the size is inversely related to density, e.g. VLDL with large size and very low-density and HDL with small size and high-density (Alaupovic, 1991; Campos et al., 2001; Dashti et al., 2011; Krauss and Burke, 1982; Lee et al., 2003; Queiroz et al., 2010; Rezaee et al., 2006).

Plasma lipoproteins are functionally tightly regulated to maintain a strict homeostatic balance between lipoproteins. Disturbances in plasma lipoprotein homeostasis result in dyslipidemia, e.g. hypertriglyceridemia, hypercholesterolemia and hyperlipidemia, which in turn have serious pathophysiological consequences like obesity, insulin resistance, type 2 diabetes and cardiovascular diseases (Campos et al., 2001; FREDRICKSON and LEES, 1965; Lee et al., 2003; Thompson, 2004).

LDL is the largest cholesterol transporter in human, highly susceptible to oxidation and considered as being a risk factor in developing coronary artery disease (CAD) (Berliner and Heinecke, 1996; Brown and Goldstein, 1997; Tymoczko, 2002), whereas human plasma very low- density lipoprotein is a very rich-triglyceride (TG) particle. In addition, VLDL plays an important role in the delivery of free fatty acid (FFA) to adipose tissue to store energy as inactive fuel in the form of

triglycerides in the lipid droplets (LD) within adipocytes or supply active energy for skeletal muscles and other tissues via FFA delivery (Merkel et al., 2002). Many studies have shown that hypertriglyceridemia (with VLDL as TG carrier) the same as hypercholesterolemia (with LDL as cholesterol transporter) is directly related to the risk of cardiovascular diseases (Austin et al., 1998; Sarwar et al., 2007). Thus, an overload of TG in VLDL can be considered as a hallmark of dyslipidemia (Adiels et al., 2008). Unlike VLDL and LDL, HDL is mainly involved in the transport of cholesterol from tissues, including the vascular wall, back to the liver for further metabolization or excretion from body, a process called reverse cholesterol transport (RCT) (Azzam and Fessler, 2012; Lewis and Rader, 2005). Thus, there exists a functional dichotomy between VLDL and LDL on one hand and HDL on the other hand and it is especially the former group of lipoproteins (VLDL and LDL) is most directly linked to disease risk. Nevertheless many aspects of VLDL and LDL biology remain unclear, hampering development of rational therapy.

Although the main function of LDL and VLDL lies in the shuttling of lipids between liver, adipocytes and the other tissues, their role in protein transport has recently gained increasing attention. For instance, it has become clear that lipoproteins transport the morphogen indian hedgehog and thus deliver an important survival signal to the vascular endothelium (Queiroz et al., 2010). Also, despite lipid cholesterol modification therapies, such as LDL-lowering or raising HDL treatments, coronary artery events still occur in a large scale. This may suggest the presence of other risk factors that exert influence on the development and progression of diseases.

There is still very poor understanding, however, about which proteins are loaded on LDL and VLDL. Identification of these proteins will facilitate efforts in understanding lipoproteins' functions in pathophysiology of disease.

This consideration prompted us to generate a catalogue of LDL and VLDL protein constituents without priory assumptions as to the proteins present in these plasma lipoproteins. To this end, we performed an in-depth proteomic analysis of purified VLDL and LDL using ORBITRAP mass spectrometry analysis of tryptic digests of the protein fraction derived from these lipoproteins. Remarkably we demonstrate the presence of a fairly large group of important mediators, including those involved in coagulation and inflammation on VLDL and LDL with highly distinct profiles. Thus our results strongly support the notion that lipoproteins exert important transport functions for proteins apart from their role in lipid transport.

MATERIALS AND METHODS

Collection of plasma, isolation of lipoprotein fractions and lipoprotein delipidation.

Blood was drawn from fasted healthy volunteers after informed consent by venipuncture into tubes containing Na-citrate as anticoagulant (this study was approved by the Medical Ethical Committee of the University Medical Center Groningen under number, METc2007.081). Subsequently,

plasma was isolated by centrifugation at 1500× g for 15 min at 4 °C and stored at - 80 °C for further analysis. To collect plasma VLDL and LDL particles, a combination of salt gradient and ultracentrifugation was applied according to Kleinveld *et al.* (Kleinveld et al., 1996) with modifications as described previously (Dashti et al., 2011; Levels et al., 2007; Queiroz et al., 2010). The method for isolation of purified and defined plasma lipoproteins was already established (Dashti et al., 2011; Queiroz et al., 2010; Rezaee et al., 2006). The collected lipoprotein fractions were subsequently delipidated with a cocktail of two volumes of ice cold solution of diethyl ether: methanol (1:3). This cocktail was incubated for 15 min at room temperature followed by 45 min on ice. The mixture was then centrifuged twice at 4000 rpm for 45 at -5 °C (Cham and Knowles, 1976). The supernatant was removed, and the pellets were dried and solubilized in appropriate buffers and detergents. The delipidation of the defined and purified plasma lipoprotein was only performed for one dimensional electrophoresis (1-DE) and in-gel tryptic digestion approach.

Protein resolution deep proteomics and bioinformatical analysis

From the delipidated lipoproteins a DTT-reduced 1.5 mg VLDL and LDL protein fractions were loaded on a 24 x 24 cm 12.5 % SDS PAGE. Each lane was divided into 40 slices. After the treatment with iodoacetamide alkylation, gel slices were subjected to in-gel tryptic digestion or in-solution tryptic digestion. Peptides were extracted and analyzed by advanced nano liquid chromatography-tandem mass spectrometry (nLC-MS/MS) (LTQ–Orbitrap-XL) on an Ultimate 3000

system (Dionex, Amsterdam, The Netherlands) and infused into the mass spectrometer via a dynamic nanospray probe. The automated gain control (AGC) was 5 x 10^5 charges and 1 x 10^4 charges for MS/MS at the linear ion trap analyzer. DDA cycle consisted of the survey scan within *m*/*z* 300–1600 at the ORBITRAP analyzer with target mass resolution of 60,000 (FWHM, full width at half maximum at m/z 400) followed by MS/MS fragmentation of the five most intense precursor ions under the relative collision energy of 35% in the linear trap. Ion selection threshold for triggering MS/MS experiments was 500 counts and analyzed according to stringent HUPO criteria in combination of Bioworks software package using Uniprot (www.ebi.uniprot.org/database/download.shtml). The Turbo- SEQUEST algorithm in the BioWorks™ 3.1 software package (Thermo Electron) and the uncompressed human database (Swiss Institute of Bioinformatics, Geneva, Switzerland) were employed to analyze the MS/MS spectra and identify proteins. MS/MS spectra were searched with a mass tolerance of 5-10 ppm (VLDL) and 10-15 ppm (LDL) in combination with the following HUPO SEQUEST criteria, which is meant for high confidence peptide identification: 1- charge state *versus* cross-correlation number (XCorr) and that is XCorr > 1.9 for singly charged ions, XCorr > 2.7 for doubly charged ions, and XCorr > 3.75 for triply charged ions, 2- deltaCn 0.1, 3- peptide probability 0.001, 4-RsP 4, and 5-final score (sf = 0.85). This strategy was only used in 1-DE and in-gel tryptic digestion approach. The setting of ORBITRAP parameters and LC were for the direct in-solution tryptic digestion of VLDL and LDL protein fractions the same as in-gel tryptic digestion setting, except we applied the new PEAKS DB software package for the

mass spectra analysis, peptide sequences and protein identification, which was recently introduced by Zhang *et* al. (Zhang et al., 2012). The false discovery rate (FDR) can be manually adjusted with the PEAKS DB software package.

KOBAS version 2.0 combined with hypergeometric test and Benjamini-Hochberg FDR correction were applied to detect the enriched pathways and disorders, which VLDL- and LDL- associated proteins may have a role (Xie et al., 2011).

RESULTS

NLC-Tandem MS analysis of a pure VLDL and LDL Fractions

The procedure for isolation of purified and defined plasma lipoproteins was already established and published. A nLC-MS/MS tandem mass spectrometry (ORBITRAP LTQ-XL) combined with both in-gel and in-solution tryptic digestion of a large scale 1.5 mg of highly purified and defined human VLDL and LDL protein fraction was applied to make an in-depth analysis of VLDL protein content. Two Bioworks (version 3.1) and PEAKS DB software packages were applied to analyze the nLC-MS/MS spectra. When using Bioworks as analysis software, the false discovery rate (FDR) was 2 % and 5 % for VLDL and LDL respectively. However, when we performed Peak software, the FDR was manually adjusted to 0. This strategy resulted in the identification of 95 proteins on VLDL and 51 proteins on LDL (Table 1) including all known VLDL- and

LDL-associated apolipoproteins and other known VLDL and LDL constituents demonstrating the reliability of our deep proteomic strategy. We further exploited the novel protein constituents carried by VLDL and LDL (table 1), which is in agreement with a protein shuttling functionality of these two lipoproteins.

Protein ID	Protein name	Protein description	Table 1 Page 1	VLDL Peptide Count	LDL Peptide Count	pI	Mw (kDa)	Gene name
P02763	A1AG1	Alpha-1-acid glycoprotein 1		--	1	4.93	23.496	ORM1
P01009	A1AT	Alpha-1-antitrypsin		1-7	10-11	5.37	46.707	SERPINA1
P04217	A1BG	Alpha-1B-glycoprotein		1	1	5.56	54.219	A1BG
P02768	ALBU	Serum albumin		75	1-5	5.92	69.321	ALB
P01019	ANGT	Angiotensinogen		4	1	5.87	53.120	AGT
P02647	APOA1	Apolipoprotein A-I		8-288	3-34	5.56	30.758	APOA1
P02652	APOA2	Apolipoprotein A-II		1-14	11-17	6.27	11.167	APOA2
P06727	APOA4	Apolipoprotein A-IV		1-245	3-4	5.28	45.371	APOA4
Q6Q788	APOA5	Apolipoprotein A-V		1-9	--	5.98	41.187	APOA5
P08519	APOA	Apolipoprotein(a)		1-4	5-22	5.58	500.995	LPA
P04114	APOB	Apolipoprotein B-100		145-3389	178-254	6.58	515.282	APOB
P02654	APOC1	Apolipoprotein C-I		4-191	4	8.01	9.326	APOC1
P02655	APOC2	Apolipoprotein C-II		5-394	3	4.64	11.276	APOC2
P02656	APOC3	Apolipoprotein C-III		4-2297	3-4	5.23	10.845	APOC3
P55056	APOC4	Apolipoprotein C-III		4-320	2-4	9.19	14.543	APOC4
P05090	APOD	Apolipoprotein D		2-40	2	5.06	21.261	APOD
P02649	APOE	Apolipoprotein E		18-9531	12-19	5.65	36.131	APOE
Q13790	APOF	Apolipoprotein F		1	1-7	5.42	35.377	APOF
P02749	APOH	Beta-2-glycoprotein 1		9	1	8.34	38.298	APOH
O14791	APOL1	Apolipoprotein L1		1-61	4-8	5.50	43.946	APOL1
O95445	APOM	Apolipoprotein M		2-10	2-6	5.66	21.239	APOM
P49913	CAMP	Cathelicidin antimicrobial peptide		1-17	1	9.48	19.289	CAMP
P10909	CLUS	Clusterin		4-37	2-4	5.88	52.461	CLU
P01024	CO3	Complement C3		2-49	8-16	6.02	187.029	C3
P02748	CO9	Complement component C9 [C9a, C9b]		1	--	5.43	63.132	C9
P0C0L4	CO4A	Complement C4-A		25-29	4-15	6.66	192.650	C4A
P0C0L5	CO4B	Complement C4-B		--	4-15	6.73	192.672	C4B
P81605	DCD	Dermcidin		1-38	1	6.09	11.276	DCD
P02765	FETUA	Alpha-2-HS-glycoprotein		--	1	5.43	39.299	AHSG
Q02985	FHR3	Complement factor H-related protein 3		--	2	7.72	37.298	CFHR3
P02671	FIBA	Fibrinogen alpha chain		3	12-13	5.70	94.914	FGA
P02679	FIBG	Fibrinogen gamma chain		4	--	5.37	51.478	FGG
Q86SQ4	GP126	G-protein coupled receptor 126		--	1	8.18	136.606	GPR126
P69905	HBA	Hemoglobin subunit alpha		5	2	8.72	15.247	HBA1
P00739	HPTR	Haptoglobin-related protein		2	2	6.63	39.004	HPR
P00738	HPT	Haptoglobin		--	2	6.13	45.176	HP
P01876	IGHA1	Ig alpha-1 chain C region		3	1	6.08	37.630	IGHA1
P01877	IGHA2	Ig alpha-2 chain C region		8	1-2	5.71	36.503	IGHA2
P01834	IGKC	Ig kappa chain C region		17	1	5.58	11.601	IGKC
Q14624	ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4		1	2-3	6.51	103.292	ITIH4
O60341	KDM1A	Lysine-specific histone demethylase 1A		--	1	6.11	92.844	KDM1A
P04180	LCAT	Phosphatidylcholine-sterol acyltransferase		5	3	5.71	49.546	LCAT
P14151	LYAM1	L-selectin		--	1	6.20	42.158	SELL
P33908	MA1A1	Mannosyl-oligosaccharide 1,2-α-mannosidase IA		--	1	6.03	72.922	MAN1A1
Q9UHG3	PCYOX	Prenylcysteine oxidase 1		2-156	3-7	5.80	56.603	PCYOX1
P27169	PON1	Serum paraoxonase/arylesterase 1		1-102	1-9	5.08	39.706	PON1
P35542	SAA4	Serum amyloid A-4 protein		4-248	3-5	9.17	14.737	SAA4
P02735	SAA1	Serum amyloid A protein		2-21	1-3	6.28	13.523	SAA1
P49908	SEPP1	Selenoprotein P		--	1	8.08	41.646	SEPP1
Q8TF72	SHRM3	Protein Shroom3		--	1	7.87	216.724	SHROOM3
P10646	TFPI1	Tissue factor pathway inhibitor		1	1	8.61	34.992	TFPI
P35030	TRY3	Trypsin-3		2	1	7.46	32.508	PRSS3
P02766	TTHY	Transthyretin		--	3-4	5.49	15.877	TTR
P04004	VTNC	Vitronectin		1-12	1	5.55	54.271	VTN

Protein ID	Protein name	Protein description	VLDL Peptide Count	LDL Peptide Count	pI	Mw (kDa)	Gene name
P30490	1B52	HLA class I histocompatibility Ag, B-52- α chain	1	--	5.85	40.496	HLA-B
P02760	AMBP	Protein AMBP [Cleaved into: α-1-microglobulin	1	--	5.95	38.973	AMBP
P04745	AMYS	Alpha-amylase 1	3	--	6.47	57.730	AMY1A
P58335	ANTR2	Anthrax toxin receptor 2	1	--	7.42	53.632	ANTXR2
P05089	ARGI1	Arginase-1	7	--	6.72	34.713	ARG1
Q9BYG0	B3GN5	UDP-GlcNAc:betaGal beta-1,3-N-acetyl- GATase 5	1	--	8.0	44.024	B3GNT5
P04003	C4BP	C4b-binding protein alpha chain	1-24	--	7.15	66.989	C4BPA
Q9H813	CA075	Transmembrane protein 206	1	--	9.01	40.017	TMEM206
P31944	CASPE	Caspase-14	6	--	5.44	27.661	CASP14
P07339	CATD	Cathepsin D	13-15	--	6.10	44.523	CTSD
P08571	CD14	Monocyte differentiation antigen CD14	2	--	5.84	40.050	CD14
P11597	CETP	Cholesteryl ester transfer protein	5-6	--	5.70	54.721	CETP
P15924	DESP	Desmoplakin	2	--	6.44	331.568	DSP
Q02413	DSG1	Desmoglein-1	4-5	--	4.90	113.675	DSG1
P08217	ELA2A	Chymotrypsin-like elastase family member 2A	3	--	8.8	28.869	CELA2A
P09093	ELA3A	Chymotrypsin-like elastase family member 3A	23	--	6.43	29.469	CELA3A
P08861	ELA3B	Chymotrypsin-like elastase family member 3B	5	--	5.85	29.244	CELA3B
P23141	ELA3B	Liver carboxylesterase 1	1	--	5.44	27.661	CES1
P49327	FAS	Fatty acid synthase	1	--	6.01	273.254	FASN
Q92496	FHR4	Complement factor H-related protein 4	1	--	5.14	37.274	CFHR4
P04406	G3P	Glyceraldehyde-3-phosphate dehydrogenase	7-10	--	8.57	36.030	GAPDH
P14136	GFAP	Glial fibrillary acidic protein	16	--	5.42	49.849	GFAP
P68871	HBB	Hemoglobin subunit beta	5	--	6.74	15.988	HBB
P02042	HBD	Hemoglobin subunit delta	1	--	7.84	16.045	HBD
P04196	HRG	Histidine-rich glycoprotein	1	--	7.09	59.540	HRG
P01857	IGHG1	Ig gamma-1 chain C region	1	--	8.46	36.083	IGHG1
P01859	IGHG2	Ig gamma-2 chain C region	1	--	7.66	35.877	IGHG2
Q14623	IHH	Indian hedgehog protein	2	--	5.44	27.661	IHH
P04206	KV3G	Ig kappa chain V-III region GOL	3	--	9.34	11.822	NA
P0CG04	LAC1	Ig lambda-1 chain C regions	4	--	7.89	11.340	IGLC1
P18428	LBP	Lipopolysaccharide-binding protein	7-11	--	6.23	53.349	LBP
P01130	LDLR	Low-density lipoprotein receptor	1	--	4.86	95.313	LDLR
Q8TDL5	LPLC1	BPI fold-containing family B member 1	1	--	6.72	52.408	BPIFB1
P61626	LYSC	Lysozyme C	17-19	--	9.38	16.526	LYZ
P01871	MUC	Ig mu chain C region	2	--	6.35	49.275	IGHM
P12882	MYH1	Myosin-1	1	--	5.58	223.006	MYH1
Q9UKX2	MYH2	Myosin-2	2	--	5.64	222.906	MYH2
Q13093	PAFA	Platelet-activating factor acetylhydrolase	10-12	--	7.23	50.045	PLA2G7
P14222	PERF	Perforin-1	1	--	8.04	61.338	PRF1
P12273	PIP	Prolactin-inducible protein	15	--	8.26	16.561	PIP
P55058	PLTP	Phospholipid transfer protein	1	--	6.53	54.704	PLTP
Q16378	PROL4	Proline-rich protein 4	1	--	6.50	15.087	PRR4
P07225	PROS	Vitamin K-dependent protein S	1	--	5.48	75.073	PROS1
P05109	S10A8	Protein S100-A8	3	--	6.50	10.827	S100A8
P06702	S10A9	Protein S100-A9	11-12	--	5.71	13.233	S100A9
O95969	SG1D2	Secretoglobin family 1D member 2	1	--	8.58	9.918	SCGB1D2
P29508	SPB3	Serpin B3	1	--	6.35	44.536	SERPINB3
Q08188	TGM3	Protein-glutamine gamma-glutamyltransferase E	1	--	5.61	76.583	TGM3
P00734	THRB	Prothrombin	1	--	5.63	69.992	F2
P02788	TRFL	Lactotransferrin	7	--	8.50	78.131	LTF
P07477	TRY1	Trypsin-1	2	--	6.08	26.541	PRSS1
P07478	TRY2	Trypsin-2	2	--	4.78	26.471	PRSS2
Q9NP71	WBS14	Carbohydrate-responsive element-binding protein	2	--	8.70	93.013	MLXIPL

Table 1 Page 2

Table 1. The total list of proteins detected in highly defined and purified VLDL and LDL fractions using nLC-MS/MS.

Protein accession number, protein name, protein description, pI (isoelectric focusing point), the Molecular weight (Mw) and gene name of that protein were presented for each protein in this table. The Mw was expressed in kDa. pI was resulted from whole amino acid sequence of each protein and derived from www.expasy.org.

Functional pathway and disorder analysis of VLDL-associated proteins

To gain more insight into the possible physiological functions of proteins present on VLDL, statistically enriched pathways and diseases for the 95 identified proteins carried by VLDL were analyzed, using KOBAS 2.0 with hypergeometric test and Benjamini-Hochberg FDR correction and considering all human genes as background. This included Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Pathway Interaction Database (PID) curated, PID BioCarta, PID reactome, BioCyc , Reactome and Protein Analysis Through Evolutionary Relationships (Panther) pathway databases and KEGG disease, Genetic Association Database (GAD), Functional disease ontology (FunDO), Online Mendelian Inheritance in Man (OMIM) and National Human Genome Research Institute (NHGRI) human disease databases. Pathways and diseases with a corrected p-value <0.05 were considered relevant and are shown in figure 1 and 2. From 33 functional pathways (Figure 1), 4 are related to lipid transport and lipid metabolism and 8 pathways are linked to coagulation, hemostasis, and immunity. These data further support the notion that VLDL not only shuttle lipids through the body, but deliver proteins involved in other physiological processes as well.

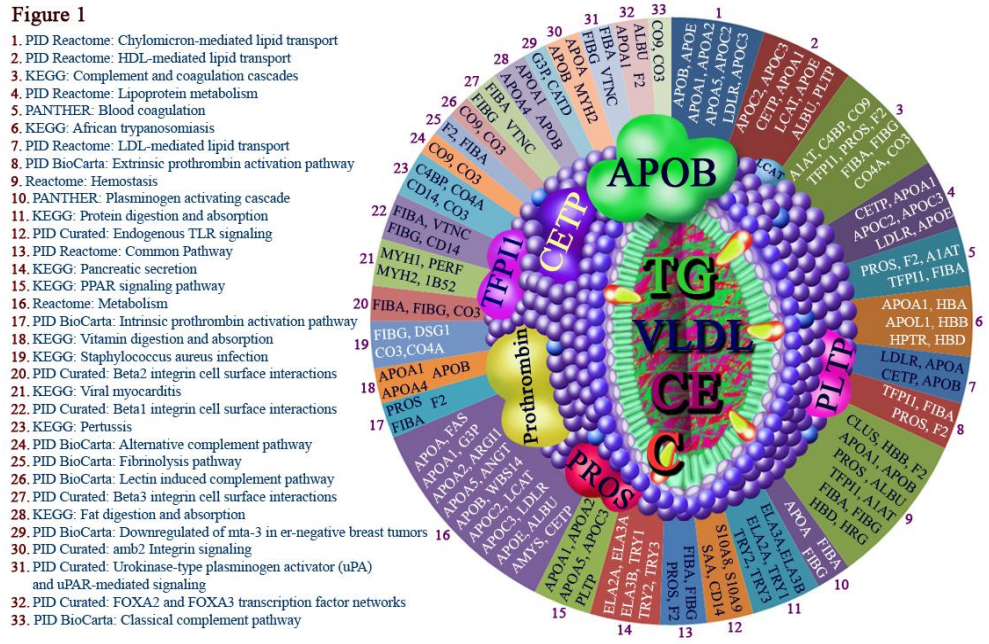

Figure 1. Enriched pathways related to the proteins present on very low-density lipoprotein (VLDL).

The abbreviations or description is: Kyoto Encyclopedia of Genes and Genomes (KEGG), Pathway Interaction Database (PID), Protein Analysis through Evolutionary Relationships (PANTHER) and Reactome is a free access database of reactions, pathways and biological functions. Only pathways with corrected (REF) p-values <0.05 are presented.

Subsequently, we investigated whether these novel protein functions could have a role for human disease (figure 2). The 28 human diseases related to proteins carried by VLDL can be divided in three main categories: 1- dyslipidemia such as hypertriglyceridemia, hypercholesterolemia and hyperlipidemia, 2- atherosclerosis and vascular disease and 3-coagulation disorders. It must be noted that atherosclerosis was considered as the most enriched disease process identified by disorder enrichment analysis, which fits well with the known involvement of lipoproteins in this disease,

although it also suggests that the contribution of VLDL to vascular disease might be somewhat underestimated.

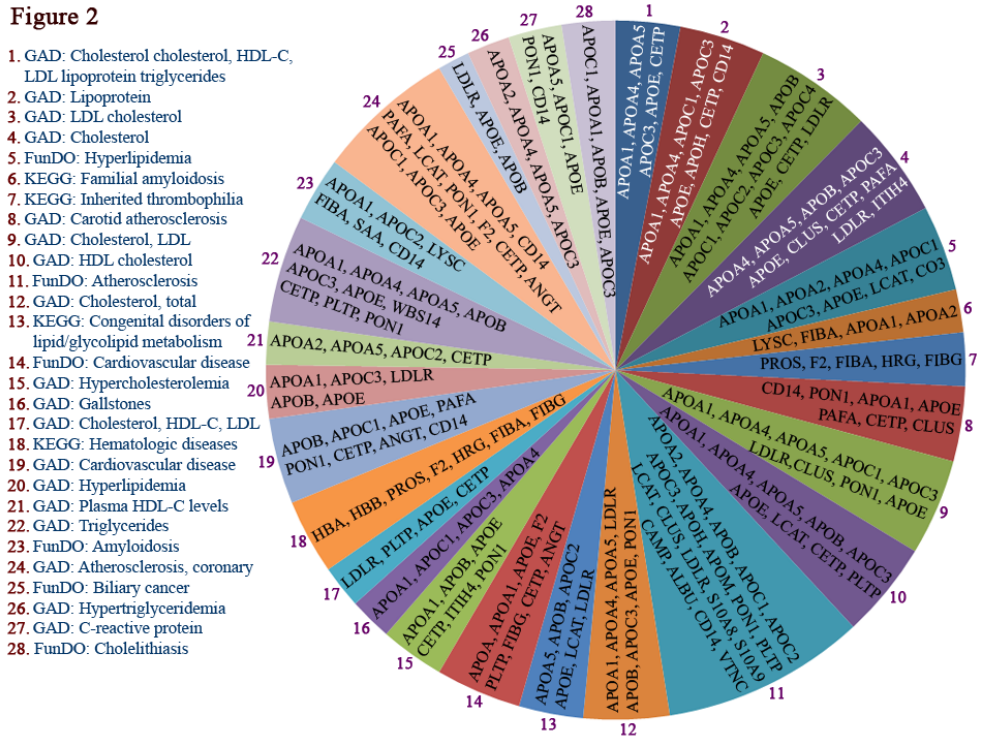

Figure 2. Enriched disorders linked to the proteins present on very low density lipoprotein (VLDL).

The abbreviations or descriptions are: Kyoto Encyclopedia of Genes and Genomes (KEGG), Genetic Association Database (GAD), and Functional disease ontology (FunDO). Only disorders with corrected p-values (REF) <0.05 were included in the analysis.

The following IDs were not recognized by KOBAS 2.0 and therefore the respective proteins were not included in the analysis: P01876, P01877, P01857, P01859, P01834, P0CG04, P01871, and P04206.

Functional pathway and disorder analysis of LDL-associated proteins

To shed more light on the possible biological and physiological functions of LDL-associated proteins, statistically enriched pathways and diseases for the 51 LDL-associated proteins were investigated using KOBAS 2.0 with hypergeometric test and Bejamini-Hochberg FD correction and considering all human genes as background. This included KEGG pathway, PID curated, PID BioCarta, PID reactome, BioCyc , Reactome and Panther pathway databases and KEGG disease, GAD, FunDO, OMIM and NHGRI human disease databases. Pathways and diseases with a corrected p-value <0.05 were considered relevant and were depicted in figure 3 (identified pathways) and figure 4 (detected disorders). The analysis of the 51 proteins present on LDL revealed 19 functional pathways of which lipid metabolism and lipid transporters, coagulation and hemostasis and complement system are the most enriched pathways. The most prominent trait (6/19 pathways) are lipid metabolism and transport pathways, in agreement of the canonical function of LDL. Nevertheless, also other functions were found, e.g. 5/19 linked to coagulation and hemostasis, thus like the protein composition of VLDL, also the protein constituents of LDL lend credit to the notion that plasma lipoproteins not only shuttle lipids through the body but are involved in protein transport as well.

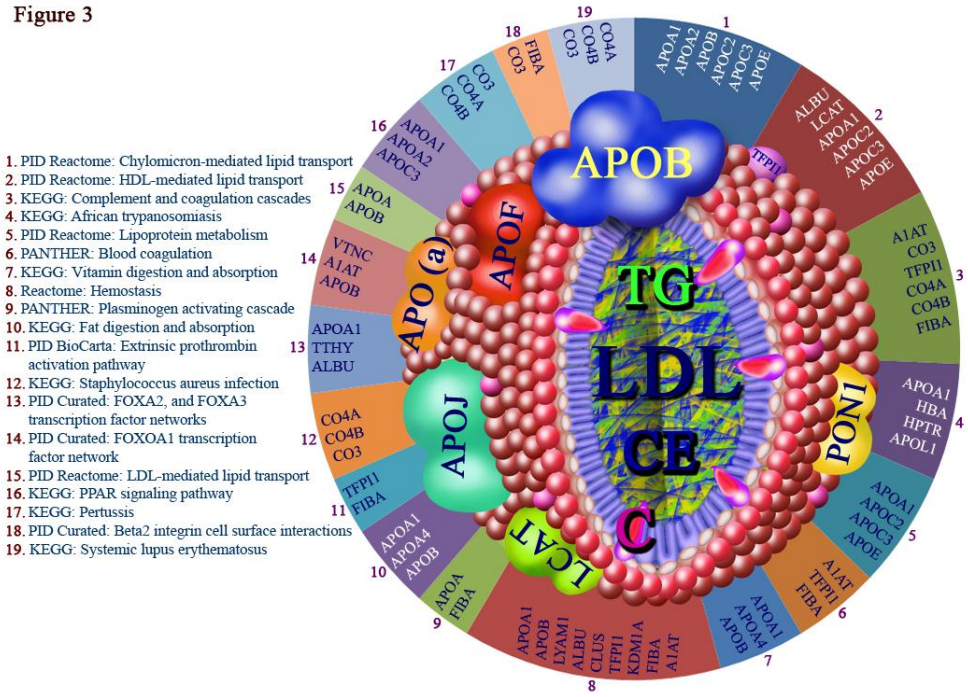

Figure 3. Enriched pathways related to the proteins present on low-density lipoprotein (LDL).

The abbreviations or descriptions are: Kyoto Encyclopedia of Genes and Genomes (KEGG), Pathway Interaction Database (PID), Protein Analysis through Evolutionary Relationships (PANTHER), and Reactome (a free access database of reactions, pathways and biological functions). Only pathways with corrected (REF) p-values <0.05 are presented.

LDL protein constituents were mapped to specific disease processes as well (**figure 4**) and suggest that LDL-carried proteins might be implicated in atherosclerosis and cardiovascular disease, hemostasis, dyslipidemia, cholelithiasis and amyloidosis diseases, not unlike the results obtained with VLDL. The results show that atherosclerosis is the most enriched disease pathway (16/51 proteins found in LDL map to this disease), suggesting that the role of LDL in vascular pathology is not only

the consequence of the LDL lipid cargo, but may involve proteins carried by this plasma lipoprotein as well.

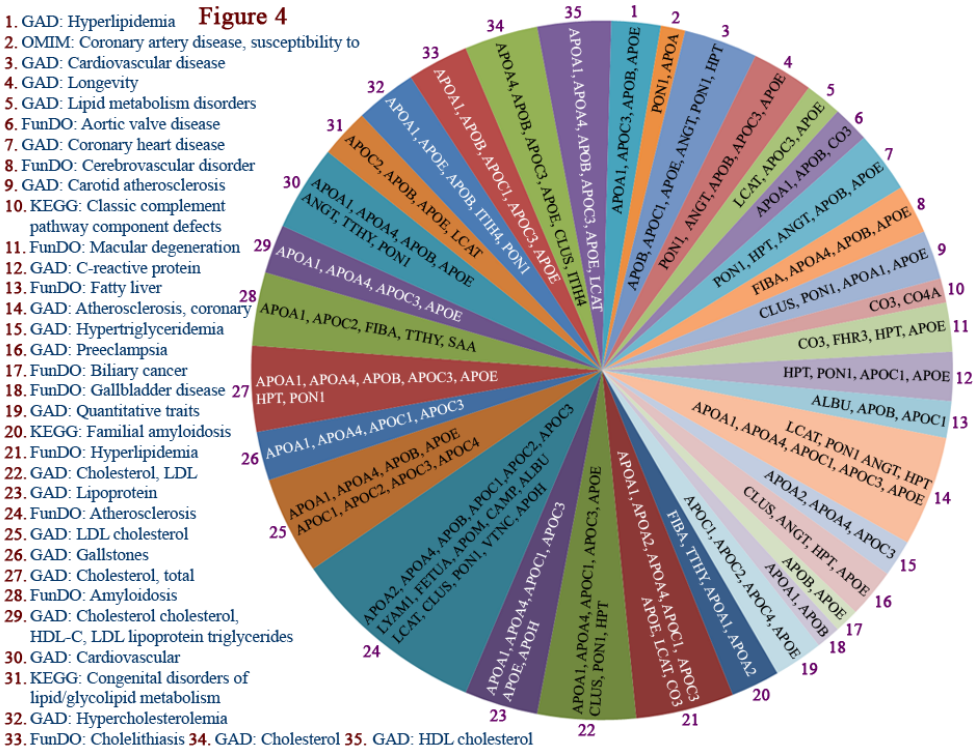

Figure 4. Enriched disorders linked to the proteins present on low-density lipoprotein (LDL).

The abbreviations or descriptions are: Kyoto Encyclopedia of Genes and Genomes (KEGG), Genetic Association Database (GAD), Online Mendelian Inheritance in Man (OMIM) and Functional disease ontology (FunDO). Only disorders with corrected p-values (REF) <0.05 were included in the analysis.

The following IDs were not recognized by KOBAS 2.0 and therefore the respective proteins were not included in the analysis: P01876, P01877 and P01834.

Differences and similarities between VLDL and LD

To obtain further insight into the specific roles that VLDL and LDL might have in pathophysiology, we compared protein cargo of these two lipoproteins (**table 1** and **figure 5).** Although a large set of proteins (39 proteins) is shared by the two particles.

44 additional proteins detected on VLDL suggest that VLDL may be more important for protein transport when compared to LDL. In general, 56 and 12 proteins were found to be unique for VLDL and LDL respectively. Presence of VLDL-specific proteins such as PLTP, CEPT, apoAV, prothrombin (F2), protein S (PROS), lysosyme, cathepsin D, and lipopolysacchariden binding protein (LBP) suggests a function for VLDL in hemostasis and defense against bacteria. Of note, complement factor H-related protein 3, complement C4-B, haptoglobin and Transthyretin were exclusively detected on the LDL particle. Therefore LDL may have a specific role in the complement system and innate immunity.

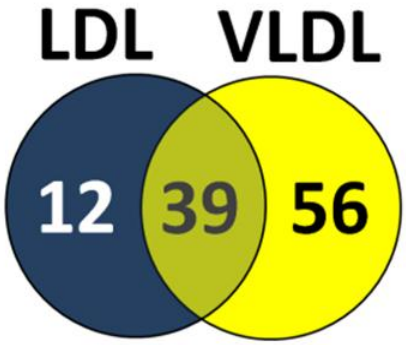

Figure 5. The comparison of proteins present on VLDL and LDL.

39 proteins are shared by VLDL and LDL particles. 45 proteins found to be unique for VLDL and 12 proteins for LDL.

DISCUSSION

In two previous studies, we reported an in-depth phospholipid and protein profiling of all defined lipoproteins and HDL respectively (Dashti et al., 2011; Rezaee et al., 2006). The established methodology to isolate defined and purified plasma lipoproteins proved to be useful for the protein analysis reported in the present study as well. We identified 95 and 51 proteins on VLDL and LDL respectively, suggesting that protein cargo is more enriched in VLDL as compared to that of LDL. We cannot completely rule out that the high sensitivity of LDL to oxidation (which is a relatively fast event following blood collection) (Berliner and Heinecke, 1996; Salvayre et al., 2002), which can result in the aggregation of LDL proteins and thus loss in LDL protein profile. Although we aimed to minimize such protein loss by preparing all lipoprotein fractions freshly before analysis by nLC-MS/MS, an effect in this area is possible. Of note, the combination of nLC-MS/MS with relatively large amounts of VLDL and LDL protein (1.5 mg) followed by loading on large size SDS-PAGE for in-gel tryptic digestion and in-solution tryptic digestion showed a very substantial increase of protein coverage of both VLDL and LDL particles particles as compared to other studies. In earlier studies, most of the identified proteins were constituted apolipoproteins and lipid transporters (Karlsson et al., 2005; Mancone et al., 2007; Sun et al., 2010). These studies have used a 2-DE approach and different cut-off filters to identify lipoprotein-associated proteins. The protein spots on the 2-DE gel were stained with Sypro ruby (which is a highly sensitive fluorescent protein Staining; 1-3 ng protein) to visualize the proteins. However, the disadvantage of such methodology is that the amount of protein derived

from spots is often not enough for reliable MS/MS analysis and heavily depends on the abundacy of protein. We employed 1-DE approach and loaded large amounts of protein (1.5 mg) to a large size SDS-PAGE (24 X 24 cm; plates were provided by General Electric) and stained with coomassie Blue. The sensitivity of this staining is very low (30-50 ng protein) but the recovery of protein derived from the visualized bands is very high and always enough for nLC-MS/MS analysis, which enabled the deep proteomic analysis reported here. The use of cut-off filters resulted in the loss of specific group of proteins as well. However, the cut-off filters were not applied in the preparation of VLDL and LDL fractions. Our data revealed that apolipoproteins and proteins involved in lipid metabolism appeared to be conserved between the different lipoproteins even though there are exceptions such as apoB, CETP, PLTP, and apoAV. Thus a homeostatic balance between lipoproteins, -and not the quantity of specific lipoproteins-, may guarantee a regulated function of lipoproteins and not the quantity of a specific lipoprotein.

Plasma lipoprotein particles are essential for lipid transport through the human body. Thus they play an important role in numerous biological processes with the implication in a plethora of diseases with atherothrombosis and type 2 diabetes as the most prominent diseases. In the past years the idea that the involvement of lipoproteins in these diseases may not solely be due to the cholesterol and triglycerides carried by these particles have been gaining ground. The current study strongly supports this notion that it identifies a fairly large set of proteins that are carried by VLDL and LDL, and many of these detected proteins are pertinent to vascular disease. In this sense the current study provides a wealth of data that may help explain the role(s) of lipoprotein in atherosclerosis, diabetes

type 2 and related diseases and can serve as a starting point for new directions of research in this area.

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Chapter 5

Human Adipocytes Hypertrophy Secrets Insulin

Run Title: Bariatric Fat Loss Surgery Heals Type 2 Diabetes

Monireh Dashti [1,2], Behiye Özcan [3, 4], Vishtaseb Khanzadeh [5], Felix Rooij[4], Arnold Spek [6], Saad Lahham [7], Johanes van der Want [1], Ajda Rowshani [8], Theo van Kooten [9], Rene Klaassen [10], Eric Sijbrands [4], Maikel P. Peppelenbosch [2], Farhad Rezaee [1,6]

[1] Department of Cell Biology, UMCG, University of Groningen, The Netherlands

[2] Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands

[3] Department of endocrinology, Erasmus Medical Center, Rotterdam, The Netherlands
[4] Dep. cardiovascular genetics & metabolic diseases, Erasmus MC, The Netherlands
[5] Dep. general Medicine, Free University MC, University of Amsterdam, The Netherlands
[6] Dep. Experimental and Molecular Medicine, Academic MC, Amsterdam, The Netherlands
[7] Department of Medical Biology, UMCG, University of Groningen, The Netherlands
[8] Department of Internal Medicine and Transplantation, Erasmus University Medical Center, Rotterdam, the Netherlands
[9] Dep. Biomedical Engineering, UMCG, University of Groningen, The Netherlands
[10] Department Gastrointestinal Surgery, Bariatric Surgery, Erasmus MC, Maas City Hospital, Rotterdam, The Netherlands

(Under review)

ABSTRACT

Objective

A triglyceride overload of lipid droplets leads to adipocytes hypertrophy, which is the main cause of obesity and its afflictions such as type 2 diabetes mellitus (T2DM). We designed the present study to investigate the role of adipocytes behind the link between obesity and insulin regulation in human.

Design

Three different models were used; *i-in vivo* (five different groups of human subjects), *ii-in vitro* (human primary preadipocytes and adipocytes), *iii-in vivo* (mRNA and protein levels of insulin1 and 2 in adipose and pancreas tissues of Prediabetes BB rats).

Results

Insulin was synthesized and secreted by both human subcutaneous (55 pmol/l) and visceral (379 pmol/l) adipocytes and low traces of insulin synthesis and no secretion were detected by both preadipocytes. Insulin release by human adipocytes was reduced approximately 3- and 4-fold upon lipopolysaccharide (LPS) and glucose treatment respectively as compared to control.

Plasma insulin levels were significantly higher in three groups of obese subjects, 1-Obese (77.00 ± 11.20), 2-Obese with diabetes (70.45 ± 10.70) and 3-Obese with diabetes and insulin injection (165·70 ± 28·70) pmol/l than lean subjects (40.20 ± 5.71 pmol/l), and significantly correlated with fat

mass ($p < 0.00003$, $r = 0.6$). After bariatric surgery, the body mass indexes (BMIs) were highly decreased from 36-47 to 26-33 kg/m^2 in obese T2DM subjects receiving insulin. Bariatric surgery rescues these patients from T2DM.

A high and low insulin production (mRNA and protein) were found in fat tissues of pancreatic beta-cells destruction and control Prediabetes BB rats respectively.

Conclusion

Human adipocytes constitutively synthesize and secrete insulin and that is biologically active with a clear physiological role in the development of T2D. These results help us to reshape our thinking about how obesity increases the risk for developing insulin resistance and T2D.

INTRODUCTION

In 1922, Banting and Macleod discovered the insulin expression in highly specialized endocrine β-cells within islets of Langerhans in pancreas (Banting et al., 1922; Banting and Best, 2007). On one hand, insulin secretion is known to be affected by immunoreactivity to insulin in type 1 diabetes in humans. On the other hand, a second defect essential for development of the insulin resistance (IR) to the type 2 diabetes (T2D) is the inability of endocrinal pancreatic β-cells to produce the required levels of insulin that maintain normal blood glucose levels. It has also been shown that the insulin sensitivity decreases in an obesity state and thereby suppresses β-cells functionality (McGarry, 2002; Olefsky and Glass, 2010). In addition, obesity is implicated in multiple pathophysiological complications such as IR, T2D and cardiovascular mortality and morbidity (Guilherme et al., 2008; Olefsky and Glass, 2010; Shoelson et al., 2006; Xu et al., 2003). Since obesity is determined by the excessive mass of adipose tissue and in particular adipocytes, adipocytes have an established and important role in the development of obesity (Greenberg and Obin, 2006; Trayhurn, 2007). Although hypertrophy of adipocytes is the main cause of obesity (de and Mozaffarian, 2008; Gustafson et al., 2009; Jernas et al., 2006), the excessive storage of energy in the form of triglycerides in lipid droplets within adipocytes is the main cause of adipocyte hypertrophy and in turn hyperplasia linked to obesity, resulting in IR. Also, the classical view of adipose tissue as a passive depot for storage of excess energy in the form of triglycerides is no longer valid and has been overturned by a novel view that adipose tissue is considered as an endocrine organ (de and Mozaffarian,

2008; Greenberg and Obin, 2006; Gustafson et al., 2009; Jernas et al., 2006; Trayhurn, 2007).

From this aspect, we would like to contend that adipose tissue is the largest endocrine organ in humans. In spite of increasing studies on the properties of adipose tissue and in particular adipocytes, the mechanisms that lead to obesity-induced pathophysiological states (such as insulin resistance) are still poorly understood. Moreover, the advent of new technology that allows the characterization of entire transcriptomes and proteomes (Adachi et al., 2007) had also led to the hope that the properties of adipose tissue might be revealed to help discover new therapeutic avenues. However, hitherto, the results have not lived up to its expected promise, prompting us to perform a well designed and in-depth study on human primary subcutaneous preadipocytes and adipocytes and visceral preadipocytes and adipocytes **(four different human primary cell types**).

Since adipose tissue is composed of different cell types (Suganami et al., 2005), the consequent cross-talk could hamper a transparent view of human adipocytes. Hence, we chose to study human primary adipocytes alone to avoid adipose tissue complexity and obtain a clear detailed picture of human adipocytes. Also, to develop successful therapeutic approaches for type 2 diabetes treatment, it is essential to unravel the role of adipocytes behind the link between obesity and insulin regulation.

MATERIALS AND METHODS

Human primary preadipocytes (subcutaneous and visceral) were purchased from PromoCell and Lonza companies. All culture media were purchased from PromoCell Company.

Human primary preadipocytes were cultured in Preadipocyte growth medium to confluence. The confluence preadipocytes were differentiated in preadipocyte differentiation media containing of 0.5 mM 3-isobutyl-1-methyxanthine, 400 ng/ml dexamethasone, 0.5 µg/ml bovine insulin, 9 ng/ml L-thyroxin, 3 µg/ml ciglitazone and d-8 µg/ml biotin for 48-72 hours. After differentiation phase, the cells were cultured in nutrition medium for 16 days to become mature adipocytes. All procedures were performed according to the instructions of PromoCell Company.

After five wash steps, the cells were incubated with fetal calf serum (FCS)-free media for 2 days. After this period, the media was collected, which contains secreted proteins of human adipocytes. The collected media were concentrated up to 800 X (8.0 ug/ul), using a speed vacuum. This collected media referred as secreted samples was stored for further experiments.

Total RNAs were isolated from human primary cells to use in mRNA expression analysis applying Nucleospin kit and secreted media were used to determine insulin protein concentrations using Dako kit. All experiments were executed according to the instructions provided by Nucleospin and Dako companies. Bioanalyser (Agilent RNA 6000 Nano kit) was applied to determine the integrity and concentrations of RNAs. RNAs were amplified using amplification Kit (Applied Biosystems). Biotinylated cRNAs were amplified using RNA amplification kit (Ambion). Washing and scanning of chips were performed according to the BeadStation 500 manual.

Probe sequence for insulin gene is:

ACCCGCCGCCTCCTGCACCGAGAGAGATGGAATAAAGCCCTTGAACCAGC and for IGF1 (insulin-like growth factor 1) gene is: **GAGGCCCAGGGGATTTTTGAAGCTGTCTTTATTCTGCCCCCATCCCAACC.** All experiments were performed according to the manual instructions provided by the companies. The specific monoclonal antibody against human insulin combined with Transmission Electron Microscopy (TEM) and/or confocal microscopy were used to visualize insulin protein in human adipocye slides. Multi-plex ELISA of IL-6, IL-8 and M-CSF proteins were purchased from Biolegend and the experiments were performed according to the Biolegend instructions. The details of protocols were described in the legend of the figures and in the results and discussion section.

Total RNAs were collected from Prediabetes BB rats (insulin dependent diabetes mellitus (IDDM) and control rats). The glucose concentration was used to monitor rats with IDDM (glucose concentration 25-30 mmol/l) and control (glucose concentration 14-16 mmol/l) rats. Total RNAs were collected from pancreas and fat tissues to make cDNAs for QPCR. The KAPA SYBR® FAST qPCR Kit (Sopachem) was used for QPCR. Total volume for QPCR was 20 µl. Since the rodents have two insulin genes (Ins1 and Ins2), the relative intensity of both Ins1 and Ins2 were measured. Insulin mRNA levels are expressed relative to β-actin mRNA levels. All primers (Tanaka et al., 1999) were HPLC purified. Primer sequences are as follows:

Rat Ins1 and Ins2 forward primers: **GCCCAGGCTTTTGTCAAACA**

Rat Ins1 reverse primer: **GTTCCCCACACACCAGGTAGAGAG**

Rat Ins2 reverse primer: **CTCCCCACACACCAGGTAGAG**

Rat β-actin forward primer: **ACGAGGCCCAGAGCAAGA**
Rat β-actin reverse primer: **TTGGTTACAATGCCGTGTTCA**

Description of population

The number of human subjects were 41 of which is divided in four different group of patients. Each group composed of 10 subjects (except diabetes group receiving insulin containing 11 subjects), independent of age and gender, as follows: **Group 1**- Lean subjects with a body mass index (BMI) between 20.1 and 25.6, **Group 2-** Oobese subjects with a BMI above 30, **Group 3-** Obese subjects with type 2 diabetes without receiving insulin and a BMI above 30 and **Group 4-** Obese subjects with type 2 diabetes, receiving insulin and BMI above 30. All patients were well-matched for their BMI. It must also be stressed that in lean group only one person has a BMI above 25 (BMI = 25.6). Also 5 type 2 diabetes patients were selected with a BMI of 36-47, which receive insulin. Blood insulin was measured before and after bariatric surgery.

Ethic

This study was approved by the Medical Ethical Committee of the Erasmus Medical Center, Medical University of Rotterdam, under MEC number: 2009-242.

RESULTS

Human primary preadipocytes were differentiated into adipocytes and the pure adipocyte fractions were assessed by monitoring morphologically and via lipid droplet labeling (Figure 1 A-B). The efficacy of differentiation reached approximately 90-95%, indicating that adipocytes could be considered as a specific homogenous cell type. As depicted in Figure 1 A, no lipid droplet was observed and/or detected in human preadipocytes. On the contrary, human adipocytes were occupied by either medium-sized or single large lipid droplets (Figure 1 B).

To confirm our microscopic approach of the adipocyte-fraction purity, the expression of five known mRNA markers of human primary adipocytes were screened. mRNA expression displayed a significant up-regulation of the five mRNA markers of both visceral and subcutaneous adipocytes, i.e. Diacylglycerol O-acyltransferase 2 (*DGAT2*), leptin (*LEP*), hormone-sensitive lipase (*LIPE*), lipoprotein lipase (*LPL*) and fat specific protein-27 (*FSP27*) (Brasaemle, 2007; Ducharme and Bickel, 2008; Ito et al., 2010; Puri et al., 2007; Puri and Czech, 2008; Puri et al., 2008; Zhou et al., 1999) (Figure 1 C-D). To check the specificity of our obtained results, *Vice versa*, the preadipocyte marker; adipocyte enhancer-binding protein 1 (*AEBP-1*) (Chung et al., 2006) was measured in all these four cell types. Indeed and as expected, presdipocyte marker (*AEBP-1)* was significantly up-regulated in both human preadipocytes as compared to human adipocytes (Figure 1 E).

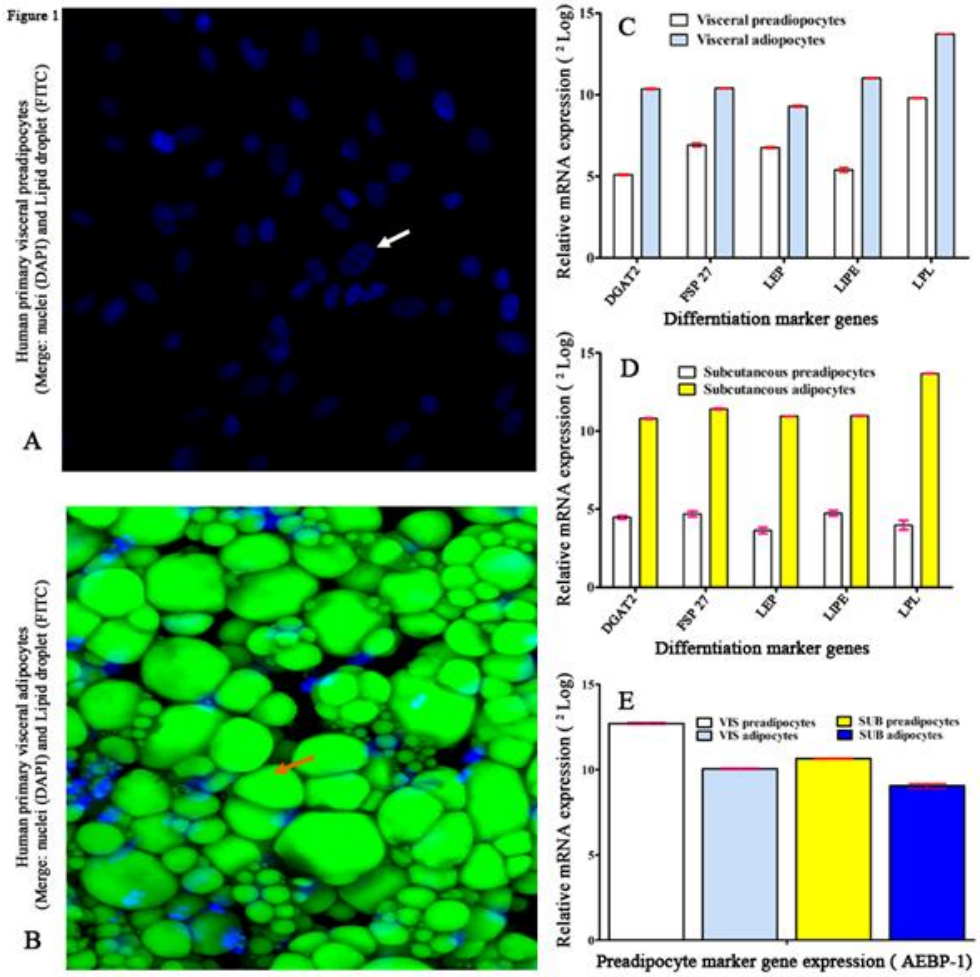


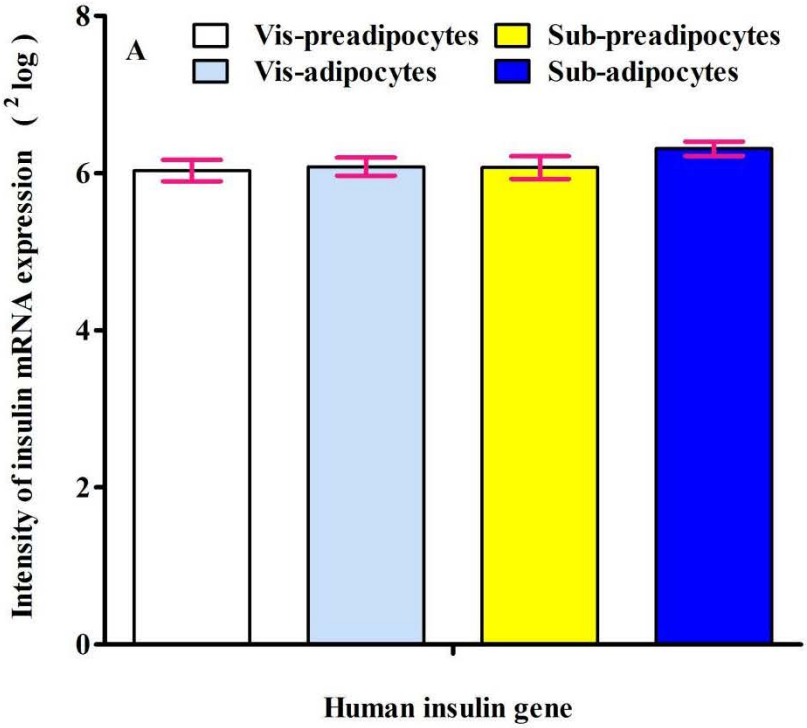
Figure 1 A-E. Differentiation of human visceral preadipocytes to adipocytes.

Panel A shows human pre-adipocytes (no lipid droplets) and **Panel B** exhibits adipocytes (with medium-sized or single large lipid droplets). In panel A, nuclei and lipid droplets were stained with DAPI and FITC respectively. Nuclei were indicated by white arrow. In panel B, lipid droplets were stained with FITC and indicated by orange arrow. Almost the whole space of adipocytes is occupied by lipid droplets. Confocal laser scan microscopy (CLSM) was applied to detect DAPI and FITC.

Panel C and D display 5 differentiation marker genes for human primary visceral and subcutaneous adipocytes respectively**.** Gene expression was expressed as ^{2}log (for

example: the difference between human subcutaneous preadipocytes and adipocytes regarding leptin (*LEP*) expression is approximately 7, thus true difference is 2^7 (=128-fold) and was shown in y-as. Diacylglycerol O-acyltransferase 2 (*DGAT2*), hormone-sensitive lipase (*LIPE*), lipoprotein lipase (*LPL*) and fat specific protein-27 (*FSP27*).
Panel E shows marker gene for human primary visceral and subcutaneous preadipocytes. Adipocyte enhancer-binding protein 1 (*AEBP-1*) was up-regulated in human preadipocytes.

After establishing our experimental model, we investigated whether human primary subcutaneous preadipocytes and adipocytes as well as visceral preadipocytes and adipocytes express insulin mRNA and secrete insulin protein. Astonishingly, all four tested cell types expressed insulin mRNA as determined by mRNA Beadarrays (Figure 2 A-B). Insulin mRNA expression was shown as ^{2}log in figure 2 A and as relative intensity in figure 2 B. As positive control, we have measured the insulin mRNA in human pancreas tissue. The relative intensity of mRNA in pancreas tissue was reached saturated state (^{2} log of relative mRNA intensity was about 14.0 ± 0.2; triplicate). Insulin mRNA expression in pancreas was approximately 256-fold higher than insulin mRNA expression in human primary preadipocytes and adipocytes. Notably, the same insulin probe sequence as we applied in human adipocytes was used to measure insulin mRNA expression in pancreas tissue.


A
Vis-preadipocytes
Vis-adipocytes
Sub-preadipocytes
Sub-adipocytes
Intensity of insulin mRNA expression (2 log)
8
6
4
2
0
Human insulin gene

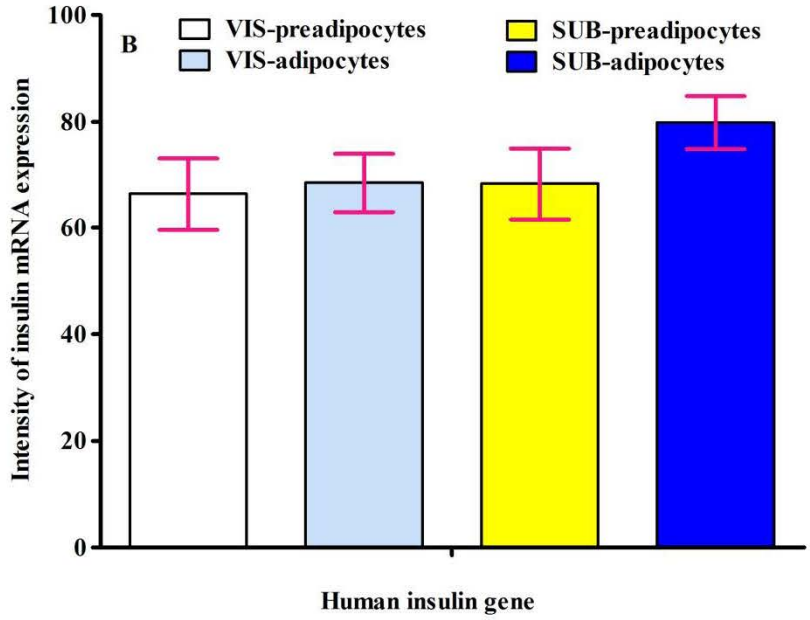

B
VIS-preadipocytes
VIS-adipocytes
SUB-preadipocytes
SUB-adipocytes
Intensity of insulin mRNA expression
100
80
60
40
20
0
Human insulin gene

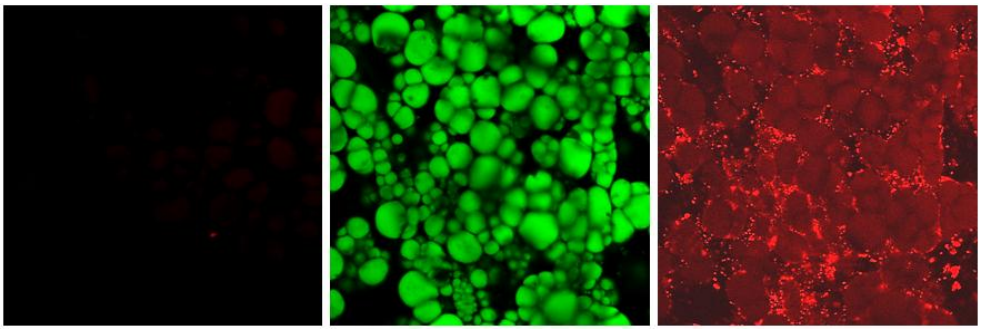
Figure 2 A-B. mRNA expression analysis of insulin in human primary subcutaneous and visceral preadipocytes and adipocytes.

Insulin mRNA expression expressed as ^{2}log in **Panel A** and as relative intensity in **panel B**. mRNA Beads approach was used to detect insulin mRNA expression. Each sample was measured 4 times by mRNA expression assay.

To determine whether the insulin mRNA is translated into protein, we applied immunofluorescent confocal laser scanning microscopy (CLSM) to human subcutaneous (preadipocytes and adipocytes) and visceral (preadipocytes and adipocytes) using a specific mouse anti-human monoclonal insulin antibody from Sigma and the detection antibody was goat anti-mouse labeled with Alexa Fluor 647. Indeed, CLSM analysis revealed the expression of insulin in the cytoplasm and on the plasma membranes of all four human cell types. Importantly, huge insulin protein expression was observed in human primary visceral and subcutaneous (not shown) adipocytes (Figure 3 A-F), while insulin protein expression in both human visceral and subcutaneous (not shown) preadipocytes was traces to very low (Figure 3 G-K). IgG1 isotype controls were all negative (preadipocytes and adipocytes) (Figure 3 A and G). To further validate our finding that human adipocytes express insulin, immunoelectron microscopy (IEM) was also applied to the primary visceral adipocytes. As expected, IEM analysis (Figure 3 M) showed insulin protein expression in human adipocytes (conjugated with 15 nm gold particles and visualized as black dots) similar to CLSM observations (Figure 3 C). IgG1 isotype control was also negative as displayed in figure 3 L.

Figure 3 A-F: Human primary visceral adipocytes

A (IgG1 + Alexa 647, Control) **B (FITC labels lipid droplets)** **C (Alexa 647 labels Insulin)**



D (DAPI, Nuclei) **E (Merge of C and D)** **F (Merge of B, C and D)**

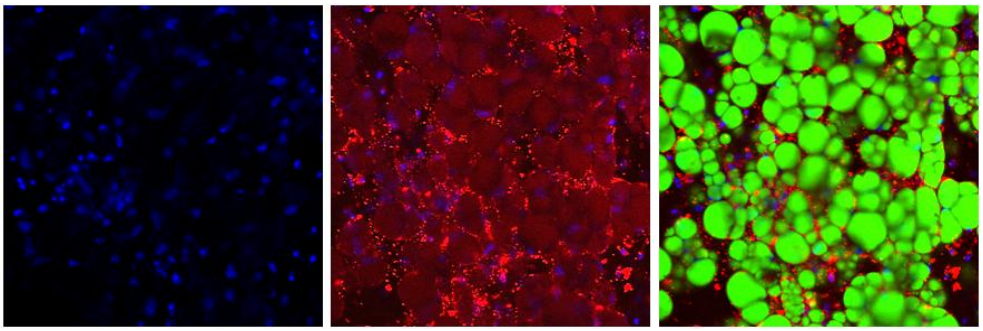


Figure 3 A-M represents Immunofluorescent confocal laser scanning microscopy (CLSM) and Immunoelectron microscopy analysis of human visceral preadipocytes and adipocytes.

From A-F, confocal microscopy analysis was applied to detect and localize insulin protein in the human primary visceral adipocytes.

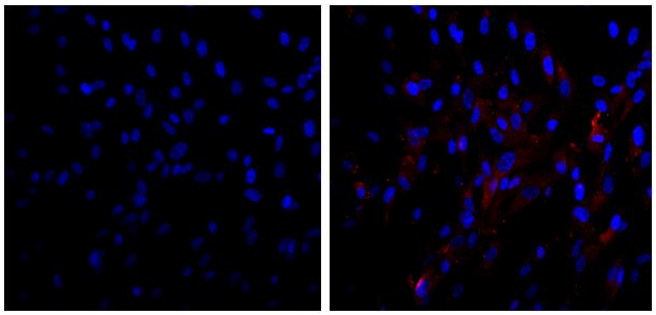
The cultured adipocytes in six-well plates were incubated with IgG1 isotype and the detection antibody Alexa 647 (**A;** considered as negative control), lipid droplets were visualized with FITC (**B;** green color); adipocyte was incubated with monoclonal antibody against human insulin (**C, red color).** Bound antibodies were detected with Alexa 647 coupled goat anti-mouse (**C**; red color); adipocytes were stained with DAPI to detect nuclei (**D**, blue color), **C (**insulin**) and D (**nuclei**)** were merged (**E),** and **B (**lipid droplets), **C** (insulin) and **D** (nuclei) exhibit a merge of all three labeling **(F).** Insulin protein was located on the surface of plasma membrane and cytoplasm **(C, E and F)** of human visceral adipocytes.

Fluorescent labeling was used for all detections. Original magnification was 400 times. The same results were found for human subcutaneous adipocytes (was not shown).

Figure 3 G-K: Huma primary visceral preadipocytes

G (Ig G1 + Alexa 647, Control) **H (FITC labels lipid droplet)** **I (Alexa 647 labels Insulin)**

J (DAPI labels Nuclei) **K (Merge of H, I and J)**



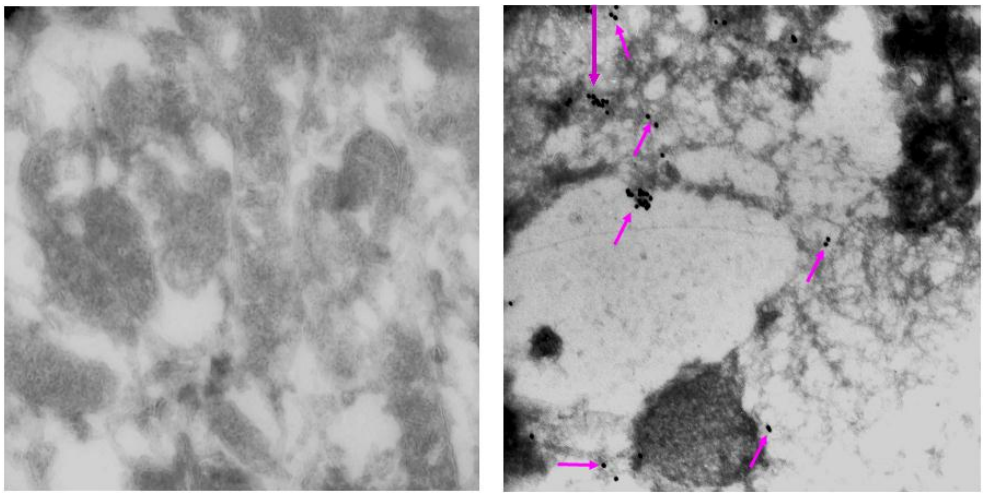
From G-K, confocal microscopy analysis was applied to detect and localize insulin protein in the human primary visceral preadipocytes.

The cultured preadipocytes in six-well plates were incubated with IgG1 isotype and the detection antibody Alexa 647 (**G;** considered as negative control), lipid droplets were stained with FITC (**H;** green color); adipocyte was incubated with monoclonal antibody against human insulin. Bound antibodies were detected with Alexa 647 coupled goat anti-mouse (**I**; red color); preadipocytes were stained with DAPI to detect nuclei (**J**, blue color). **K (**merge**)** exhibits a merge of all three labeling; **H (**lipid droplets), **I** (insulin) and **J** (nuclei)**.** Insulin protein was located in cytoplasm and on the surface of plasma membrane **(I and K)** of human visceral preadipocytes. Fluorescent labeling was used for all detections. Original

magnification used was 400 times. The same results were found for human subcutaneous preadipocytes (was not shown).

Figure 3 L-M Human primary visceral adipocytes

L- Control (Magnification 45000 X) **M- Insulin (Magnigication 45000 X)**



Labeled with gold-conjugated goat anti-mouse

From L-M, Immunoelectron microscopy analysis of human visceral adipocytes.

Panel L exhibits labelling without specific primary monoclonal antibody against human insulin, but with IgG isotype 1 (considered as control). **Panel M**: labelling with specific primary monoclonal antibody again human insulin detected with gold-conjugated goat anti-mouse. Black dots represent insulin bound gold particles (15 nm).

To investigate, whether insulin is secreted from human primary subcutaneous and visceral cells, insulin protein levels were measured in the media containing the secretion from all four cell types, using DAKO Insulin ELISA KIT. As depicted in figure 4 A-B, insulin protein was detected in the media of human primary visceral (379 pmol/l) (Figure 4 A) and subcutaneous (55 pmol/l) (Figure 4 B) adipocytes. Notably, no insulin protein was detected in both human primary preadipocytes (Figure 4 A-B).

To further establish the link between fat mass and insulin secretion levels, we chose to stimulate all four cell types with LPS and 25 mM glucose. Hence, to substantiate our findings, insulin levels were measured in the secretion of all four cell types treated with Lipopolysaccharide (LPS) (200 ng/ml). Insulin levels were also measured in human primary visceral preadipocytes and adipocytes as well as human subcutaneous preadipocytes treated with 25 mM glucose. Upon LPS treatment, insulin protein levels secreted by human subcutaneous and visceral adipocytes were reduced approximately three-fold (Figure 4 A-B). Treatment of human primary visceral adipocytes with 25 mM glucose for 24 h resulted in an approximately four-fold less insulin protein concentrations than the control (untreated with glucose) (Figure 4 A).

To determine, whether the lack of undetectable insulin secretion is a specific feature of human preadipocytes, IL-6 and IL-8 protein concentrations was measured in the secretion of only human subcutaneous preadipocytes treated with and without LPS. IL-6, IL-8 and M-CSF proteins were notably detected and measured in the secretion of human subcutaneous preadipocytes without LPS treatment (2.5 pg/ml), (0.0 pg/ml) and (10.5 pg/ml) and treated with LPS (499 pg/ml), (3610 pg/ml) and (97.7 pg/ml) respectively (Figure 4 C).

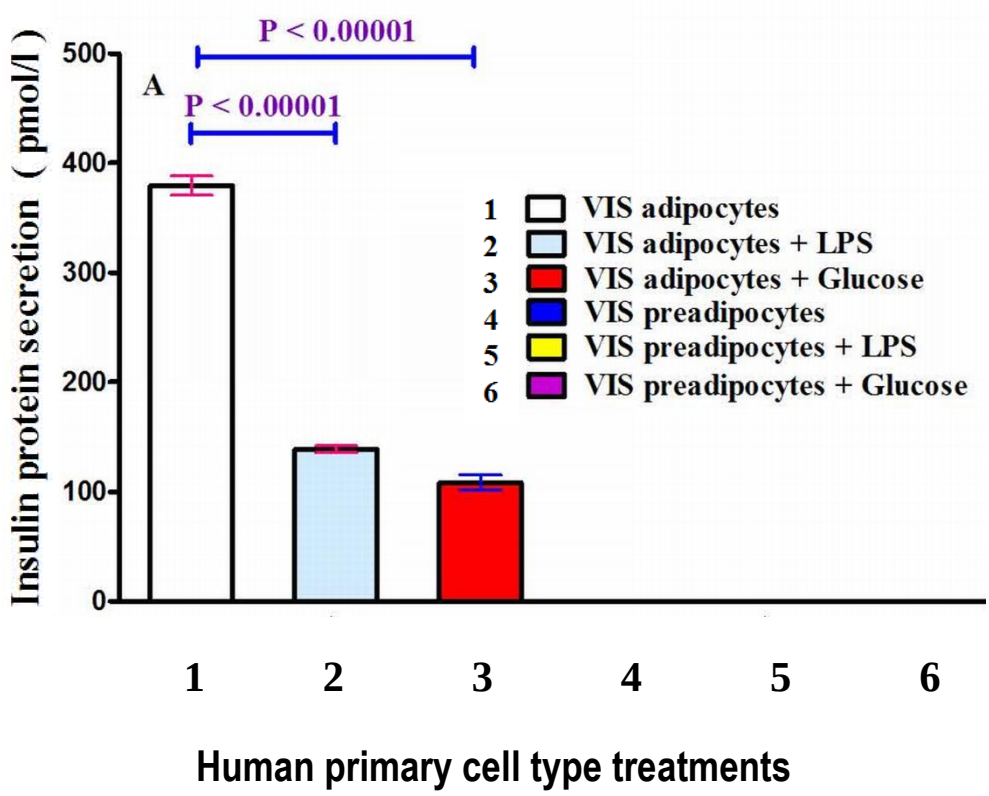


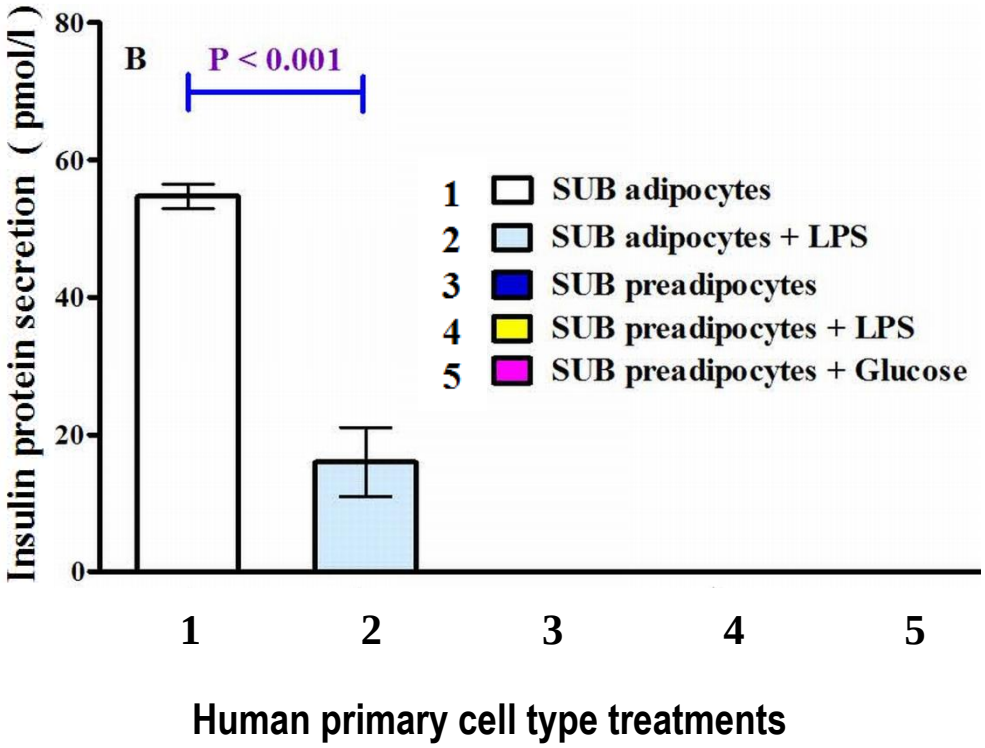
Figure 4 A-B. ELISA analysis of secreted fractions collected from with and without LPS and 25 mM glucose treated human primary visceral and subcutaneous preadipocytes and adipocytes.

DAKO insulin ELISA kit was applied to measure insulin concentrations in the secreted fractions. Two independent ELISA was used to measure insulin concentrations in secreted fractions; insulin concentration in secreted samples was performed respectively in single and duplicate by ELISA.

A and B show ELISA of insulin respectively in human primary visceral and subcutaneous preadipocytes- adipocytes-secreted fractions. Insulin concentrations were expressed in pg/ml as shown in Y-axis. X-axis displays human primary visceral adipocytes and preadipocytes treated with and without lipopolysacchariden (LPS) and 25 mM glucose.

panel A; Visceral cells: the following bars represent: white bar (human visceral adipocytes-secreted fraction), light blue bar (human visceral adipocytes-secreted fraction treated with

LPS (200 ng/ml)), red bar (human visceral adipocytes-secreted fraction treated with 25 mM glucose), dark blue bar (human visceral preadipocytes-secreted fraction), yellow bar (human visceral preadipocytes-secreted fraction treated with LPS (200 ng/ml)) and violet bar (human visceral preadipocytes-secreted fraction treated with 25 mM glucose).



Panel B; Subcutaneous cells: the following bars represent: white bar (human subcutaneous adipocytes-secreted fraction), light blue bar (human subcutaneous adipocytes-secreted fraction treated with LPS (200 ng/ml)), dark blue bar (human subcutaneous preadipocytes-secreted fraction), yellow bar (human subcutaneous preadipocytes-secreted fraction treated with LPS (200 ng/ml)) and violet bar (human subcutaneous preadipocytes-secreted fraction treated with 25 mM glucose). The p-value obtained from an independent two-tailed test samples (T Test). $P < 0.05$ was accepted as statistically significant.

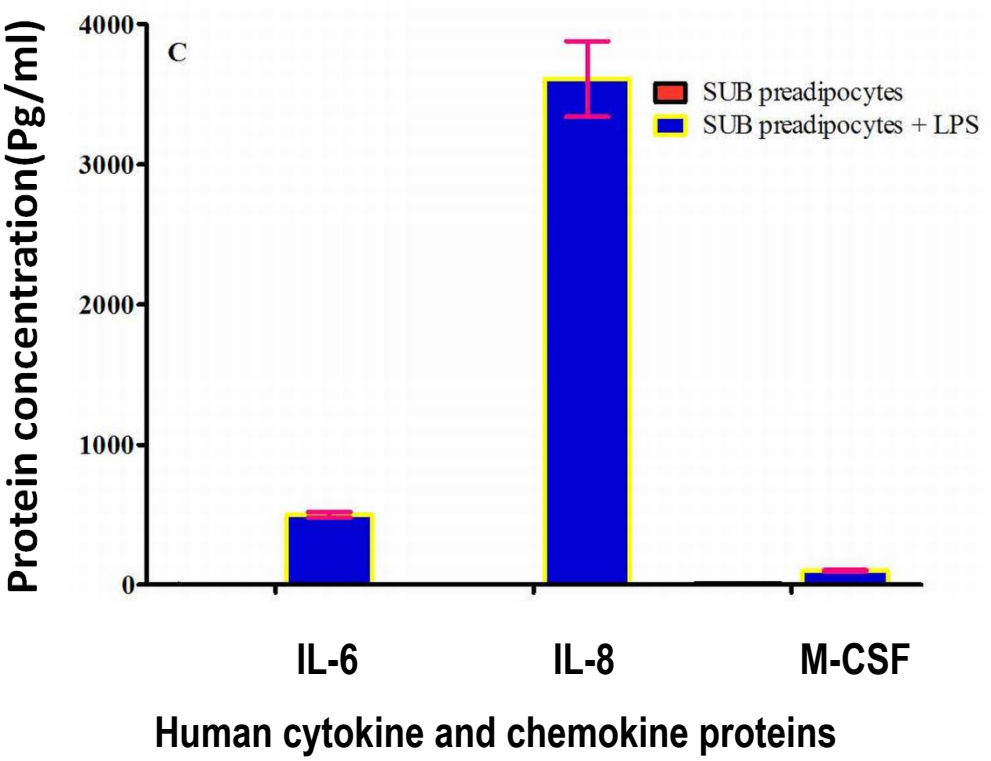


Figure 4 C. IL-6, IL-8 and M-CSF analysis in human primary subcutaneous preadipocytes.

DAKO insulin ELISA kit was applied to measure insulin concentrations in the secreted fractions. Two independent ELISA was used to measure insulin concentrations in secreted fractions; insulin concentration in secreted samples was performed respectively in single and duplicate by ELISA.

Panel C; IL-6, IL-8 and M-CSF were measured in human primary subcutaneous preadipocytes, using Bio-plex according to instructions provided by Bio-Legend. Red bars represent human preadipocytes without LPS treatment and dark blue bars show human preadipocytes treated with LPS. The concentrations of these three cytokine and chemokines were expressed as pg/ml shown in Y-axis. Samples were measured in duplicate.

It is easy to speculate how insulin resistance may result as a consequence of obesity. This could be true if the following hypothesis***, insulin protein concentrations are positively correlated with body fat mass***, is also true.

To test this hypothesis, blood insulin concentrations were measured in 41 human subjects divided into four groups of 10 (except diabetes group receiving insulin containing 11 subjects), independent of age and gender, as follows: **Group 1-** Lean subjects with a body mass index (BMI) between 20.1 and 25.6, **Group 2-** Oobese subjects with a BMI above 30, **Group 3-** Obese subjects with type 2 diabetes without receiving insulin and a BMI above 30 and **Group 4-** Obese subjects with type 2 diabetes, receiving insulin and BMI above 30. All patients were well-matched for their BMI. It must also be stressed that in lean group only one person has a BMI above 25 (BMI = 25.6).

Interestingly, the lowest blood insulin concentrations were found in lean subjects as compared with the other three groups (Figure 5 A) and blood insulin concentrations were positively and highly significant correlated with body fat mass ($p < 0.00003$, $r = 0.6$, One way ANOVA) as shown in figure 5 B. As expected, the highest blood insulin concentrations were observed for obese subjects with diabetes receiving insulin as therapy (Figure 5 A).

To confirm (additionally) and validate the above suggested hypothesis, we investigated whether human obese subjects with type 2 diabetes receiving insulin can be rescued and repaired via a reduction of weight loss (or BMI) in these human subjects. For this reason, the bariatric surgery was applied and that is an established surgery to treat the obese subjects. The BMI (based on fat mass) of five human subjects (independent of age and gender but well a BMI above 35) with diabetes, which use insulin to adjust their blood glucose were three times measured; **1-** before bariatric surgery is considered as basis point, **2-** after surgery and **3-** 6 months after the second

measurement. As depicted in figure 5 C, the BMI of human subjects were above 35 of which three subjects above or equal to 45, one 36 and one 39. After bariatric surgery (done by president of bariatric surgery in The Netherlands), the BMI was tremendously reduced in all subjects as shown in figure 5 C. That was even more amazing to observe that BMI of three human subjects were reduced to below 30 (one subject just above 25; lean value), indicating that these three subjects are not anymore considered as obese subjects. The BMI of the other two patients were continually reduced by approximately 30%. A weight loss of 40-60 kg was observed in all subjects. Moreover, although all five human subjects do not need any more insulin injection and that is not necessary to measure insulin in these subjects, the insulin was measured in human plasma of these five subjects and we have found a concentration range (50-350 pg/ml).

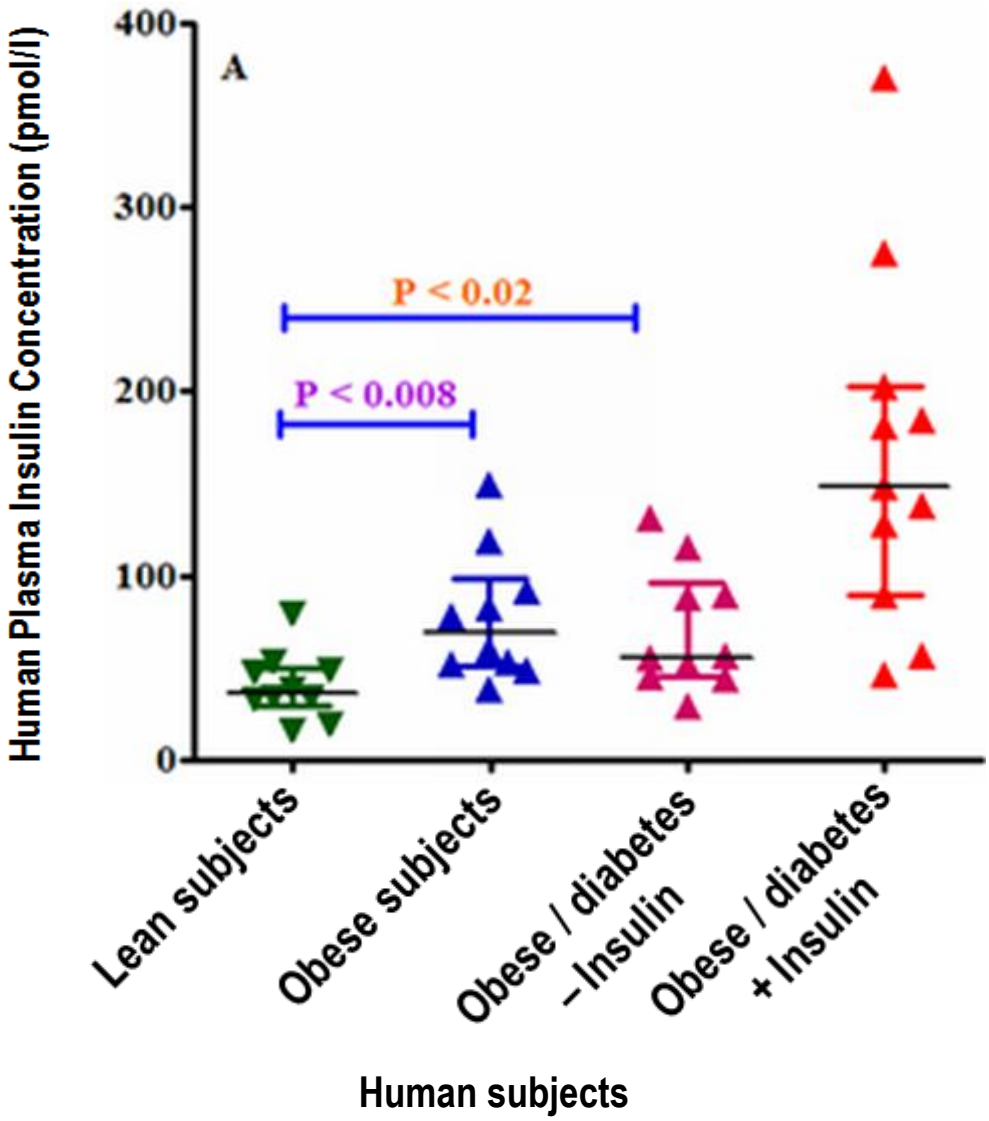


Figure 5 A-C. The measurement of insulin concentrations in 5 different groups of human subjects.

Figure 5 A. DAKO insulin ELISA kit was applied to measure insulin concentrations in the plasma of 41 human subjects categorized in four groups based on Body Mass Index (BMI); 1- Lean subjects (BMI, 20.1-25.6, only one patient above 25), 2- Obese subjects (BMI, 32.5-39.5), 3-Obese subjects with diabetes without receiving insulin (BMI, 32.1-40) and obese subjects with diabetes, receiving insulin (BMI, 33.3-38). BMI is expressed as mass

(kg)/(height (m))^2. The p-value resulted from one-way analysis of variance (ANOVA). P < 0.05 was accepted as statistically significant. Insulin concentrations were expressed as pmol/l shown in Y-axis.

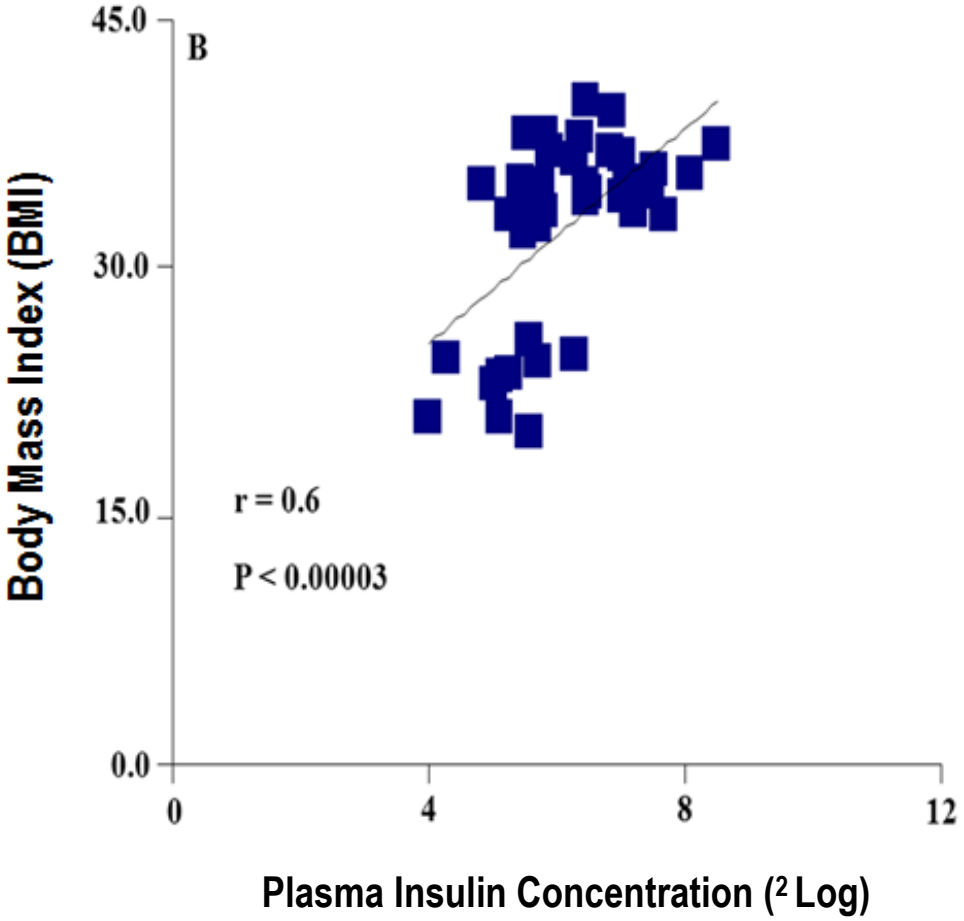


Figure 5 B. Correlation between plasma insulin concentrations (*n* = 41 human subjects) and Body Mass Index (BMI). BMI is expressed as mass (kg)/(height (m))^2. The p-value resulted from one-way analysis of variance (ANOVA). P < 0.05 was accepted as statistically significant. Insulin concentrations were expressed as pmol/l shown in Y-as and BMI was expressed as ^{2}log shown in X-axis.

There was a strong positive correlation between plasma insulin concentration and human BMI (P < 0.00003, r = 0.6).

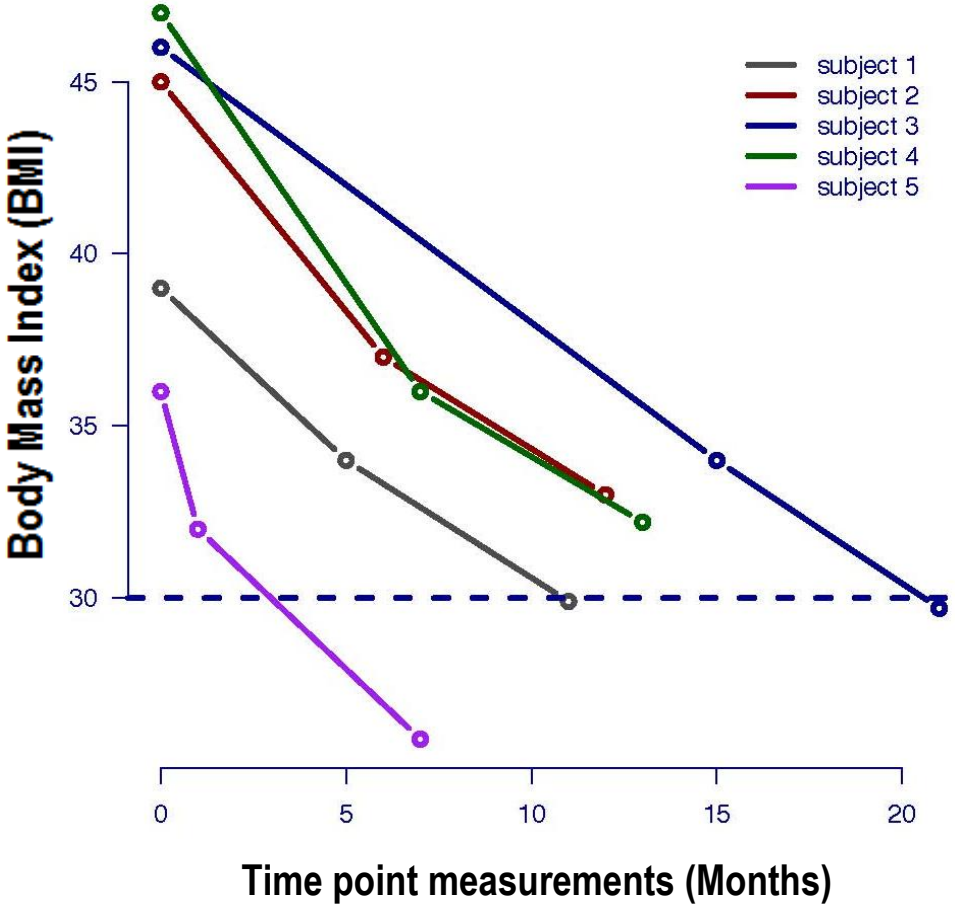


Figure 5 C. The bariatric surgery of 5 human subjects with type 2 diabetes receiving insulin injection. The BMI was measured three times in 5 patients; 1- before bariatric surgery (first time point), 2- after bariatric surgery (second time point), and 3-6 months after second point. BMI was sharply decreased after surgery. The BMI was calculated as Kg / m^2 shown in Y-axis. The time point 0 is considered as before surgery. The x-axis shows the BMI measurements in different time points.

To establish the assumption that the production of insulin by human adipocytes is independent of pancreas and provide direct evidence for adipocyte insulin production in a physiological context, we must have a model that islets of Langerhans- associated beta cells are selectively

destroyed or not functional any more. Based on search machine, prediabetic BB (Bio-Breeding) rats are considered as a good and established model for studying insulin-producing β cell destruction located in the islets of Langerhans in pancreas during the development of IDDM (Hoch et al., 2008; Lacasa et al., 2007; Lee et al., 2008; Quackenbush, 2002). For this reason, we have chosen for Prediabetes BB rats to investigate whether our assumption is true. Since these rats develop spontaneously IDDM during puberty and high glucose (25-30 mmol/l) is an indicator for the presence of IDDM in these rats, we also measured the glucose in these rats before isolation of fat and pancreas tissues. Prediabetes BB rats with a glucose concentration of 14-16 mmol/l was used as control in our experiments. As depicted in figure 6 D and G (in figure 6 D-G), immunofluorescent confocal microscopy (IFCM) analysis of rat-derived pancreas tissue cryostats using insulin-specific polyclonal antibodies revealed that islets of Langerhans-associated beta cells were destroyed and not detectable in the rats with IDDM. That is the reason why we could not detect any insulin labeling in these IDDM rats. This finding is in agreement with all other studies, which beta cells were destructed in these rats and not present or detectable. However, islets of Langerhans-associated beta cells were easily detected in control rats by using insulin-specific antibodies and Alexa 633 as detection antibody as shown in figure 6 L and N.

To confirm that the islets of Langerhans-associated beta cells were indeed destroyed and not detectable, we also applied immunofluorescent confocal microscopy analysis of rat-derived pancreas tissue cryostats using glucagon-specific antibodies. As indicated in figure 6 (D and G, H and K), islets of Langerhans well detected using glucagon-specific antibodies in combination with alexa 488. This indicates that α cells in pancreas, which produce glucagon, were present and detectable. Interestingly, as shown in figure 6 H,

four beta cells were detected. This confirmed that almost all Langerhans-associated beta cells were destroyed and also proves that our strategy is specific and works. As negative control, cryostat slide labeled with IgG isotype combined with alexa 633 and DAPI (nuclei staining). We did not observe any signal resulted from IgG or detection antibody (alexa 633) as displayed in figure 6 A. A substantial production of insulin at the protein level was observed in both fat tissues derived from IDDM and control rats (Figure 6 O-Q and R-T), applying the same insulin-specific antibodies combined with IFCM.

Although IDDM rats, which do not have beta cells and as consequence there is no insulin production, fat tissue of both IDDM and control rats produce clearly and convincingly insulin as shown in figure 6 (O and Q, R and T) and this insulin production is independent of pancreas-associated islets of Langerhans beta cells. Notably, the staining of insulin in fat tissues of IDDM was much stronger than insulin staining in the fat tissues of control rats.

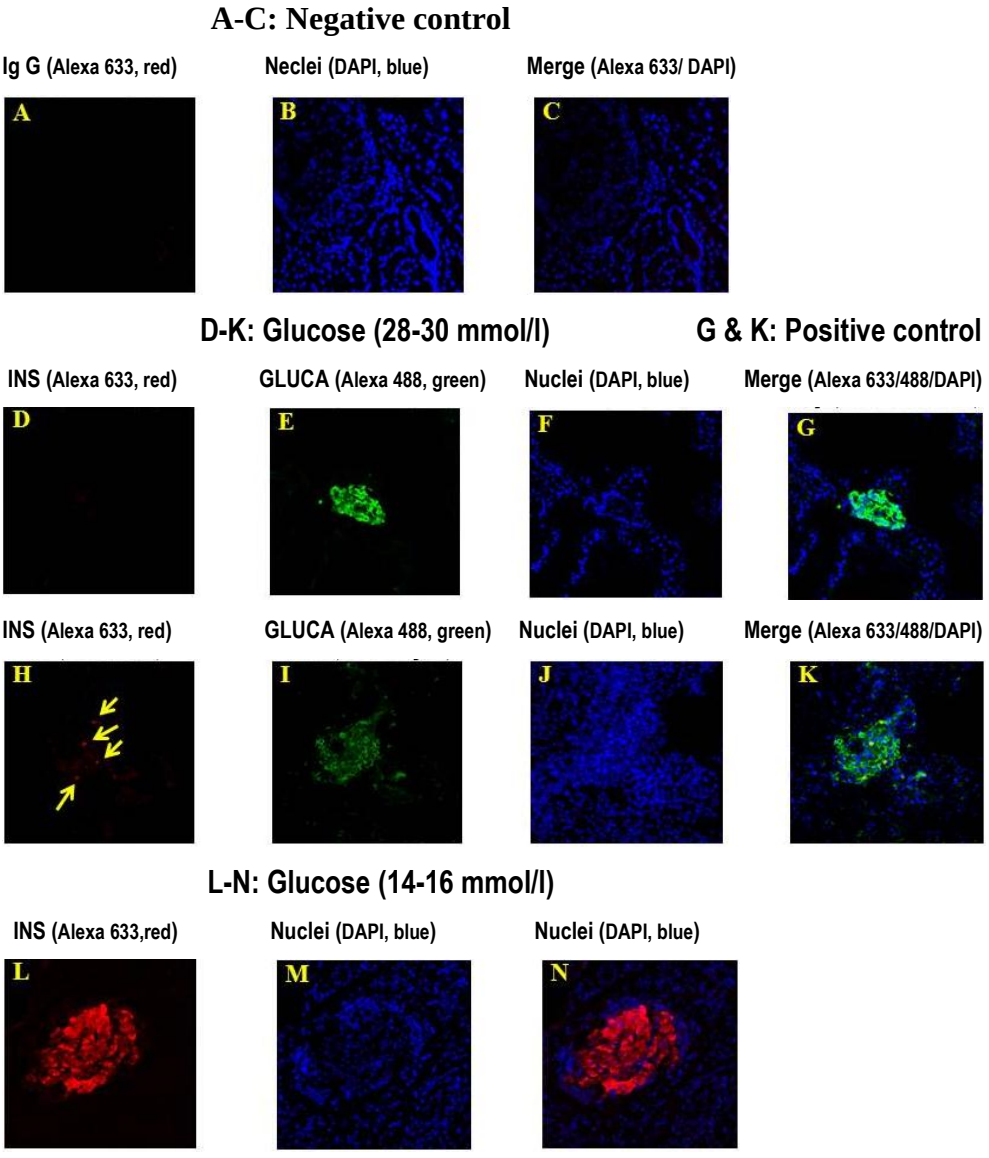


Figure 6 A-N. The localization and visualization of Insulin and Glucagon in the pancreas tissue cryostats of Prediabetes BB rats using confocal microscopy strategy.

Confocal microscopy analysis was used to localize Insulin and Glucagon in insulin dependent diabetes mellitus (IDDM) and control rats. The glucose concentration of control

rats were between 14 and 16 mmol/l. The glucose concentration in IDDM rats were between 25-30 mmol/l. Cryostats were made from pancreas tissues of these rats. The thickness of pancreas cryostats was 5 µm. A cryostat slide was stained only with IgG isotype and alexa 633 coupled goat anti-guinea pig (**panel A**); considered as a negative control). This slide was not incubated with primary polyclonal antibody. The nuclei were stained with DAPI.

Other cryostats were also stained with Polyclonal antibody against rat insulin and bound antibodies were displayed with alexa 633 coupled goat anti-guinea pig (**panel D** and **G**; **H** and **K**; **L** and **N**; red color). Other cryostats were also stained with Polyclonal antibody against rat glucagon and bound antibodies were displayed with alexa 488 coupled goat anti-rabbit (**panel E** and **G**; **I** and **K**; green color). DAPI was used to stain and detect nuclei in all tissues; **F** and **G**; **J** and **L**; **N** and **O**; dark blue color. Fluorescent labeling was used for all detections.

Insulin was not detected in IDDM rats but glucagon (green fluorescent) well. Insulin was found to be positive in islets of Langerhans-associated beta cells in control rats. In an islet of Langerhans, four beta cells were found to be positive for insulin as indicated by 4 light yellow arrows. Glucagon as positive control was found to be positive in the pancreas-associated islets of Langerhans beta cells in both IDDM and control rats.

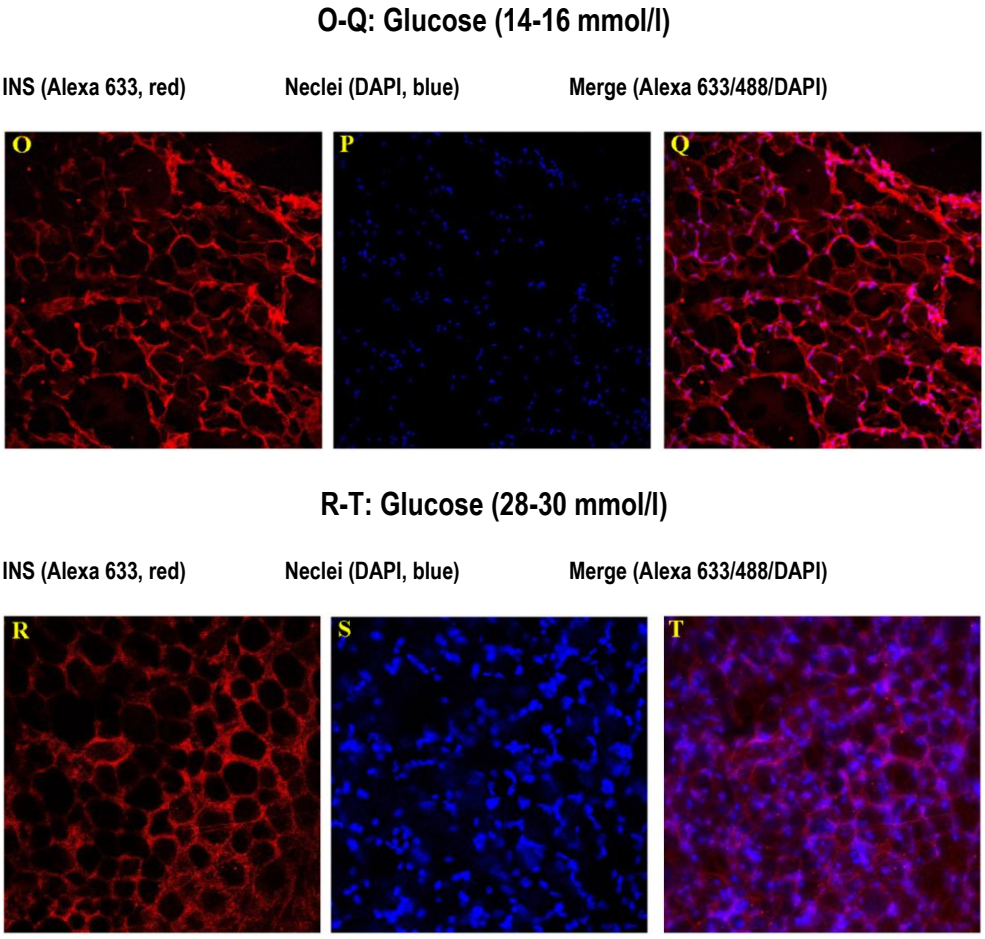
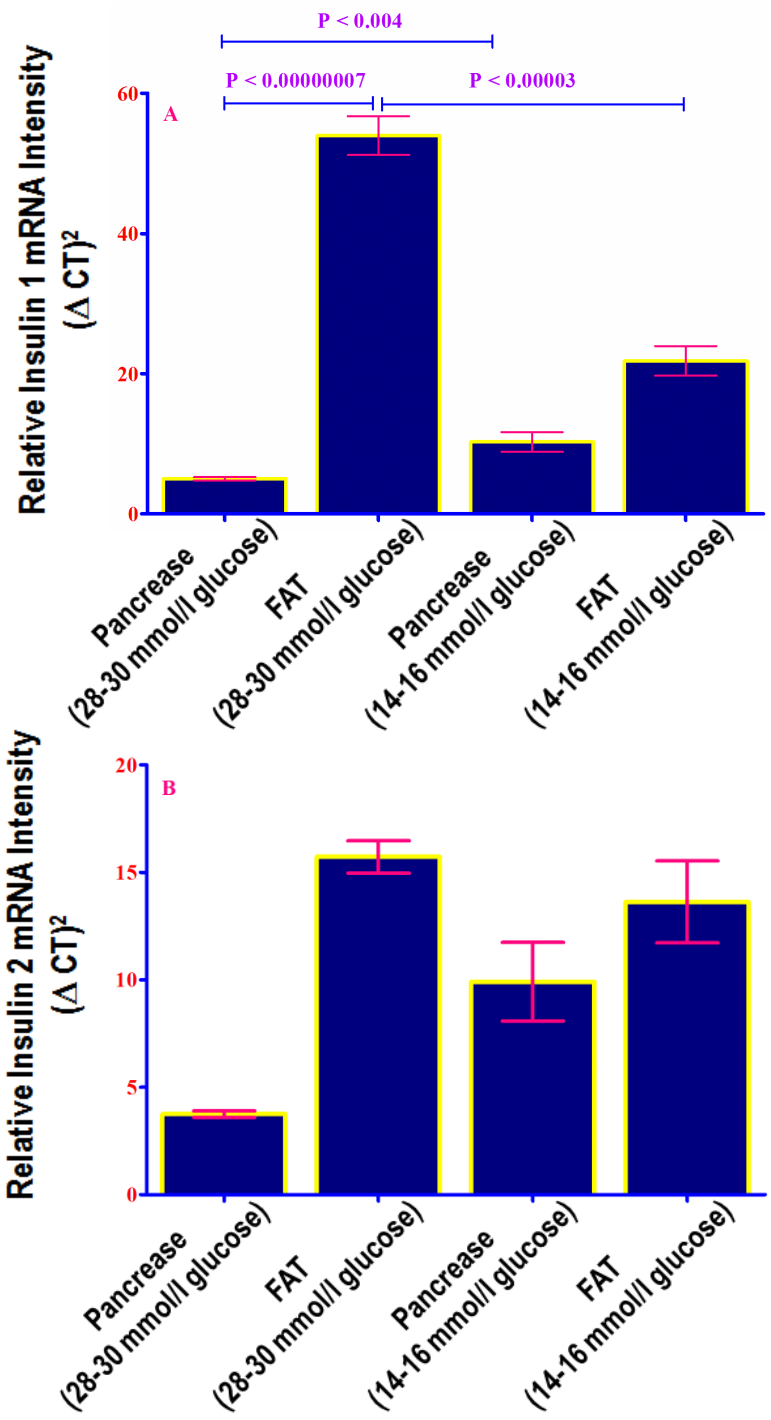


Figure 6 O-T. The localization and visualization of Insulin in the fat tissue cryostats of Prediabetes BB rats using confocal microscopy strategy.

Confocal microscopy analysis was used to localize Insulin in IDDM and control rats. Cryostats were made from fat tissues of these rats. The thickness of fat cryostats were 15 µm. Fat cryostats were also stained with Polyclonal antibody against rat insulin and bound antibodies were displayed with alexa 633 coupled goat anti-guinea pig (**panel O**, **Q**, **R** and **T**). DAPI was used to stain nuclei in all slides. Insulin was found to be positive in both IDDM and control rats.

To confirm the data obtained by IFCM analysis of pancreas cryostats and fat tissues, the total RNAs were collected from the same tissues derived from the same rats for QPCR. Since the rats have two insulin genes; Ins1 and Ins2, the both Ins1 and Ins2 mRNAs were also measured in pancreas and fat tissues. Intriguingly, the relative intensity of Ins1 and Ins2 mRNA were the highest in the fat tissues and lowest in pancreas tissues of IDDM rats as depicted in figure 7 A. Surprisingly and unexpectedly, in IDDM rats, which have no or very low number of beta cells, the mRNA expression of Ins1 is very high in the fat tissue of IDDM rats (figure 7 A), whereas control rats, which have enough β cells, the mRNA expression of Ins1 is low in the fat tissues of control rats. Although in general the relative intensity of Ins1 mRNA expression is higher than Ins2 mRNA expression, the pattern of Ins1 and Ins2 mRNA expression in these tissues were clearly similar (figure 7 A and B).



Prediabetes BB rats tissues (Pancrease and FAT)

Figure 7 A-B. mRNA expression analysis of insulin1 and insulin2 in Prediabetes BB rats.

A, mRNA expression of insulin1 (Ins1) and **B**, insulin2 (Ins2) were measured in pancreas and fat tissues of IDDM and control rats, using QPCR with Taqman 7900HT. Relative intensity of mRNA was expressed as $(\Delta CT)^2$. Two independent biological replicates were used in mRNA expression analysis. The p-value obtained from an independent two-tailed test samples (T-Test). $P < 0.05$ was accepted as statistically significant.

DISCUSSION

This study was designed to unravel the role of adipocytes behind the link between obesity and insulin regulation in human. The purity and specificity of preadipocytes and adipocytes fractions were determined using two independent techniques: confocal microscopy and illumine mRNA expression,

All adipocytes marker genes are involved in the lipid metabolism and hydrolyzation of triglycerides. Importantly, the up-regulation of these differentiation markers in human adipocytes confirmed the observed differences between preadipocytes and adipocytes with respect to lipid droplets. Since both adipocyte and preadipocyte markers responded as expected (based on published data), this indicated that our approach resulted in the specific homogenous fractions of all four cell types (human subcutaneous and visceral preadipocytes and adipocytes).

In an established model, insulin mRNA expression was measured. Interestingly, all four cell types synthesize insulin. The gene expression was expressed as ^{2}log to suppress false positive results derived from intrachip comparison as well as the up-regulation and down-regulation of genes are

treated in a similar fashion (Naito and Okada, 1975). Stringent criteria were applied to ensure that the insulin expression observed in the human primary subcutaneous (preadipocytes and adipocytes) and visceral (preadipocytes and adipocytes) was not an artifact. To this end, human primary subcutaneous (preadipocytes and adipocytes) and visceral (preadipocytes and adipocytes) RNAs were isolated from two independent technical replicates and each replicate was performed in duplicate and each sample was measured in Illumina in duplicate. Finally, each cell type was measured ***four times***. Notably, human primary subcutaneous and visceral cells were derived from two different subjects to ensure biological variance. The mRNA expression data convincingly showed the expression of insulin gene in ***all 16 measurements (preadipocytes and adipocytes of both subcutaneous and visceral cells)***. Finally, it is important to stress that the differences between human primary preadipocytes (subcutaneous and visceral) and adipocytes (subcutaneous and visceral) with respect to the expression of five known mRNA markers of adipocytes and preadipocyte marker, is an indication of the specificity of insulin mRNA expression by preadipocytes and adipocytes. Immunofluorescent confocal laser scanning microscopy confirmed the expression of insulin mRNA at protein levels. Intriguingly, a huge insulin protein staining was shown to be in cytoplasm and plasma membrane of human visceral and subcutaneous adipocytes, but very low traces in human visceral and subcutaneous preadipocytes. The former could be due to the presence of large lipid droplets (Fat storage depot) within adipocytes and the latter appeared to be due to the lack of lipid droplets in human preadipocytes. Regarding stringent criteria, immunoelectron microscopy was also applied to confirm the results obtained by immunofluorescent microscopy. Indeed, immunoelectron microscopy showed a huge staining of insulin protein in human adipocytes

the same as observed in immunofluorescent microscopy. Astonishingly, we observed the secretion of insulin protein by both human adipocytes and no insulin secretion by both human preadipocytes. Hence, it seems reasonable to conclude that we could not detect insulin in human preadipocytes-secreted fractions due to the absence of lipid droplets, which is considered to be an energy storage depot in the form of fat. Although Kojima *et al.* (Kojima et al., 2004) suggested the extrapancreatic insulin production by other tissues, however, only in diabetes states, our data confirmed not only the concept suggested by Kojima *et al.* but also the insulin production by adipocytes in normal state. Although insulin is similar to insulin-growth factor 1 (IGF-1) in sequence and structure, which lays out the ground of cross-reactivity of insulin mRNA with that of IGF-1, the selected probe sequence for IGF1 gene was different from insulin probe sequence (only 28% sequence homology between two probe). This indicates that insulin probe is specific for insulin gene and do not react with IGF1.

As expected, a reduction of insulin secretion was observed upon treatment of human adipocytes with LPS or glucose. Based on published data, both LPS (Feingold et al., 1992; Hikawyj-Yevich and Spitzer, 1977; Manickam et al., 2010) and 25mM glucose (Heyduk et al., 2010) induce inflammation, which in turn results in an increased lipolysis (Dyrberg et al., 1988; Like et al., 1982; Marliss et al., 1982). As a consequence, fat mass homeostasis would be disrupted and in line with our initial observations (shown in results), no insulin protein secretion by human preadipocytes (lacking lipid droplets). Amazingly, the results obtained by visceral adipocytes treated with glucose showed almost the same pattern as beta cells treated with glucose *in vitro* (Wallis et al., 2009). Importantly, no insulin protein was also detected in both human primary preadipocytes treated with LPS and 25 mM glucose. This was expected since there is a lack of lipid droplets in the

preadipocytes for LPS and glucose to act on. Again, this phenomenon is in concurrence with our initial suggestion showing a strong correlation between lack of fat in preadipocytes and absence of insulin secretion and very low (traces) expression of insulin as well as a strong correlation between present of huge fat droplets in adipocytes and both the secretion of insulin protein and high expression of insulin.

Hence, the stimulation of cells with 25 mM glucose results in an inflammatory state similar to the effect of LPS, as discussed above, and this similarity extends to insulin levels. Since these proteins were clearly and convincingly expressed by human preadipocytes, the absence of insulin secretion by human preadipocytes is a specific phenomenon and therefore human preadipocytes (precursor of adipocytes) can be considered as a good negative cell control. This is very important to stress that this experiment was performed in the same samples as the stimulation of all four cell types with LPS and glucose. The reason why we have chosen to measure IL-6 and IL-8 (Feingold et al., 1992) is that these cytokines have already been established to be secreted by human preadipocytes and we assumed that this could also hold true for preadipocytes.

On the one hand, the lack of lipid droplets in preadipocytes was associated with very low expression of insulin protein and no insulin protein secretion. On the other hand, human mature adipocytes contained either medium-sized or single large lipid droplets associated with increased insulin protein levels within the adipocytes and a convinced secretion of insulin protein. These results encourage us to postulate that there could be a relationship between the concentration of insulin and body fat mass.

90 years after the discovery of insulin, we have made the astonishing discovery that ***human primary adipocytes constitutively produce and release low but not trivial amounts of insulin***. This intriguing discovery

helps us **to reshape** thinking that explains how obesity could increase the risk for developing IR and in turn T2DM. This re-evaluation of current knowledge lays the foundation for another point of view that increased background levels of circulating insulin resulting from an increased size of the adipose compartment (lipid droplets) in obese individuals would make it more difficult for even healthy endocrine beta cells of pancreas to produce effective changes in blood insulin concentrations.

Since blood insulin levels were high in all human obese subjects as compared with lean subjects and showed a highly significant positive correlation, our suggested hypothesis that blood insulin concentrations increase with the increase of body fat mass is indeed true. It is then possible to suggest that insulin resistance may result as a consequence of obesity. This positive correlation appears to be in apparent agreement with our *in vitro* observations where we were unable to detect insulin protein by human primary preadipocytes (no lipid droplet, thus, traces of insulin expression and no secretion). Finally, it must be stressed that our hypothesis regarding insulin and lipid droplets was also confirmed with *in vivo* published studies by Puri *et al.*(Puri et al., 2007; Puri and Czech, 2008; Puri et al., 2008). In this very structured study, they have shown that FSP27 protein promotes energy reservoir in the form of triglycerides within lipid droplets and knock out of FSP27 gene in mice led to the increase of lipolysis and as consequent the enhancement of insulin sensitivity, protecting mice from diet-induced obesity and in insulin resistance.

Amazingly, a reduction of fat mass (fat mass BMI) was found in 5 patients with type 2 diabetes after bariatric surgery. Intriguingly, the fat loss (weight loss) rescued and recovered these human subjects from type 2 diabetes and all patients do not receive any more an insulin injection, ensuring "***a great feel of freedom and relief***". These data indicate that fat loss in these

subjects restore the ability of pancreas-associated β-cells produce sensitive insulin to adjust blood glucose without external insulin injection, confirming our above suggested hypothesis in this study. This finding suggests a direct physiological role of insulin produced by fat tissue.

Importantly, immunofluorescent confocal microscopy analysis of Prediabetes BB rats-derived pancreas and fat tissue cryostats using insulin and glucagon-specific antibodies showed in diabetes rats: 1- beta cells destruction in islets of Langerhans (no insulin detection in pancreas), but huge insulin staining in fat tissue 2- alpha cells well present (glucagon), and control rats: 1- intact beta cells, huge insulin staining in islets of Langerhans, less insulin staining in fat tissue. These data proves that our strategy is specific and the model rat is a right choice. Amazingly, the similar results were observed for insulin mRNA levels in the same tissues. Since insulin mRNA and protein expression were tremendously higher in fat tissues of diabetes rats than control rats, these results provide a direct evidence for physiological role of fat tissue insulin production.

The results presented in this study indicate that the constitutive release of insulin by the endocrine adipocyte compartment apparently smothers the dynamic regulation of insulin levels by the beta cells of the endocrine pancreas and thus provides a plausible mechanism as to how obesity (Fat Mass) could cause insulin resistance. This model also creates possibilities to provide a solution to obesity-linked type 2 diabetes resulting from fat-associated hyperinsulinemia through targeted down-regulation of insulin production in the adipocyte compartment in order to restore the function of the endocrine pancreatic β cells as the major determinant of circulating insulin levels and allow insulin sensitivity to recover.

This study provides a novel knowledge about the role of adipocytes in insulin regulation in humans and opens a new avenue towards the therapy of type 2 diabetes.

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Chapter 6
Non-Clasical Coagulation Factor Synthesis Sites: A Study in Nine Human Primary Cell Types

Monireh Dashti [1,2], Vishtase Khanzadeh [3], Clark J. Zeebregts [4], Arnold Spek [5], Eric Sijbrands [6], Maikel P. Peppelenbosch [1, 2], Farhad Rezaee [1, 5]

[1] Department of Cell Biology, UMCG, University of Groningen, The Netherlands
[2] Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands
[3] Department of internal Medicine, Free University MC, Amsterdam, The Netherlands
[4] Department of Surgery, Division Vascular Surgery, UMCG, Groningen, The Netherlands.
[5] Department of Experimental & Molecular Medicine, Academic MC, Amsterdam, The Netherlands.
[6] Department of cardiovascular genetics and metabolic diseases, Erasmus MC, Rotterdam, The Netherlands.

(Under review)

ABSTRACT

The coagulation/fibrinolysis system is essential for wound healing after vascular injury. According to the literature, the synthesis of majority coagulation factors is restricted to the liver, platelets and the endothelium. We decided to challenge this classical interpretation by measuring coagulation factors in nine human primary cells. Factor X (FX) mRNA was expressed by fibroblasts, visceral preadipocytes/adipocytes and hepatocytes, but not in macrophages or other cells. All cells expressed FVIII except endothelial cells. Fibroblasts, endothelial cells and macrophages synthesized thrombomodulin (TM), but not FV. Interestingly, vascular-related cells (platelets/monocytes) that synthesized FV did not express FX and vice versa Monocytes express FV, FVIII and FXIIIA, which may ensure optimal activation of FX and stabilization of the fibrin clot. Monocytes also express thrombomodulin, which may destabilize the fibrin clot. Overall, the canonical view of coagulation factor production may need revision because many cell types do express coagulation factors.

INTRODUCTION

Atherothrombosis is a complex of thrombus and atherosclerotic plaque and a major cause of death worldwide (Rauch et al., 2001). The capacity of the coagulation system to repair blood vessel damage provoked by loss of vascular integrity (e.g., trauma or oxidized low-density lipoprotein (LDL) (Li and Mehta, 2005) as shown in Figure 1) is critically dependent on the presence of coagulation factors. This dependence is emphasized by the serious health problems observed in patients suffering from the congenital absence of such factors and modulators (e.g., thrombomodulin or protein C), which normally ensure rapid and appropriate fibrin clot formation. Upon vascular injury, tissue factor (TF) expressed by the damaged endothelial layer and TF bearing-cells activate the initiation phase of coagulation pathway and forms a complex with circulating factor VIIa (FVIIa) (Mackman, 2009), resulting in the activation of FX (See **Figure 1** for an illustration of the vascular endothelial cell layers including coagulation factors and cells, and a summary of the results of this paper). In addition, FVIII is an important cofactor that plays an unambiguous role in the activation of FX in the tenase complex (Factor IXa, FVIII and FX) (Fay, 2004; Saenko and Scandella, 1997; Webster et al., 1976). It must be noted that the prothrombinase complex, a target of both coagulation pathways, plays a major role in the formation of the fibrin clot, and that the FX and its cofactor, FV, are key components of the complex. The liver and platelets (PLTs) are two major sites of FV synthesis and the liver is a major site of FX synthesis (Bouchard and Tracy, 2001; Mackman, 2009; Nicolaes and Dahlback, 2002; Rauch et al., 2001; Webster et al., 1976); Based on the literature, FX could be expressed by other cells (Scotton et al., 2009).

Moreover, although numerous studies show that FVIII forms a complex with von Willebrand factor (vWF) released from endothelial cells (ECs), no studies have been conducted to determine whether other circulating cells, such as platelets and monocytes (MONOs), synthesize FVIII. During the inflammation reaction following bacterial infection or tissue damage, the activation of coagulation and the formation of fibrin, which is the final event in the coagulation cascade, represent a defense mechanism that walls off infection and permits repair of the vascular wound (Bouchard and Tracy, 2001; Nicolaes and Dahlback, 2002).

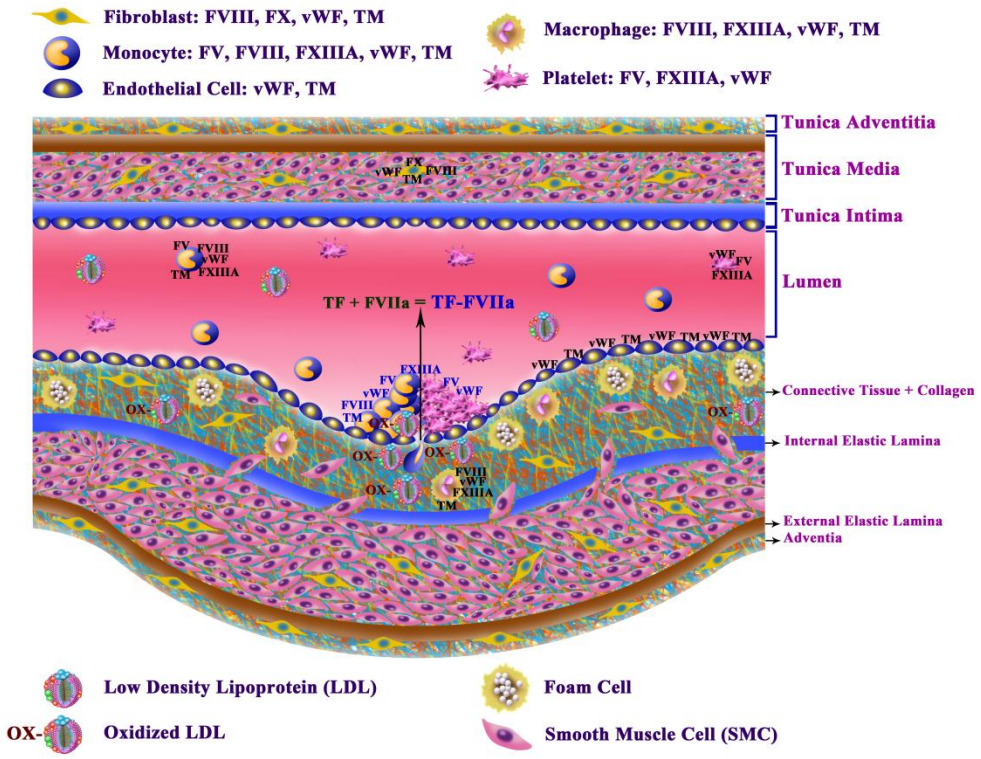


Figure 1. General structure of blood vessels and the factors involved in coagulation. The layers above the lumen show an intact blood vessel while those below the lumen show a blood vessel after injury.

The blood vessel is divided into four major sections 1) tunica adventitia, 2) tunica media, 3) tunica intima and 4) lumen. The tunica adventitia is composed of fibroblasts, connective tissue and collagen and is separated from the tunica media by the external elastic lamina. The tunica media contains fibroblasts, smooth muscle cells (SMCs), connective tissue and collagen, and is isolated from the tunica intima by the internal elastic lamina. The tunica intima, which is bordered on one side by the tunica media and on the other side by the lumen, contains endothelial cells, and in the case of new intima, the internal elastic lamina is damaged and therefore they contained all contents found in tunica media.

In a normal vascular system, blood cells circulate in a regulated way such as monocytes, platelets etc. After a vascular injury, the wound healing and repair process will be immediately activated. However, that is also established that a chronically vascular injury was caused by OX-LDL particles as shown in figure 1. After a vascular injury, TF will be expressed by damaged tissues and TF bearing-cells and form a complex with the activated FVII (FVIIa) present in circulation as shown by black arrow from the site of injury (indicated in the middle of lumen). At the same time, monocytes and platelets arrived very fast at the site of the injury to stop bleeding and repair the injury via the direct activation of coagulation cascade components (FV/FVIII). These two cofactors are involved in an optimal activation of FXa. Both platelets and monocytes ensure the stability of fibrin network via cross linking with FXIIIA, which delivered by these two cell types. Thrombomodulin (TM) was also expressed by monocytes, macrophages, endothelial cells and fibroblasts. When monocytes enter into the tissue at the site of injury, they were converted to macrophages. Macrophages do not express FV and FX, while fibroblasts express FX but not FV.

Although the nature and origin of inflammation and that of fibrin formation are completely different, inflammation and fibrin formation are very closely linked: inflammation induces the coagulation cascade while coagulation modifies the inflammation state or restrains inflammation after completion of wound healing. Coagulation is required after inflammation to eliminate the infection, as was nicely postulated by Mackman (Mackman, 2009).

Thus, since defects in coagulation lead to complications in a plethora of inflammatory conditions, knowledge of the regulatory mechanisms of coagulation factor production is of major importance for our understanding of vascular disease, cancer and other pathologies.

In general, the canonical view is that coagulation factor production is restricted to three compartments: the liver, where most coagulation factor production takes place, the endothelium, which is characterized, for example, by high levels of vWF, and the platelets, which despite being devoid of a nucleus seem to be capable of substantial coagulation factor production. No systematic investigation of the capacity of other cell types to produce coagulation factors has ever been undertaken. Hence, we decided to establish ultrapure human primary cell cultures including stem cells, endothelial cells (ECs), fibroblasts, macrophages, monocytes, preadipocytes, adipocytes hepatocytes and platelets to assess coagulation factor production.

MATERIALS AND METHODS

Nine human primary cell types were used in this study, including Stem cells, fibroblasts (epidermal), endothelial cells (Human Umbilical Vein Endothelial Cells = HUVEC), monocytes (CD14 cells), macrophages (monocytes differentiated by phorbol myristate acetate = PMA), preadipocytes (Lonza), adipocytes (preadipocytes differentiated to adipocytes) and hepatocytes (Becton & Dickinson (BD) Company). The cells were cultured in the presence or absence of LPS (200 ng/ml). Cells were exposed to LPS for 24 h. Subsequently, total RNA was collected. The mRNA expression of the prothrombinase complex (FV and FX), tenase

complex (FVIII), FVIII-stabilized protein (vWF), fibrin clot stabilizer (FXIIIA) and thrombin: thrombomodulin complex (THR:TM complex), which induces protein C, was measured in the 16 cell types (each expression was measured three times). Platelet-associated proteins, collected from a highly pure platelet population isolated by iodixanol gradient, were analyzed by LC-MS/MS as described by Birschmann *et al.* (Birschmann et al., 2008) (two independent isolations and two independent LC-MS/MS analyses were performed). Blood was collected from fasted healthy volunteers by venipuncture into tubes containing the anticoagulant EDTA. All healthy volunteers provided informed consent. Subsequently, different iodixanol concentrations were prepared and layered within 3-ml tubes. The gradients were then centrifuged. Highly purified Platelet fractions were isolated from the tubes by aspiration through a needle. Subsequently, Platelet -associated proteins were trypsinized at 37 °C for 24 h to convert proteins to peptides. After 12 h of incubation, 100 ng of trypsin was added to the fraction to ensure the complete conversion of proteins into peptides. After washing the peptide fraction through a C18 column, 5 μl of the purified Platelet -associated protein (peptide) fraction was assayed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). LTQ ORBITRAP-XL (Thermo Fisher) was used for MS.

This study was approved by the Medical Ethical Committee of the University Medical Center Groningen with the reference number METc2007.081).

RESULTS

To investigate whether other human cells, besides the canonical sites of coagulation factor production, express clotting factors(s), FV mRNA expression was measured. As depicted in Figure 2A, FV mRNA was expressed at high levels in human monocytes and in human hepatocytes, with hepatocytes, as the canonical site of FV expression, serving as a positive control. Remarkably, FV was not detected in other cells, including human macrophages, which are derived from monocytes (Figure 2A). Because FV is an FX cofactor, the expression of FX was assessed in all cells. As shown in Figure 2B, FX was only expressed in fibroblasts, visceral preadipocytes, and adipocytes, and the expression in these cells was as strong as in hepatocytes (positive controls). FVIII, another FX cofactor, was also analyzed. FVIII was expressed in all cell types except traces in endothelial cells (Figure 2C).

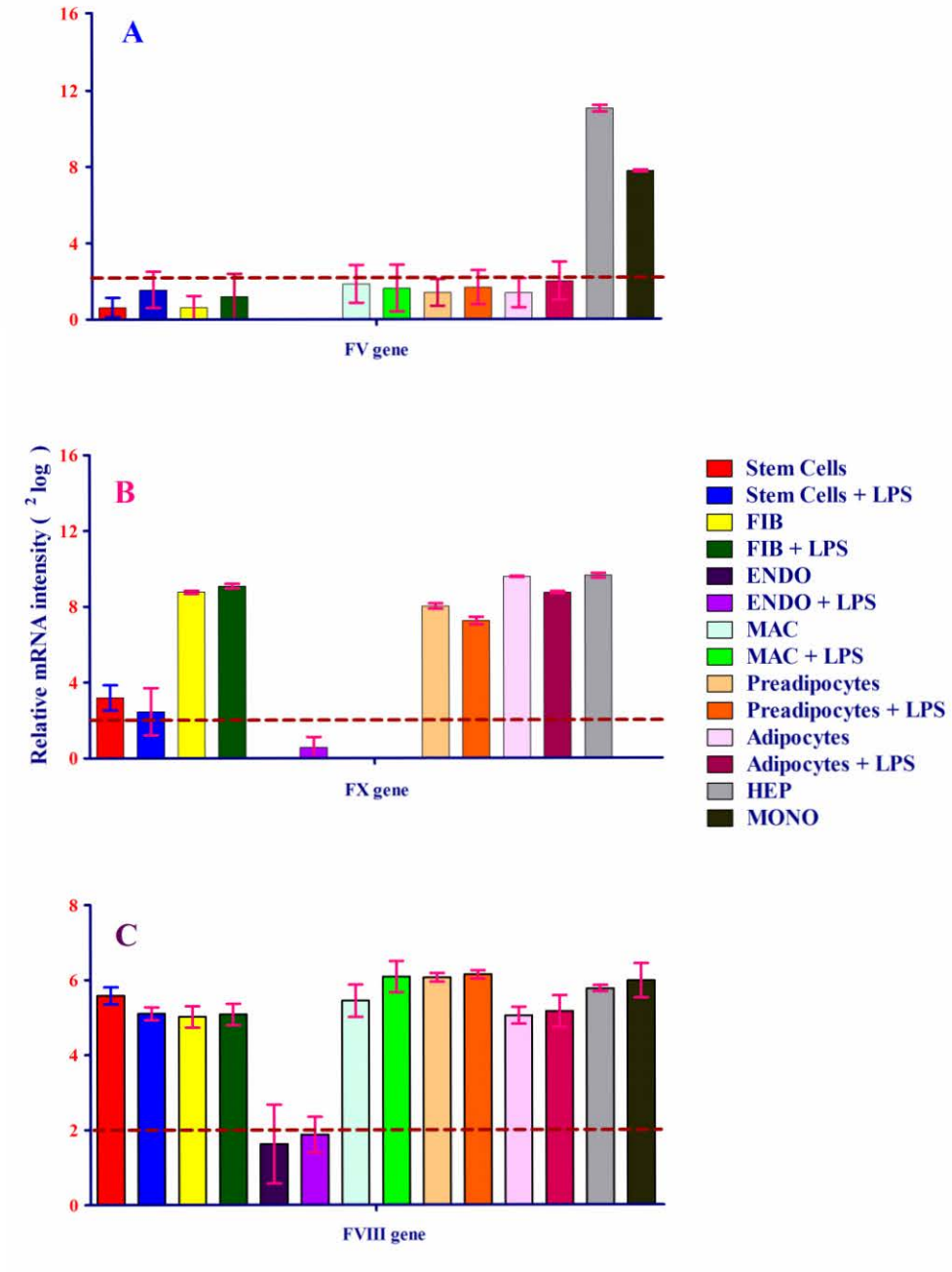

A
16
12
8
4
0
FV gene
B
Relative mRNA intensity (^{2}log)
FX gene
Stem Cells
Stem Cells + LPS
FIB
FIB + LPS
ENDO
ENDO + LPS
MAC
MAC + LPS
Preadipocytes
Preadipocytes + LPS
Adipocytes
Adipocytes + LPS
HEP
MONO
C
8
6
4
2
0
FVIII gene

Figure 2 A-C. Expression of FV (A), FX (B) and FVIII (C) by eight human primary cell types.

Cells include stem cells, fibroblasts, endothelial cells, macrophages, preadipocytes and adipocytes, hepatocytes and monocytes with and without exposure to LPS. The level of each mRNA was measured in triplicate.

Figure 2A shows the expression of the FV gene by human monocytes (dark grey bar) and hepatocytes (light grey bar). Monocytes and hepatocytes were not exposed to LPS. Hepatocytes were used as a positive control for coagulation factor expression. Human primary hepatocytes died upon exposure to LPS. Monocytes were not exposed to LPS because LPS treatment results in the differentiation of monocytes into macrophages. mRNA expression was expressed as ^{2}log values on the y-axis. Relative mRNA intensity was obtained from three measurements.

Figure 2B shows the expression of the FX gene by human fibroblasts, preadipocytes, adipocytes and hepatocytes. Gene expression was expressed as ^{2}log values on the y-axis. Relative mRNA intensity was obtained from triplicate. Hepatocytes served as a positive control for coagulation factor expression.

Figure 2C shows the expression of the FVIII gene by human primary cells. FVIII mRNA expression was expressed as ^{2}log values on the y-axis. FVIII mRNA expression was observed in all cells tested except in endothelial cells. Relative mRNA intensity was obtained from three measurements. Hepatocytes served as a positive control for coagulation factor expression.

vWF forms a complex with FVIII, which stabilizes FVIII. As FVIII is also a member of the tenase complex, the expression of vWF was also investigated in all nine human primary cell populations. As shown in Figure 3A, vWF expression was very high in endothelial cells. In addition, mRNA analysis showed that vWF was also synthesized by fibroblasts, macrophages, preadipocytes, hepatocytes and monocytes; however, the expression of vWF

was inhibited in preadipocytes upon exposure to lipopolysaccharide (LPS) (Figure 3A).

After analyzing these coagulation factors that activate prothrombin (inactive form of thrombin), the mRNA expression of FXIIIA, a fibrin clot stabilizer, and that of thrombomodulin, a potent thrombin cofactor, were studied. As displayed in Figure 3B, FXIIIA was synthesized at a high level by monocyte-derived macrophages and monocytes, and was weakly expressed by hepatocytes. FXIIIA expression was not detected in other cells (Figure 3B).

The mRNA expression of thrombomodulin was very high in bone marrow-derived atem cells, as depicted in Figure 3C. In addition, thrombomodulin was strongly expressed in endothelial cells, macrophages, monocytes and, to a lesser extent, in fibroblasts; however, as with FXIIIA expression in macrophages, thrombomodulin expression was inhibited after exposure of macrophages to LPS. No thrombomodulin expression was detected in visceral preadipocytes, adipocytes or hepatocytes.

Figure 3 A-C.

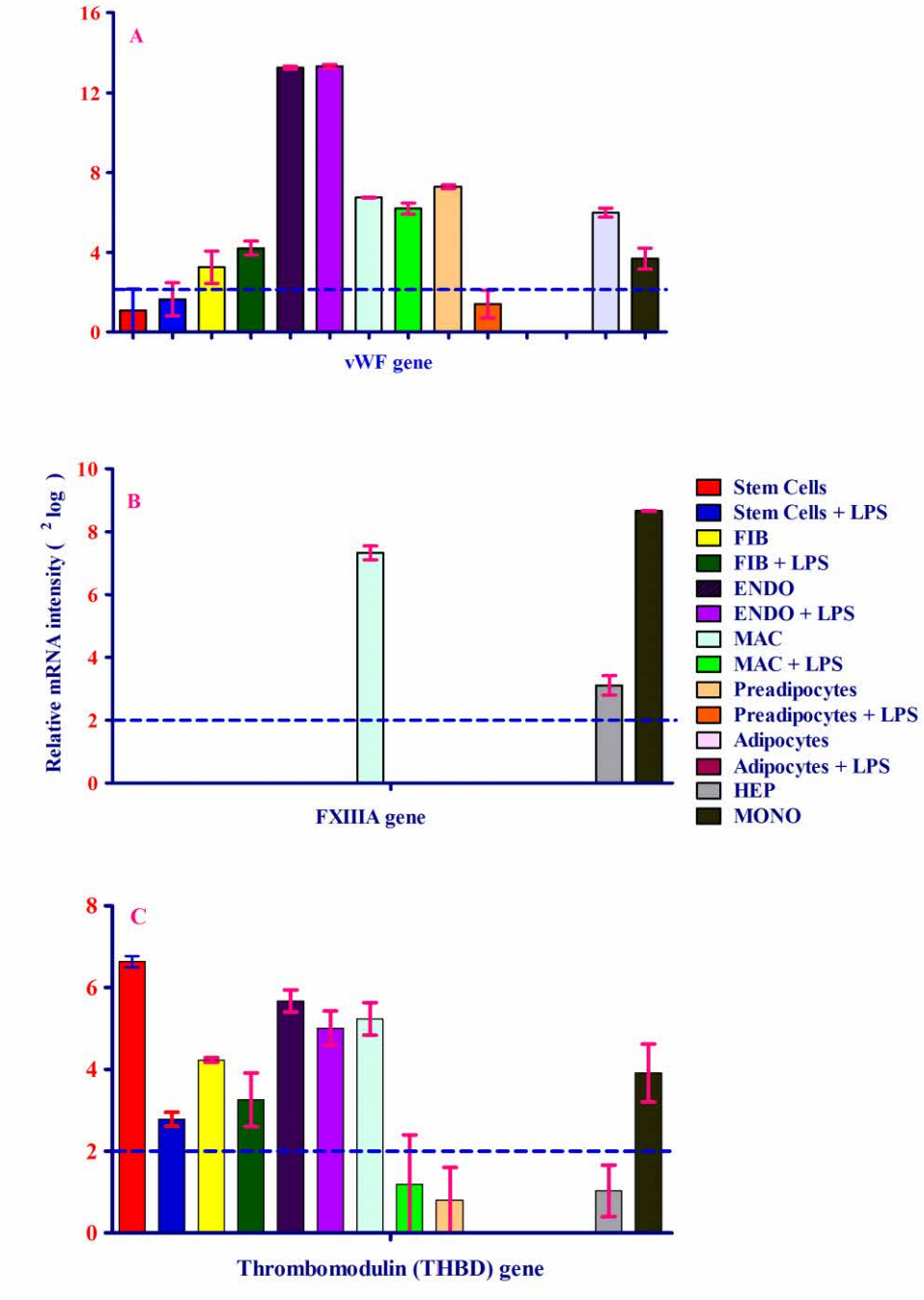

A
16
12
8
4
0
vWF gene
B
Relative mRNA intensity (2 log)
10
8
6
4
2
0
FXIIIA gene
Stem Cells
Stem Cells + LPS
FIB
FIB + LPS
ENDO
ENDO + LPS
MAC
MAC + LPS
Preadipocytes
Preadipocytes + LPS
Adipocytes
Adipocytes + LPS
HEP
MONO
C
8
6
4
2
0
Thrombomodulin (THBD) gene

Figure 3. mRNA expression of vWF, FXIIIA and Thrombomodulin in eight human primary cell types treated with or without LPS.

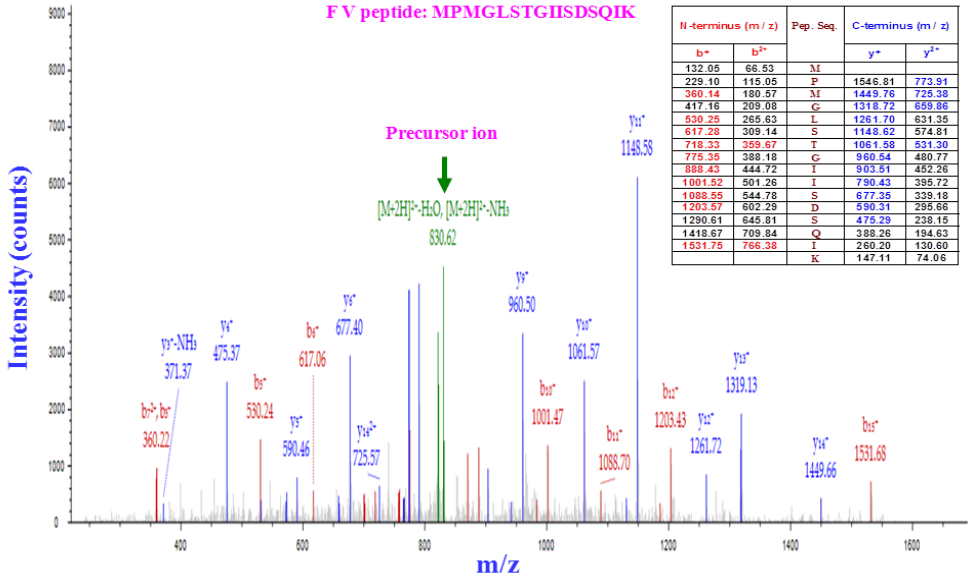
Figure 3A vWF mRNA expression was expressed as ^{2}log values on the y-axis. vWF mRNA expression was detected in all cells tested, except preadipocytes and adipocytes. The highest level of expression of vWF mRNA was observed in endothelial cells. Relative mRNA intensity was obtained from three measurements. Hepatocytes served as a positive control for coagulation factor expression. The level of each mRNA was measured three times.

Figure 3B. mRNA expression of FXIIIA in eight human primary cell types treated with or without LPS. FXIIIA mRNA expression was synthesized only by macrophages and monocytes. LPS inhibited mRNA expression in macrophages. FXIIIA mRNA expression was expressed as ^{2}log values on the y-axis. Relative mRNA intensity was obtained from three measurements. Hepatocytes served as a positive control for coagulation factor expression. The level of each mRNA was measured three times.

Figure 3C. mRNA expression of thrombomodulin (TM) in eight different human primary cells treated with or without LPS. Thrombomodulin mRNA expression was observed in all cells except hepatocytes, preadipocytes and adipocytes (^{2}log lower than or close to 1). Thrombomodulin mRNA expression was inhibited by LPS. Thrombomodulin mRNA expression was expressed as ^{2}log values on the y-axis. Relative mRNA intensity was obtained from three measurements. Hepatocytes served as a positive control for coagulation factor expression. The dashed line represents the threshold.

To gain more knowledge regarding the expression of coagulation factors by other cells, human platelets were screened for the expression of coagulation factors. platelets were isolated by iodixanol gradient centrifugation and the expression of coagulation factors was assessed by LC-MS/MS. Orbitrap LTQ-XL (LC-MS/MS) analysis showed that platelets synthesize FV, FXIIIA and vWF, as shown in Figure 4, 5 and 6 respectively.

Figure 4.

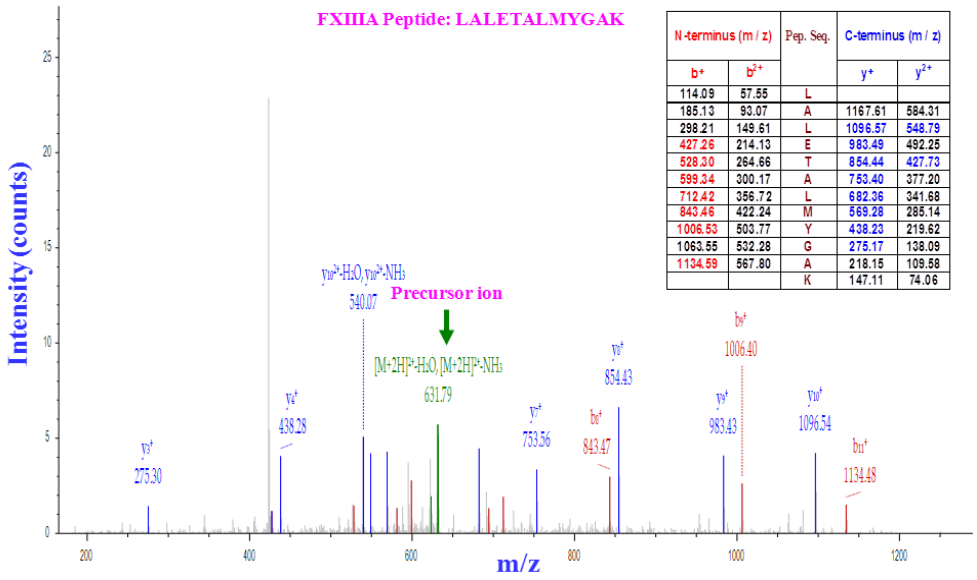


N-terminus (m/z)		Pep. Seq.	C-terminus (m/z)	
b+	b2+		y+	y2+
132.05	66.53	M		
229.10	115.05	P	1546.81	773.91
360.14	180.57	M	1449.76	725.38
417.16	209.08	G	1318.72	659.86
530.25	265.63	L	1261.70	631.35
617.28	309.14	S	1148.62	574.81
718.33	359.67	T	1061.58	531.30
775.35	388.18	G	960.54	480.77
888.43	444.72	I	903.51	452.26
1001.52	501.26	I	790.43	395.72
1088.55	544.78	S	677.35	339.18
1203.57	602.29	D	590.31	295.66
1290.61	645.81	S	475.29	238.15
1418.67	709.84	Q	388.26	194.63
1531.75	766.38	I	260.20	130.60
		K	147.11	74.06

Figure 4. Proteomic analysis of FV synthesis by platelets.

After one-step iodixanol gradient centrifugation, purified platelet fractions were collected and subjected to LC-MS/MS (LC-Tandem mass spectrometry). Results are displayed graphically with the intensity expressed as the ion count (y-axis) and plotted against the m/z ratio of the ions (x-axis). The precursor ion scan mass spectrum of the FV tryptic digest resulted in an m/z of 830.62 as shown in green. The MS/MS product ion spectrum obtained from the fragmentation of this precursor ion at m/z 832•62 with b-ions (red peaks) and y-ions (blue peaks) corresponds to the sequence **MPMGLSTGIISDSQI** from the tryptic FV peptide **MPMGLSTGIISDSQIK,** as shown in the table (**Figure 4, inset**). Five peptides matched the FV protein sequence. Each sample was subjected twice to LC-MS/MS.

Figure 5.



N-terminus (m / z)		Pep. Seq.	C-terminus (m / z)	
b^+	b^{2+}		y^+	y^{2+}
114.09	57.55	L		
185.13	93.07	A	1167.61	584.31
298.21	149.61	L	1096.57	548.79
427.26	214.13	E	983.49	492.25
528.30	264.66	T	854.44	427.73
599.34	300.17	A	753.40	377.20
712.42	356.72	L	682.36	341.68
843.46	422.24	M	569.28	285.14
1006.53	503.77	Y	438.23	219.62
1063.55	532.28	G	275.17	138.09
1134.59	567.80	A	218.15	109.58
		K	147.11	74.06

Figure 5. Proteomic analysis of FXIIIA synthesis by platelets.

After one-step iodixanol gradient centrifugation, purified platelet fractions were collected and subjected to LC-MS/MS (LC-Tandem mass spectrometry). Results are displayed graphically with the intensity expressed as the ion count (y-axis) and plotted against the m/z ratio of the ions (x-axis). The precursor ion scan mass spectrum of the FXIIIA tryptic digest resulted in an m/z of 631.79 as shown in green. The MS/MS product ion spectrum obtained from the fragmentation of this precursor ion at m/z 931•79 with b-ions (red peaks) and y-ions (blue peaks) corresponds to the sequence L**ALETALMYGA** from the tryptic FXIIIA peptide **LALETALMYGAK,** as shown in the table (**Figure 5, inset**). Five peptides matched the protein sequence of FXIIIA. Each sample was subjected twice to LC-MS/MS.

Figure 6.

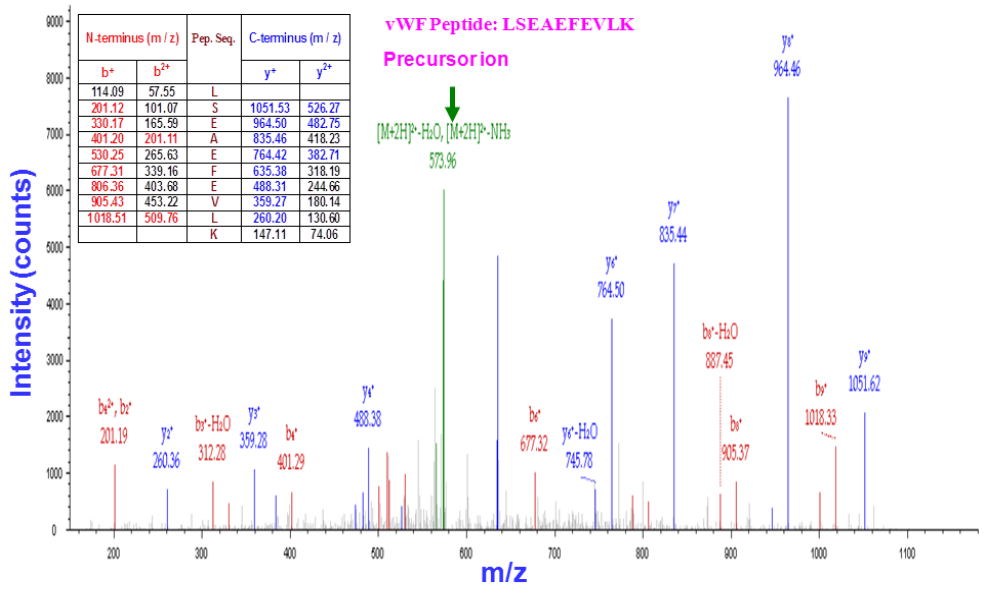


Figure 6. Proteomic analysis of vWF expression by platelets.

After one-step iodixanol gradient centrifugation, purified platelet fractions were collected and subjected to LC-MS/MS (LC-Tandem mass spectrometry). Results are displayed graphically with the intensity expressed as the ion count (y-axis) and plotted against the m/z ratio of the ions (x-axis). The precursor ion scan mass spectrum of the vWF tryptic digest resulted in an m/z of 573.96, as shown in green. The MS/MS product ion spectrum obtained from the fragmentation of this precursor ion at m/z 573•96 with b-ions (red peaks) and y-ions (blue peaks) corresponds to the sequence L**SEAEFEVL** from the tryptic vWF peptide **LSEAEFEVLK,** as shown in the table (**Figure 6, inset**). Six peptides matched the sequence of vWF. Each sample was subjected twice to LC-MS/MS.

DISCUSSION

The classical view of coagulation factor production holds that production of these factors is restricted to three compartments in order of importance: the liver, the endothelium and platelets. To investigate whether other cells are novel sites of expression of FV, FVIII, vWF and FX, the mRNA expression of these factors was investigated in nine human primary cell populations before and after exposure to LPS, with the exception of monocytes and hepatocytes which were not studied after LPS exposure. Human hepatocytes cannot survive exposure to LPS, and the exposure of monocytes to LPS results in their differentiation into macrophages. Human primary hepatocytes served as a positive control in our experiments. LC-MS/MS-based proteomic analysis of a highly purified human platelet population, isolated by iodixanol gradient centrifugation, was also performed.

As shown in **Figures 2A–C** and **3A–C,** coagulation factor expression was highly cell type-restricted and the liver and, to a lesser extent, the endothelium and platelets was responsible for the production of coagulation factors and modulators such as FVIII, vWF and thrombomodulin. Thus, the canonical view that restricts coagulation factor production to these three cell types appears to be correct; however, important discrepancies from the canonical interpretation of human coagulation factor production were observed for FV, FX and thrombomodulin.

Since FV is the most important FX cofactor and is expressed only by monocytes and hepatocytes (positive control cells), mutations resulting in the absence or dysfunction of activated FV (FVa) lead to hemorrhagic disease; likewise, mutations resulting in excessive longevity of the active mutations are associated with thrombosis. In particular, the R506Q mutation

(also known as FV Leiden) is associated with thrombophilia and activated protein C (APC) resistance, and is a significant clinical problem. Since monocytes expresses FV and bone marrow produces monocytes, congenital problems in FV production may be rationally treated by allogeneic bone marrow transplantation.

Although the formation of fibrin is crucial during vascular injury, a rapid response and acute fibrin clot formation are essential for the prevention of bleeding and for vascular wound healing. Since we showed that coagulation factor FV is expressed by monocytes, monocytes can no longer be considered merely as a membrane surface for activated coagulation factors to be primed by tissue factor: monocytes should be considered as "emergency" cells directly involved in the FX activation (FXa) via FV delivery. FV can also be activated by elastase and cathepsin G, which are present in monocytes (Allen and Tracy, 1995). In this context, a sharp increase in thrombin generation is expected, and this increase is predicted to continue until the wound is repaired.

Interestingly, almost all components except FV are present in plaques to form prothrombinase complex. Notably, monocytes and platelets are only circulating cells, which supply FV to activate FX. monocytes are not tissue resident cells, but circulating monocytes that express FV, however, when monocytes cross the endothelium, as shown in **Figure 1**, they differentiate into macrophages (tissue cells) and lose FV expression. Since coagulation factors need to be released into the blood, the expression of FV by circulating monocytes suggests that an important function of monocytes may be to maintain FV plasma levels. The possibility that monocytes are a main source of FV in plasma may explain why previous attempts at rectifying genetically aberrant FV expression with liver transplantation

failed. This suggests that co-transplantation of bone marrow (which produces monocytes) could constitute a viable therapeutic approach.

Lipids, collagens, calcium, and inflammatory cells are well-established components of atherosclerotic plaques (Rogers et al., 2000). In addition, fibroblasts play an important role in wound healing via synthesis of collagen and extracellular matrix (Midwood et al., 2004). It is fascinating that the expression of FX is absent in cells that express FV, and *vice versa* (hepatocytes are not in direct contact with blood vessels, hence these cells were excluded from this observation) (**Figure 2A and B**). We propose naming this phenomenon the *FV/FX expression law*. Proteomic analysis of highly purified platelets from human plasma revealed expression of FV and vWF, but not FVIII or FX. Intriguingly, both monocytes and platelets express FV and vWF, but not FX. The previously established expression of FV and vWF by platelets (Alberio et al., 2000; Allen and Tracy, 1995; Etingin et al., 1993; Mackman, 2009; Nesheim et al., 1982) was confirmed in our study. Although FVIII was detected in all cells tested, surprisingly only a trace amount of FVIII expression was observed in endothelial cells. Monocytes are different from fibroblasts and macrophages with respect to the expression of FV and FX. According to the *FV/FX expression*, FV is a direct and crucial cofactor for FX activation. Moreover, fibroblasts and macrophages are both present in tissue and matrix; fibroblasts express FX but macrophages do not express FV. Monocytes and monocyte-derived macrophages show differences only in the expression of FV and FX among all the coagulation factors tested. These data indicate that the formation of the prothrombinase complex is highly regulated and rapid.

Since human platelets and endothelial cells do not express FVIII, monocytes appear to be the only source of FVIII at the site of vascular injury, where FVIII forms a complex with vWF released by endothelial cells, platelets

and/or monocytes. These results reinforce the notion that monocytes may be directly involved in the process of coagulation. Since FXIIIA (Hevessy et al., 1996; Muszbek et al., 2007) plays an unambiguous role in the stabilization of the fibrin clot during wound healing and because thrombomodulin, an anticoagulant, functions in a complex with thrombin and is important for the degradation and lysis of fibrin after vascular repair, cells were also screened for the expression of FXIIIA and thrombomodulin. FXIIIA mRNA was expressed by monocytes, and proteomic analysis of highly purified platelets revealed that these cells also synthesized FXIIIA protein. It has already been established that FXIIIA plays a highly important role in the stabilization of the fibrin clot by crosslinking fibrin (Hevessy et al., 1996; Muszbek et al., 2007). Notably, both platelets and monocytes (Poon et al., 1989) express FXIIIA and a logical explanation for this novel finding could be that these two cell types supply FXIIIA for the crosslinking of fibrin. The expression of FXIIIA by these two cell types also suggests that monocytes and platelets are not only involved in priming coagulation but also take part in the final event of fibrin stabilization.

Interestingly, this study also showed that monocytes and macrophages, but not platelets, expressed thrombomodulin. Thrombomodulin has been shown to be present on the surface of endothelial cells (Esmon and Owen, 1981; Esmon et al., 1982; Esmon et al., 1983). Based on these data, we suggest that both platelets and monocytes arrive at the site of injury and synergistically initiate primary hemostasis and, as a final event, stabilize the fibrin clot to prevent bleeding and repair the wound. Thus, platelets appear to have a function in the coagulation process, but because of the synthesis of thrombomodulin by monocytes, these monocytes appear to deliver additional thrombomodulin to complex with thrombin and activate protein C after completion of wound healing.

The data obtained in this study suggest that the classical hypothesis needs to be revised and that a new hypothesis (see Figure 1) for vascular injury and coagulation is required. Monocytes have a direct and dual role in coagulation and in the up-regulation of APC via forming of thrombin:thrombomodulin (THR:TM complex). The first function of monocytes is to express FV, FVIII and FXIIIA to ensure optimal activation of FX and stabilization of fibrin. The second function of monocytes, which occurs after the interruption of vascular bleeding and completion of wound healing by both platelets and monocytes, is to produce thrombomodulin: monocyte-derived thrombomodulin forms a complex with thrombin (THR:TM complex), which destabilizes the fibrin clot by breaking down FXIIIA and, in turn, activates protein C by approximately 1000-fold (Esmon et al., 1982). This reaction prevents further formation of fibrin and FV by inhibiting the thrombin-mediated activation of these two coagulation factors. It is assumed that these reactions need to be very rapid. These data suggest that monocytes provide thrombomodulin as a potent thrombin cofactor in the APC negative feedback cycle to inhibit thrombin formation and thus its function. It must be noted that the THR:TM complex can only be formed after bleeding has stopped at the site of vascular injury.

Moreover, the THR:TM complex abolishes the activation of platelets by inhibiting thrombin, as shown by Esmon *et al.* (Esmon and Owen, 1981; Esmon et al., 1982; Esmon et al., 1983). It is also very interesting to note that both FXIIIA and thrombomodulin mRNA levels retuned to the baseline level after exposure to LPS. The response of the coagulation factors to LPS indicates that they are biologically active. The reduction of both FXIIIA and thrombomodulin upon exposure to LPS suggests complex formation or protein-protein interactions between FXIIIA and thrombomodulin. Taken

together, these data suggest that these coagulation factors are constitutively expressed by these human primary cells.

Overall, our data show that the canonical view of coagulation factor production needs to be revised and that many more cell types are involved in this process. This study provides evidence, for the first time, for a novel role for monocytes in the coagulation system. In addition, our results point to the existence of an *FV/FX expression*, and open a new chapter in our understanding of the coagulation process that could potentially lead to advances into the investigation of the development of atherothrombosis.

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Chapter 7
General Summary and Discussion

Monireh Dashti [1,2], Maikel P. Peppelenbosch [2]
Farhad Rezaee [1]

[1] Department of Cell Biology, UMCG, University of Groningen, The Netherlands
[2] Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands

Triglyceride overload of the lipid droplets in adipocytes results in hypertrophy of these cells.This is the main characteristic of obesity, which is considered the risk number one of health world-wide and its afflictions such as type 2 diabetes mellitus (T2DM). A disruption in energy metabolism provokes a low grade chronic inflammation in adipose tissue (AT), subsequently fostering pathophysiological diseases such as insulin resistance (IR) and T2DM (Guilherme et al., 2008). Based on classical view, necrotic adipose tissue adipocytes (ATA) activates adipose tissue macrophages (ATM), which in turn are the major mediators of the inflammation state (Ye and Keller, 2010). Through this thesis I contribute to defining a novel hypothesis, which is recently gaining ground: 1- Adipocytes synthesize and secret immune-associated components (Nishimura et al., 2009) 2- Metabolic dysfunction in adipose tissue contributes to obesity (Guilherme et al., 2008; Wajchenberg, 2000) 3- The adipocytes of obese prime inflammation via their immune functionality (Meijer et al., 2011) 4- The landscape effect of the inflamed adipose tissue drives residential macrophages to acquire activated macrophage characteristics (Balistreri et al., 2010; Guilherme et al., 2008) 5- The activated (also called migrated) macrophages establish a vicious circle that exacerbates the inflammatory pattern in the adipose tissue started by adipocytes (Meijer et al., 2011). The proinflammatory cytokines production of inflamed adipose tissue has two main pathological features responsible for two basic medical problems in the body: 1. Energy metabolism disorders in the adipocytes, which lead to an increased release of free fatty acids (FFAs) to the circulation, resulting in dyslipidemia and atherosclerosis (Wellen and Hotamisligil, 2005; Ye and Keller, 2010). 2- Interaction with the insulin pathway and induction of insulin resistance and hyperglycemia (Xu et al., 2003). The proinflammatory cytokines released to the circulation

produce systemic chronic inflammation in the peripheral tissues and consequently insulin resistance in these tissues (Guilherme et al., 2008). Insulin resistance in the skeletal muscle, as the main energy consumer in the body, is the main reason of hyperglycemia. The systemic inflammation also induces endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) production, which enhance the insulin resistance (Hotamisligil, 2006; Wellen and Hotamisligil, 2005; Ye and Keller, 2010). Importantly, the mechanisms responsible for induction of insulin resistance remain poorly understood, but the constitutive insulin production by the adipocytes, I describe in this thesis, will reduce the relative size of changes in insulin levels in the blood as provoked by the endocrine pancreas and thus certainly a factor here (chapter 5).

The two main pathophysiological properties that determine differences in the susceptibility of individuals to obesity include the interaction of genetic background with the environment (e.g. gene-environment and gene-gene interaction) (Guilherme et al., 2008; Hummasti and Hotamisligil, 2010; Wellen and Hotamisligil, 2005). Adipose tissue is considered as the main key to the regulation of the energy metabolism in body (Watt and Spriet, 2010). Dysregulation of this energy metabolic system is the basis of the age-related diseases, including metabolic syndrome, atherosclerosis, osteoporosis, neurodegeneration, stem cell malfunction, and cancer (Balistreri et al., 2010; Saely et al., 2012; Ye and Keller, 2010) and these influences have been investigated ad nauseam. A neglected factor in the etiology of the age-related diseases, however, is hedgehog signaling. Hedgehog signaling is able to control energy metabolic system via influence on all cells of the adipose tissue like adipocytes (Cousin et al., 2007; Dwyer et al., 2007; James et al., 2010; Moldes et al., 2003), fibroblasts (Choi et al., 2010; Takebe et al., 2011; Zeisberg et al., 2007), stem cells (Fontaine et al.,

2008), and endothelial cells (Kanda et al., 2003; Luo et al., 2009). In this thesis, I propose it to constitute the first real anti-geriatric factor (Al-Aly et al., 2007; Dang et al., 2002; Guan et al., 2009; Omoigui, 2007; Schieffer et al., 2004; Shao et al., 2007; Shao et al., 2011; Thomas et al., 2000). Importantly, adipose tissue is a main source of Indian hedgehog in the body and loads it on VLDL (Queiroz et al., 2010).

Since adipose tissue is the major organ in the storage of energy and tightly under balance, an energy metabolism disorder affects this tissue as first tissue target and makes it highly vulnerable to damage (Watt and Spriet, 2010). Thus, adipose tissue plays an important role in whole-body energy regulation (Guilherme et al., 2008). Adipose tissue is composed of many different cell types with a continuous communication mediated by many factors such as AMP-activated protein kinase-(AMPK)-LKB1signaling and insulin signaling between these cells to maintain a regulated function for adipose tissue in a normal state. Also, AMPK activity and to a lesser extent in Akt/PKB guide almost all energy-dependent processes in adipose tissue and that modulation of activity of the nutrient signaling offers a wide range of option to deal with metabolic diseases (Lim et al., 2010; van et al., 2011). Preadipocytes and adipocytes constitute 10-15% and 65-75% of the cells in adipose tissue respectively (Otto and Lane, 2005; Tchkonia et al., 2007).

In the normal state, the preadipocyte fraction of adipose tissue is continuously stand-by to differentiate, if required, to adipocytes throughout life. The studies in this thesis describing the differences in coagulation factor production between pre-adipocytes and adipocytes further add to the massive support that the functionality of both cell types is fundamentally different. Preadipocytes have a closer genome profile to the immune cells than adipocytes and therefore they are considered as the linker between the metabolic and immune system in the adipose tissue (Charriere et al., 2003).

Plasma lipoproteins (PLPs) are considered as a vehicle to transport lipids (cholesterol, triglycerides, and cholesterol esters) in our circulation. Chylomicrons, very low-desity lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) are the major constituents of Plasma lipoproteins. Plasma lipoproteins are functionally tightly regulated to maintain a strict homeostatic balance between lipoproteins. Disturbances in plasma lipoprotein homeostasis result in dyslipidemia, e.g. hypertriglyceridemia, hypercholesterolemia, and hyperlipidemia (Wung et al., 2006), which in turn have serious pathophysiological consequences like obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (Brunzell et al., 2008). In addition, VLDL plays an important role in the delivery of free fatty acid (FFA) to adipose tissue to store energy as inactivefuel in the form of triglycerides in the lipid droplets (LD) within adipocytes or supply active energy for skeletal muscles and other tissues via FFA delivery. In addition, these structures seem capable of transporting hydrophobic hormones through the aqueous environment, Hedgehog (see above) a major example. Progress in this field was hampered by lack of insight into the constituents of these particles. The studies described in this thesis on plasma lipoproteins phospholipid composition and the protein constituents of VLDL and LDL will facilitate future research.

Indeed, since plasma lipoproteins structure and pathophysiological character are similar to those of lipid droplets (LDs) (Cermelli et al., 2006; Farese, Jr. and Walther, 2009; Guo et al., 2009), we also studied the composition of plasma lipoproteins. Both Plasma lipoproteins and LDs contain a hydrophobic core containing cholesterol (C), triglycerides (TG), and cholesterol esters (CE), which are surrounded by hydrophilic monolayer membrane of phospholipids (PL). Thus the only differences observed between plasma lipoproteins and LDs are restricted to the protein fraction,

which consist of LD coating proteins involved in the balancing lipolysis and adipogenesis e.g. prilipin, adipophilin, adipose triglyceride lipase (ATGL), fatty acid binding protein-4 (FABP-4), and many other proteins (Ray et al., 2009). The proteins, which are present on LDs and in cytoplasm of all cells and in particular adipocytes (can be divided in two groups with respect to function: 1- the proteins, which protect LDs from breakdown (e.g. prevent hydrolysis) such as perilipin (constitute 80% of LDs) and FABP-4 and 2- proteins, which stimulate the breakdown of LDs. The former proteins appeared to be directly associated with LDs and latter proteins seems to translocate from cytoplasm towards LDs such as hormone sensitive lipase (HSL) and adipose triglyceride lipase, after appropriate stimulation (Brasaemle, 2007; Chung et al., 2006; Ducharme and Bickel, 2008; Ito et al., 2010; Meijer et al., 2011; Puri et al., 2007; Puri and Czech, 2008; Puri et al., 2008; Ray et al., 2009; Zhou et al., 1999). Apolipoproteins play a major role for the normal and regulated function of plasma lipoproteins (Mahley et al., 1984; Shachter, 2001; Wroblewska, 2009). Apolipoprotein A-I and apolipoprotein B are the major proteins for HDL and triglyceride-enriched lipoproteins (e.g. Chylomicrons, VLDL and LDL) respectively (Davidson and Thompson, 2007; Olofsson et al., 2007). VLDL is able to stimulate coagulation via extrinsic pathway (contact pathway) and activate coagulation factor VIII via the intrinsic coagulation pathway (Olufadi and Byrne, 2006; Silveira et al., 1994). Thus adipose tissue is intimately involved in the regulation of coagulation at many levels, not only through production of the factors involved (chapter 6) but also by their relationship with plasma lipoproteins. Since both PLPs and adipose tissue -associated cells, e.g. endothelial cells, fibroblasts, macrophages and adipocytes as well as bone-derived stem cells, play a role in the stimulation and activation of coagulation cascade, we also decided to screen major coagulation proteins

in these PLPs and adipose tissue-associated cells as well as many other cells (chapters 6). I expect that the non-caniconical production of coagulation factors further helps to explain the relative poor results obtained by treating genetic deficiency for coagulation factors with liver transplantation.

Although the main function of LDL and VLDL lies in the shuttling of lipids between liver, adipocytes and the other tissues, their role in protein transport has recently gained increasing attention. An in depth analysis was executed to screen proteins and major phospholipids associated with VLDL, LDL and HDL, using nLC-MS/MS (chapters 3 and 4). We identified presence of 95 VLDL and 51 LDL associated proteins including all known apolipoproteins and lipid transport proteins, and intriguingly a set of coagulation, complement system and antimicrobial proteins. Prothrombin, protein S, fibrinogen γ, PLTP, CETP, CD14 and LBP were present on VLDL but not on LDL. Prenylcysteine oxidase 1, dermcidin, Cathelicidin antimicrobial peptide, TFPI-1 and Fibrinogen a chain were associated with both VLDL and LDL (chapter 4). Although we have observed differences between VLDL and LDL with respect to proteins, the majority of aopliporteins and proteins involved in lipid transport and metabolism were conserved in all lipoproteins. We have also found that major phospholipids are conserved in lipoproteins as well. This study provides a catalogue of protein constituents of the human plasma lipoprotein network and provides strong support for the notion that protein shuttling through this system is involved in the regulation of biological processes such as coagulation, atherosclerosis, defense against bacteria and energy metabolism. Since phosphatidylethanolamine (PE) and phosphatidylinositol (PI) are present specifically on lipoproteins, it is then possible to manipulate plasma lipoproteins specifically and selectively as well.

The role of adipocytes in energy metabolism is clear and an excessive energy storage in adipocytes LDs leads to the hypertrophy of these cells, which is the main cause of obesity and its associated morbidity and mortality. However, the link behind adipocytes and insulin regulation is not clear. Hence, we have measured the insulin production at both gene and protein levels in human primary subcutaneous and visceral preadipocytes and adipocytes. As determined by mRNA Beadarrays, insulin mRNA expressed in all four tested cell types, which is also confirmed by immunofluorescent confocal laser scanning microscopy (IFCLSM) and Immunoelectron microscopy at protein levels. Importantly, huge insulin protein expression was observed in human primary visceral and subcutaneous adipocytes but not in preadipocytes, which appeared to be due to the lack of LDs (chapter 5). Plasma insulin levels were significantly higher in three groups of obese subjects (e.g. T2D, T2D receiving insulin, and obese subjects) than lean subjects. After bariatric surgery the BMIs were highly decreased from 36-47 to 26-33 kg/m^2 in obese subjects with T2DM receiving insulin injection. These patients do not receive any more insulin injection. A high and low insulin production (mRNA and protein) were found in fat tissues of pancreatic beta-cells destruction and control Prediabetes BB rats respectively. A fat mass reduction via bariatric surgery heals T2DM and a high insulin production in fat tissues of Prediabetes rats with destructed beta-cells. These phenomenones ensure a clear physiological role for adipose tissue insulin production. This study opens a novel avenue towards T2DM therapy.

As we expected, the synthesis of coagulation factors were not restricted to the classical tissues (e.g. liver, endothelial cells and platelets). Both monocytes and platelets express FV, FXIIIA and vWF, whereas FX mRNA were expressed only (except hepatocytes) by fibroblasts, Preadipocytes and

adipocytes. In addition, monocytes express FVIII and thrombomodulin. All cells express FVIII except endothelial cells, which express traces amount of FVIII. Fibroblasts, endothelial cells, and macrophages synthesize TM, but not FV. Since monocytes and platelets express FV and FXIIIA, both cells prime coagulation activation and stabilize the fibrin clot. Based on our results, each vascular cell that expresses FV does not synthesize FX and *vice versa*. We called this phenomenon *FV/FX expression*. After wound healing, fibrin clot could be destabilized via break down of FXIIIA by monocytes-supplied thrombomodulin. The thrombomodulin-thrombin complex also activates protein C, which removes the destabilized fibrin clot from circulation. This study provides evidence for the dual role of monocytes in coagulation/fibrinolysis systems (chapter 6). Our data also suggest that congenital problems in FV production can be rationally treated by allogeneic bone marrow transplantation.

In toto, this thesis touches on many aspects of adipose tissue physiology and its relation to the plasma lipoproteins compartment. A picture emerges that the adipocytes are a much more complicated in the context of biochemically, immunologically and endocrinologically active cell type and much more complicated as than we assumed earlier. Our observations may reflect the evolutionary origin of the adipocyte: no liver is present in invertebrates and the metabolic functionality of this organ in vertebrate metabolism is carried out by the adipose tissue. Whether this is the true background of the unexpected versatility of the adipocyte, but as a whole the current thesis would support such a view.

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Chapter 8
Appendixes

Nederlandse Samenvatting Voor Niet-Ingewijden

List of Publications

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Nederlandse Samenvatting Voor Niet-Ingewijden

Geachte lezer, in dit proefschrift heb ik aannemelijk pogen te maken dat het vetweefsel niet louter een passieve voorraad van reserves is, maar ook een belangrijk endocrien en immunologisch orgaan is dat uitgebreid communiceert met en zelfs in bepaalde mate aansturing geeft aan de andere organen van het lichaam. Dit laatste gebeurt onder andere via het zogenaamde plasma lipoproteïne compartiment, een groep van deeltjes die hydrofobe moleculen, zoals cholesterol, triglyceriden of steroide hormonen, door de waterige omgeving van het bloed kan transporteren. Het vetweefsel fungeert als een soort coördinatie-centrum voor het transport middels lipoproteïnen door het bloed, hydrofobe moleculen worden bijvoorbeeld uit de lipoproteïnen opgenomen en opgeslagen in het vetweefsel, terwijl weer andere hydrofobe biomoleculen op de lipoproteïnen geladen worden voor functies elders in het lichaam. De complexe metabole rol van het vetweefsel doet denken aan die van de lever, en inderdaad is in de meerderheid van het dierlijk leven geen aparte lever aanwezig en worden de functies die in ons lichaam de lever uitvoert volbracht door het vetweefsel (denk bijvoorbeeld aan insekten).

Ik ben dit proefschrift begonnen met een uitgebreide beschrijving en samenvatting van de literatuur m.b.t. tot de histologie en moleculaire biologie van het vetweefsel, hoe dit weefsel betrokken is bij het regelen van de energievoorziening van ons lichaam. Daarnaast ga ik uitgebreid in op ziektebeelden die aanpalend zijn m.b.t. het disfunctioneren van het vetweefsel, in het bijzonder type 2 diabetes maar ook vaatlijden. Deze ziektebeelden hangen in het bijzonder samen met obesitas en ik geef een korte samenvatting in het begin van dit proefschrift van hoe er tegen deze

samenhang wordt aangekeken. Ik denk echter dat zowel het belang van het vetweefsel voor fysiologie als pathologie als ook de complexiteit van de onderhavige interacties veelal onderschat wordt.

Dit laatste punt illustreer ik in hoofdstuk 2, waar ik in ga op Hedgehog signalen. Hedgehog is een belangrijk morphogen tijdens de embryonale ontwikkeling. Ook wordt onderkend dat in het volwassen organisme Hedghog signalen actief blijven terwijl recentelijk duidelijk is geworden dat voornamelijk het vetweefsel de hydrofobe Hedgehog moleculen blijft produceren en dit Hedgehog via het plasmalipoproteïne compartiment elders in het lichaam afgeleverd kan worden. Ik beargumenteer in een literatuurstudie dat Hedgehog productie door het vetweefsel een anti-geriatisch signaal geeft, dus dat in contrast tot de algemene gedachte dat Hedgehog vooral belangrijk is bij de start van het leven (de embryologische ontwikkeling) juist ook aan het einde van het leven een belangrijke functie heeft. In denk dat deze studie fraai illustreert hoe complex maar ook hoe belangrijk de functie van het vetweefsel en het geassocieerd lipoproteïne transport in de fysiologie is.

Om deze functionaliteiten verder in kaart te brengen ga ik in hoofdstuk 3 en hoofdstuk 4 gedetailleerd in op de samenstelling van het lipoproteïne compartiment. Ofschoon het belang van deze structuren voor ziekte en gezondheid al sinds jaar en dag onderkend wordt, is het merkwaardig onduidelijk wat zich nu precies in de verschillende lipoproteïnen bevind. Met behulp van de meest moderne biomics technieken probeer ik in dit proefschrift hierin verandering aan te brengen. In hoofdstuk 3 geef ik een volledige beschrijving van alle fosfolipide (fosfolipioom) bestandelen van de verschillende lipoproteïnen terwijl in hoofdstuk 4 alle eiwitten die terug te vinden zijn in het verylow and low-densitity lipoprotein (VLDL and LDL, de zogenaamde slechte cholesterol) worden gekarakteriseerd en

gerelateerd en fysiologie en pathofysiologie. Uit dit werk wordt het rijke spectrum aan biologische functies waarin het vetweefsel via het lipoproteïne compartiment betrokken is, verder duidelijk.

Maar het vetweefsel kan ook belangrijke niet-hydrofobe moleculen uitscheiden. In hoofdstuk 5 documenteer ik zorgvuldig dat het vetweefsel constitutief insuline produceert. Het gevolg is dat dikke mensen een hoog basaal niveau van insuline hebben, wat het moeilijker maakt voor de endocriene pancreas middels dynamische insuline secretie te reageren met een relevante verandering in insuline niveau op een verandering in de bloedglucosewaarde. Ik bediscussieer deze bevindingen daarom ook in de context van type 2 diabetes (insuline-resistente diabetes).

Een laatst voorbeeld van de complexe functie van het vetweefsel wordt gegeven in hoofdstuk 6. Hier laat ik zien dat niet alleen de lever een belangrijke bron is van stollingsfactoren (zoals algemeen gedacht), maar dat het vetweefsel hier ook een rol heeft. Wederom wordt het belang van het metabolisme in de vetcellen blijkbaar vaak onderschat.

In Hoofdstuk 7, worden een algemene samenvatting en discussie van de bevindingen uit dit proefschriftloop geprsenteerd. Het laatste hoofdstuk wordt dan beëindigd met appendix.

Uiteindelijk kader ik mijn bevindingen in m.b.t. de al aanwezige literatuur en ga dieper in op het werk wat nog noodzakelijk. Ik denk wel, lezer, dat ik u met dit proefschrift overtuigd heb van het belang van het vetweefsel in de energiehuishouding en fysiologie in haar algemeenheid. Ook hoop ik een eerste tip van sluier te hebben kunnen oplichten over hoe complex deze functie eigenlijk is.

List of Publications

Hedgehog Signaling as an Antagonist of Ageing and Its Associated Diseases

Monireh Dashti, Maikel P. Peppelenbosch and Farhad Rezaee

BioEssays,Volume 34, Issue 10, October 2012

A Phospholipidomic Analysis of All Defined Human Plasma Lipoproteins

Monireh Dashti, Willem Kulik, Frans Hoek, Enno C. Veerman, Maikel P. Peppelenbosch & Farhad Rezaee

Nature Scientific report, 7 November 2011

An In-depth Proteomic Analysis of Human Plasma Very Low-Density Lipoprotein (VLDL) and Low-Desity Lipoprotein (LDL)

Monireh Dashti1, Vishtase Khanzadeh, Clark J. Zeebregts, Arnold Spek, Eric Sijbrands, Maikel P. Peppelenbosch1, Farhad Rezaee

Under review

Non-classical Coagulation Factor Synthesis: A Study in Nine Human Primary Cell Types

Monireh Dashti, Vishtase Khanzadeh, Clark J. Zeebregts, Arnold Spek, Eric Sijbrands, Maikel P. Peppelenbosch, Farhad Rezaee

Under review

Human Adipocytes Hypertrophy Secrets Insulin.

Run Title: Bariatric Fat Loss Surgery Heals Type 2 Diabetes

Monireh Dashti, Behiye Özcan, Vishtaseb Khanzadeh, Felix Rooij, Arnold Spek, Saad Lahham, Johanes van der Want, Ajda Rowshani, Theo van Kooten, Rene Klaassen, Maikel P. Peppelenbosch, Eric Sijbrands and Farhad Rezaee

Under review

Donor Chimera Model for Tolerance Induction in Transplantation

Sub title: Organs of genetically modified animal fetus may be better for transplantation

Monireh Dashti, Maikel P. Peppelenbosch and Farhad Rezaee

Under review

Role of Adipose Tissue in Pathophysiology of Energy Metabolic System Disorders

Monireh Dashti, Maikel P. Peppelenbosch and Farhad Rezaee

In preparation

A Quick Look at Biochemistry; Respiratory System and Carbohydrates

Monireh Dashti

In preparation

Nutrient Signaling in Adipose tissue and Its Consequences for Metabolic Disease

Monireh Dashti, Maikel P. Peppelenbosch and Farhad Rezaee

Under review

Evolutionary Conserved Nutrient- and Immune- Homeostasis; Energy Metabolism System Disturbance and Atherosclerosis

Sub Sitle: Lipid, Hedgehog, Inflammatory and Coagulation Pathways are Involved in Atherosclerosis

Monireh Dashti

In preparation

Lipid Syndrome: A Novel Interpretation in Pathophysiology of Metabolic Diseases

Monireh Dashti

In preparation

Analgesia in Experimental Small Bowel Transplantation; Towards a Practical Protocol

M. Dashti Rahmatabady, A. Zandvoort, C. M. A. Thuring, A. Smit – v. Oosten, R. Ploeg, M. P. Peppelenbosch, H. Leuvenink, G. Dijkstra, V. Nieuwenhuijs.

Proceeding article of the XI International Small Bowel Transplant (September 2009, Bologna, Italy)

Curriculum Vitae

Dr Monireh Dashti Rahmatabadi was born on 25th October 1971 in Tehran-Iran. She started her Medical study in Mashhad University of Medical Science (MUMS), between September 1992 and Augustus 1999. The subject of her thesis book was "Study of pityrosporum ovale prevalence among the patients with acne vulgaris in comparison with control group" under supervision of Dr. F. Pezeshkpour. She had 3 years experiences of medical work as a General Practitioner in clinic afterward. She had been started her PhD study in 15th January 2008 till 6th Sep 2012 in Immunology department under supervision of Prof. Dr. Maikel Peppelenbosch on different subjects including ubiquitination and Rac1 activity in crohn's disease, comparison of the sedation protocols of Paracetamol and Temgesic medications in rats. My main project, which is the contents of this book on the adipose tissue, coagulation and lipoproteins and hedgehog signaling subjects started from 29th June 2009 under direct supervision of Dr F. Rezaee. The main theme of these subjects is energy metabolism and the age-related diseases mainly metabolic syndrome (MS) and its related complications including type 2 diabetes and atherothrombosis. The results of these studies are published (or under review) as different experimental and review papers, which are listed at the end of this book. The lab experiences, which is earned during this period are cell culturing, tissue staining (electronic and confocal microscopy), mRNA extraction, Elisa, western blotting, immmunoprecipitation, pull down assay, actin filament staining, monocyte isolation from blood, Mixed Lymphocyte Reaction (MLR), Rac1 activity assay and in lower level PCR, gel electrophoresis,

DNA extraction, designing the primer, Mass Spectrometry (MS) and FACS analysis using Calibur program.

Monireh Dashti Rahmatabadi,

6th september, 2012

Abbreviations

ABCA1: ATP-binding cassette transporter A1

ACE: Angiotensin converting enzyme

AEBP: Adipocyte enhancer-binding protein

AICAR: 5 aminoimidazole 4 carboxamide 1 dribofuranoside

Akt/PKB: Protein kinase B

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

ANS: Autonomic nervous system

Arrb2: Arrestin β2

Apo: Apolipoprotein

AT: Adipose tissue

ATG13: Autophagy gene

ATM: Adipose tissue macrophages

AT1R: Angiotensine type 1 receptor

AT1-R: Angiotensin II, type-1 receptor

BAT: Brown adipose tissues

BMI: Body mass index

BMP: Bone morphogenetic proteins

BNDF: Brain-derived neurotrophic factor

Boc: Brother of Cdo (members of the Ihog family) in mammals

Boi: Brother of Ihog in Drosophila

Bax: Pro-apoptotic relative

cAMP: Cyclic AMP

CL: Cardiolipin

CE: Cholesterol ester

CEBPα: CCAAT/enhancer binding protein alpha

CETP: Cholesteryl ester transfer protein

Ci-FL (Ci-155A): Cubitus interruptus full-length zinc-finger TF in Drosophila (155 kb intact active form of the Ci TF)

Ci-75 R: 75 kb cleaved processed form of Ci TF

CKI: Casein kinase I

CLSM: Confocal laser scanning microscopy

CM: Chylomicrons

CNS: Central nervous system

Cos2: Costal2 atypical kinesin-like protein (KIF7 in vertebrates)

CREBP: cAMP response element binding protein

DAG: Diacylglycerol

DAPI: Nuclei staining

DGAT2: Diacylglycerol O-acyltransferase 2

DMPC: 1,2-dimyristoyl- sn-glycero-3-phosphocholine

Dhh: Desert hedgehog

Disp: (D12) receptor: 12-pass SSD TM Dispatched protein

EBP1: ErbB-3 binding protein

EEL: External elastic laminaes

ELISA: Enzyme-linked immunosorbent assay

eLF-4E: Eukaryotic initiation factors-4E

ER stress: Endoplasmic reticulum stress

ER: Endoplasmic reticulum

FFA: Free fatty acid

FGF: Fibroblast growth factotor

FITC: Fluorescein isothiocyanate

FN-III: Fibronectin type 3-like domain

FSP27: Fat specific protein 27

Fu: Fused

Fu5AH: Rat hepatoma cell line

F VII: (Coaculation) factor VIIa

GATA 2 and 3: Anti-adipogenic transcription factors

GIP: Gastric inhibitory polypeptide

Gli- FL (Gli-A): Full lenght activator form of Kru¨ppel-like zinc-finger TF in mammals; Targets for Hedgehog signaling

Gli R: Cleaved and repressor form of Gli TF

GLP-1: Glucagon-like peptide-1

GLUT: Glucose transporter type

GPCR: G protein-coupled receptor

GSK: Glycogen synthase kinase

Hh: Hedgehog

HDL: High-density lipoprotein

HMGCoA reductase

HPGC: High performance gel-permeation chromatography

HPLC: High-performance liquid chromatography

HSC: Hedgehog signaling complex

HSL: Hormone sensitive lipase

ICAM: Intercellular adhesion molecule

IDDM: Insulin dependent diabetes mellitus

IDL: Intermediate-density lipoproteins

IEL: Internal elastic laminaes

IFT: Intraflagellar transport protein

Ihh: Indian hedgehog

IL-6: Interleukin 6

IEM: Immunoelectron microscopy

IFCLSM: Immunofluorescent confocal laser scanning microscopy

IFCM: Immunofluorescent confocal microscopy

IGF: Insulin-like growth factor

Ig-like domain: Immunoglubuline-like domain

Ihog proteins: Interference hedgehog co-receptor with Ptc

IR: Insulin resistance, Insulin receptor

IRS: Insulin receptor substrate

KATP: ATP-sensitive K+ channels

Kif7: Kinesin family member 7

LBP: LPS-binding protein

LC-ESI-MS: Liquid chromatography-electrospray ionization/ mass spectrometry

LCFA: long chain fatty acids

LD: Lipid droplet

LDL: low-density lipoprotein

LDL-C: LDL cholesterol

LEP: Leptin

LIPE: Hormone-sensitive lipase

LKB1: liver kinase B1

LOD: Limit of detections

LPL: lipoprotein lipase

LPS: Lipopolysacchariden

MAG: 2-monoacylglycerol

mCM: Mature chylomicron

MCP: monocyte chemoattractant protein,

M-CSF: Macrophage colony-stimulating factor

mRNA: Messenger RNA

MSC: Mesenchymal stem cells

m TOR: Mammalian target of rapamycin

mTORC1: mTOR complex 1

nCM: Nascent chylomicron

NEFA: Non-esterified fatty acids

NO: Nitric oxide

NFkB: Nuclear factor-KappaB

ox-LDL: Oxidized LDL

MAPK: Mitogen-activated protein kinases

P70S6K: P70 ribosomal S6 kinase

p16 (INK4a): Cyclin-dependent kinase (CDK) inhibitors

PC: protein C

PC: Phosphatidylcholines

PDK: Phosphoinositide dependent kinases

PE: Phosphatidylethanolamine

PI: Phosphatidylinositol

PI3K: Phosphatidylinositol 3 kinase

PJS: Peutz–Jeghers syndrome

PKB/Akt: Protein kinase B

PLFF: Plasma lipoprotein free fraction

PLTP: Plasma phospholipids transfer protein

PPARγ: Peroxisome proliferator-activated receptor gamma

Ptc (Drosophila) / Ptch1 (Mammals) receptor (12 TM): 12-pass TM Patched core Hh protein containing SSD

RAS: Renin-angiotensin system

rCM: Remnant chylomicron

RCT: Reverse cholesterol transport

RheB: Ras homolog enriched in brain

ROS: Reactive oxygen species

P70S6K: P70 S6 kinase

PKA: Protein kinase A

SC: Subcutaneous

SCF complex: Skp, Cullin, F-box containing complex for ubiqutination

Shh: Sonic hedgehog

SM: Sphingomyelin

SMCs: Smooth muscle cells

Smo receptor: 7-pass TM smoothened protein

SR-BI: Scavenger receptor class B type I

SREBP-1c: Sterol regulatory element binding protein 1c

SSD: Sterol-sensing domain

Su(fu): Suppressor of fused

T2DM Type 2 diabetes mellitus

TAG: Triacylglycerol

TF: Tissue factor

TF: Transcription factor

TG: Triglycerides

TGFβ: Transforming growth factor beta

TM: Thrombomodulin

TM: Transmembrane spanning receptor

TNFα: Tumor necrosis factor alpha

TSC: Tuberous sclerosis complex

tPA: Tissue plasminogen activator

UCP-1: Uncoupling protein 1

ULK1: Unc-51-like kinase 1

VCAM: Vascular cell adhesion molecule

VDCC: Voltage-dependent Ca^{2+} channels

VEGF: Vascular endothelial growth factor

VLDL: Very low-density lipoprotein

Vis: Visceral

WAT: White adipose tissues

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Monireh Dashti Rahmatabadi,

2012, Rotterdam, The Netherlands

PhD Portfolio Summary

PhD student: Monireh Dashti Rahmatabadi University: Erasmus Medical Center, Dep. Gastroenterology and Hepatology PhD period: January 2008 till September 2012	Promotor: Prof. dr. M. P. Peppelenbosch Copromotor: Dr. F. Rezaee

PhD Training

General Courses	Year	Workload (Hours/ECTS)
- Good Research Practices: GCP/CLP	2008	2
- Safe Microbiological Techniques	2009	2
- GUIDE Introduction	2009	1
- Bioinformatics	2009	1.5
- Techniques in Molecular Biology (MOBITEC)	2010	4
- Advanced Drug Delivery and Drug Targeting	2010	4
- Presentation Skills	2010	2
- Epidemiology and applied statistics	2010	4
- Publishing using word	2011	1

Presentations

- Oral and poster presentation in GUIDE Annual Meeting, June 10th, 2010	2010	6

International Conferences

- TTS/ ESOT basic Science Symposium, March 12th-15th 2009, Brussels, Belgium	2009	6
- XI International Small Bowel Transplant Symposium, September 9th-12th, 2009, Bologna, Italy	2009	6

Teaching Activities

- **PhotoShop**	2011	2

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