Diabetes-induced cardiovascular disease

A literature study on the emergence of cardiovascular disease in diabetes mellitus type II patients.

AUGUST 2011 **Pjotr Postma**

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Figure frontpage:	Pandora's Box by Gabriel Rosetti
	One of my references (Diabetes Mellitus and Cardiovascular Disease: Pandora's box has been opened. 2004, Jarret Berry et. al) made me decide to choose this picture. I think Pandora's Box is a nice metaphor for diabetes mellitus II, its growing incidence and its possible future consequences in society.

Acknowledgements

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Abstract

In this paper a broad overview of the onset of cardiovascular disease through diabetic-induced mechanisms is given. Risk factors for DMII, like obesity, a bad diet and stress are accompanied with oxidative stress. Oxidative stress leads to insulin resistance because of impairment in the signaling cascade of the insulin receptor. Insulin resistance leads to hyperglycemia and hyperglycemia results in the glycation of proteins in plasma and the extracellular matrix. The glycated proteins will eventually crosslink with each other leading to the formation of advanced glycation end-products (AGE). The receptor for AGE is RAGE and stimulation of it leads to the stimulation of NF-kB. NF-kB is a transcription factor complex that can induce expression of more RAGE, free radicals, adhesion molecules, chemokines and cytokines. An inflammatory response in the endothelial cells will thus occur, leading to the attraction of monocytes, platelets and cytokines. Possible inhibitors of inflammation, such as NO, are eliminated by the oxidative stress occurring in DMII. This leads to a pro-atherogenic phenotype. Platelets in diabetes are hyperactive so clotting reactions will occur accelerated. The upregulated expression of coagulation factors also contribute to amplification of clotting reactions. Altered levels of other plasma components like TF and PAI-I will further contribute to the pro-thrombotic state. All of these symptoms initially caused by DMII will lead to a higher probability of developing cardiovascular disease.

Summary

Diabetes mellitus type II is a disease with an increasing incidence worldwide. The disease has a lot of dangerous effects that, in time, may lead to (deathly) cardiovascular disease. Cardiovascular disease is the leading cause of death worldwide already, and the upcoming DMII epidemic will probably amplify this position. The direct effects of DMII are not deathly in most cases, but DMII slowly influences the vasculature and the composition of blood leading to pathological cardiovascular consequences.

This paper consists mainly out of two parts; a literature study on diabetes-induced cardiovascular disease and a practical pilot project that I have conducted in a two-week internship. In the pilot project I tried to elucidate the effect of hyperglycemia on blood flow through vessel walls and bridged DMII to cardiovascular disease.

Working on this paper involved a lot of independent reading and writing, taking weekly feedback into account. In the small internship, I did a lot of independent work as well, under guidance of my supervisor and people from the division Hematology and Thrombosis of the UMC Utrecht. It was a very educational period and I have enjoyed working on this interesting and relevant subject.

List of used abbreviations

POAD	Peripheral occlusive arterial disease
CVD	Cardiovascular disease
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
oxLDL	Oxidized low-density lipoprotein
DMII	Diabetes mellitus type 2
DMI	Diabetes mellitus type 1
IRS-1	Insulin receptor substrate-1
GLUT4	Glucose transporter type 4
PI3-kinase	Phosphatidylinositol 3-kinase
TNF-α	Tumor necrosis factor α
IL-6	Interleukin-6
ECM	Extracellular matrix
AGE	Advanced glycation end product(s)
RAGE	Receptor advanced glycation end product
A_{1c}	Glycated hemoglobin
NF-kB	Nuclear factor-kB
HMGB-1	High mobility group box-1
Mac-1	Macrophage-1 antigen
sRAGE	Soluble RAGE
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
VCAM-1	Vascular cell adhesion molecule-1
ICAM-1	Inter-Cellular Adhesion Molecule 1
VEGF	Vascular endothelial growth factor
TF	Tissue factor
MMP	Metalloproteinase
vWF	von Willebrandfactor
AT-III	Anti-thrombin III
tPA	Tissue plasminogen activator
PAI-1	Plasmin activation inhibitor-1
cAMP	Cyclic AMP

TxA2	Thromboxane A2
GPVI	Collagen-receptor glycoprotein VI
CD40L	CD40 ligand
sCD40L	Soluble CD40 ligand
G6P	Glucose 6- phosphate
IHD	Ischemic heart disease
ACS	Acute coronary syndromes
ADP	Adenophospate

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Preface:

Atherosclerosis and thrombosis

Blood supply through circulation is of great importance in most living organisms. Oxygen is transported by hemoglobin in erythrocytes and is crucial in nearly all metabolic reactions. Oxygen transport to tissues thus consolidates organ function and maintains a healthy homeostasis. Nutrients, hormones and cells of the immune system are transported through the circulation as well. A smooth flow of blood is thus indispensible to survive.

Atherosclerosis is a disease in which blood vessels slowly get occluded by a process of arterial thickening thereby depriving a smooth flow of blood. In latter stadiums, very little or no oxygen is able to be transported towards the regarding tissues anymore, leading to hypoxia; a state of oxygen-deprivation. This is a pathological condition with cell death and eventually loss of organ-function as a result. The occlusion can be initiated by the formation of growing atherosclerotic plaques in an inflammatory response in the vessel wall. Atherosclerosis is a process that often starts in adolescence but can proceed for decades before symptoms occur. [1]

Haemostasis, the prevention of excessive blood loss when vessel wall damage occurs is crucial for survival. Platelets and coagulant factors will adhere and interact to form a network of different proteins to form a plug in the damaged vessel wall, thereby slowly repairing the damage in a complex reaction. Occlusion of vessel walls may also occur by thrombosis; a condition in which blood components create clots even when no vessel damage is present. Thrombi may eventually rupture and become embolisms; thrombi that travel through circulation and get stuck by adhering to a vessel wall. In some cases this can have a sudden death as a consequence. Thrombosis is a great risk factor for cardiovascular death. [1]

Atherosclerosis thus occurs as a result of inflammatory reaction on vessel walls and thrombosis as a result of abnormal clotting of blood components. Atherosclerosis and thrombosis may occur in every artery in the human body resulting in different symptoms depending on the location. In the limbs, occlusion of vessels will lead to an impaired blood supply to the extremities and this is called peripheral occlusive arterial disease (POAD). But in the heart or brain, hypoxia can have fatal effects: heart infarction, brain infarction and stroke.

Cardiovascular disease (CVD) is a synonym for symptoms that may occur by obstruction of vessels, in cases of atherosclerosis and thrombosis. Currently, cardiovascular disease is the leading death cause in the world, especially in industrialized parts of the world. About one-third of deaths yearly worldwide are caused by cardiovascular disease. This one-third accounts for up to 17 million individuals. [2]

Onset of atherosclerosis and thrombosis

Cholesterol is an important sterol that contributes to cell membranes in the body. Cholesterol is transported through the body by two lipoproteins; low-density lipoprotein (LDL) and high-density lipoprotein (HDL). LDL carries cholesterol from the liver to tissues and HDL carries cholesterol from tissues to the liver. So HDL is responsible for the removal of cholesterol from tissues, whereas LDL is responsible for the delivery of cholesterol to tissues. LDL is pathological in high levels and is associated with atherosclerosis. [3]

LDL can become oxidized by free radicals and this oxidized LDL (oxLDL) adheres to vessel walls causing damage. As a result, monocytes are attracted to the place of damage in an immune response. At the place of damage, the monocytes will migrate to the subendothelial space and transform into macrophages. The macrophages then phagocytose oxLDL but they are not capable of processing oxLDL intracellularly. The macrophages loaded with oxLDL and cholesterol are called foam cells, because of their microscopic visual appearance (see figure 1).

Accumulation of foam cells in the subendothelial space leads to the formation of a 'fatty streak'. Formation of the fatty streak is the first step in atherosclerosis. The foam cells will eventually burst because of the excessive amount of intracellular oxLDL, resulting in even more free cholesterol and oxLDL in the vessel wall. This stimulates an ongoing immune response and attracts more monocytes in a self-sustaining process creating an atherosclerotic plaque. Smooth muscle proliferation in the tunica intima occurs because of the inflammatory response and this leads to a fibrous cap around the atherosclerotic plaque. Because of this fibrous cap, the plaque will stiffen and become rigid. (see figure 1)

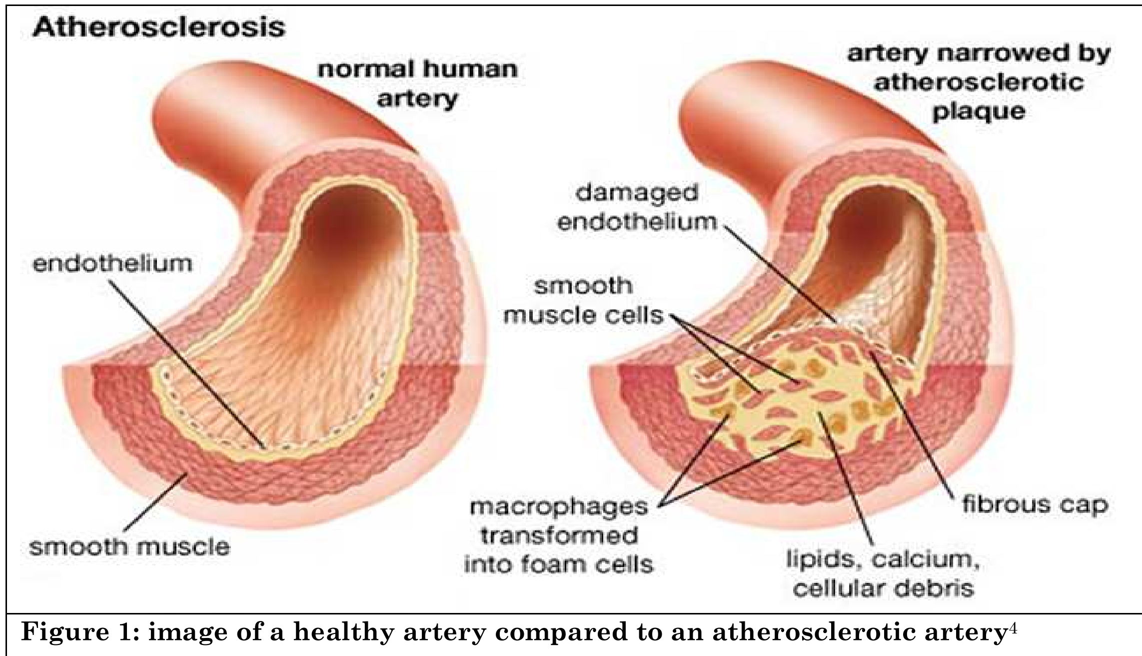


Figure 1: image of a healthy artery compared to an atherosclerotic artery[4]

Thrombosis occurs when platelets and coagulant factor form a fibrin meshwork even when no vessel wall damage is present. This may occur in diseases when activity of platelets or coagulant factors are altered, leading to hypercoagubility. Some auto-immune diseases and genetic disorders are known to alter the composition of blood leading to a pro-thrombotic environment in circulation.

Risk factors

Thrombosis arises difficultly in arteries with a high blood-pressure, because the different coagulant factors will interact less easy under shear stress. However, in arteries with atherosclerosis in which flow of blood is deprived already, thrombosis may occur accelerated.
Some genetic disorders are known to increase the risk of thrombosis as well, by altering activity of platelets or coagulant factors.
Risk factors for atherosclerosis include smoking, abdominal obesity, hypertension, a bad diet, low level of exercise and diabetes. [2] These risk factors are closely related to each other and in many cases mutually present in individuals. They are all typical for a bad lifestyle and this is why cardiovascular disease is the major cause of death in the western world. In this paper, the role of one specific risk factor is elucidated. This specific risk factor is the metabolic disease diabetes mellitus type II.

1.1 Diabetes mellitus type II

Diabetes mellitus type II (DMII) is a metabolic disease in which glucose metabolism is impaired. DMII is epidemically growing in incidence worldwide. At the moment, 246 million people worldwide are suffering from DMII and 7 million new cases occur every year. In India, a country that is developing very fast, the prevalence of diabetes is expected to rise from 50.8 to 87.0 million between 2010 en 2030. In China, the prevalence of diabetes is expected to rise from 43.2 to 62.6 million patients between 2010 and 2030 [5] .These numbers show that 'westernization' of countries leads to increased occurrence of DMII in their population.

Direct symptoms of DMII may include frequent urination, blurry vision, excessive thirst, decreased healing of wounds and an itchy skin. However, many people with DMII are not aware of having the disease, thus symptoms may not always occur. [6]

80 tot 90% of the diabetic patients are suffering from DMII (instead of DMI). DMI is a congenital disease while DMII is idiopathic. Every year, 3.8 million diabetic patients die of the possible consequences of diabetes, mostly cardiovascular disease [5].

1.2 Insulin and insulin resistance

Glucose is the most important molecule in contributing energy in metabolic processes through the synthesis of ATP in the mitochondria, in a process that is called the Krebs-cycle. In diabetes, glucose levels in circulation are greatly elevated because glucose uptake in cells is impaired.

Insulin is a protein that is important in mediating the transport of glucose into cells through activation of a glucose transporter (see figure 2). Insulin is made in the ß-cells of the Islets of Langerhans in the pancreas. In diabetes mellitus type I (DMI), these Islets of Langerhans are incapable of secreting insulin because of an auto-immune reaction against them. In DMII, insulin levels are initially normal but cells get insensitive towards insulin in a gradual manner overtime.

The insulin receptor is a tetrameric transmembrane protein with an intrinsic tyrosine kinase activity. At cellular level, binding of insulin to the insulin-receptor leads to activation of insulin receptor substrate-1 (IRS-1). This activation stimulates the expression of glucose

transporter type 4 (GLUT4), which is a glucose transporter on the cell membrane and is responsible for the uptake of glucose in muscle and fat cells, the main stores of glucose (see figure 2). [7, 8]

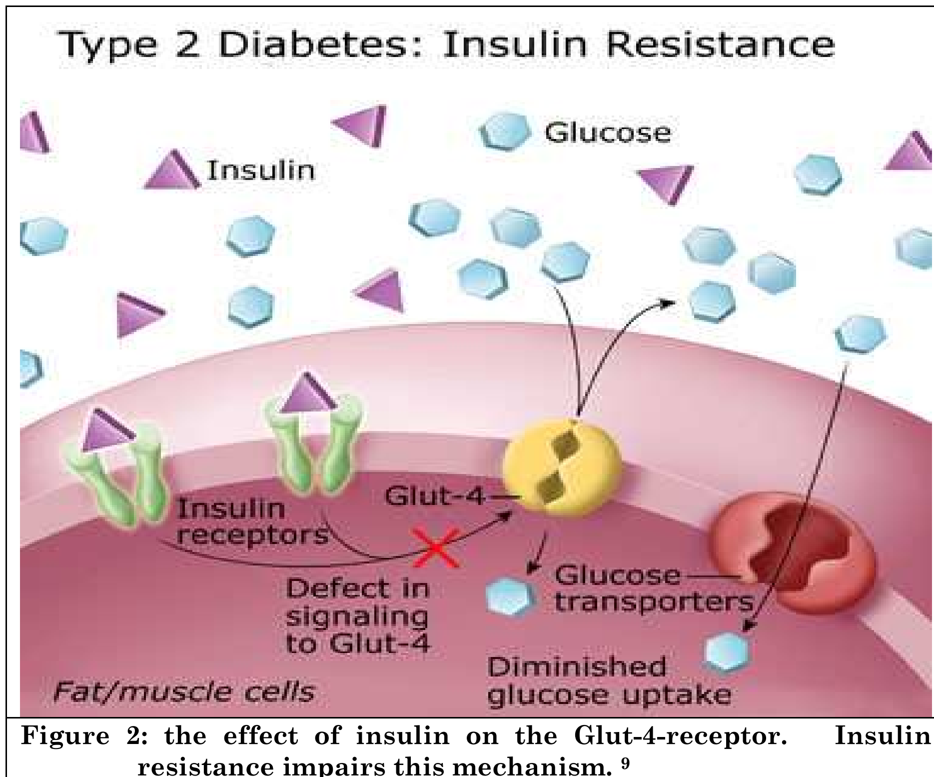


Figure 2: the effect of insulin on the Glut-4-receptor. Insulin resistance impairs this mechanism. [9]

The reason for insulin resistance is an alteration of the insulin receptor signaling and this may be caused by oxidative stress and pro-inflammatory cytokines. [10] Indeed, increased levels of oxLDL, which is an oxidative stress marker, were found in insulin resistant patients in a large cohort study. [8]

Phosphorylation of certain intracellular serine domains, especially ser994, of the insulin receptor is found in obese rat models with insulin resistance [11]. This phosphorylation leads to impairment of the signaling cascade. The insulin signaling cascade is a complex route and activation leads to a number of pathways with different effects(see figure 3). The insulin receptor, IRS-1, phosphatidylinositol 3-kinase (PI 3-kinase) and Akt may be affected in insulin resistance, thus signaling possibly fails at different levels (see figure 3). [10]

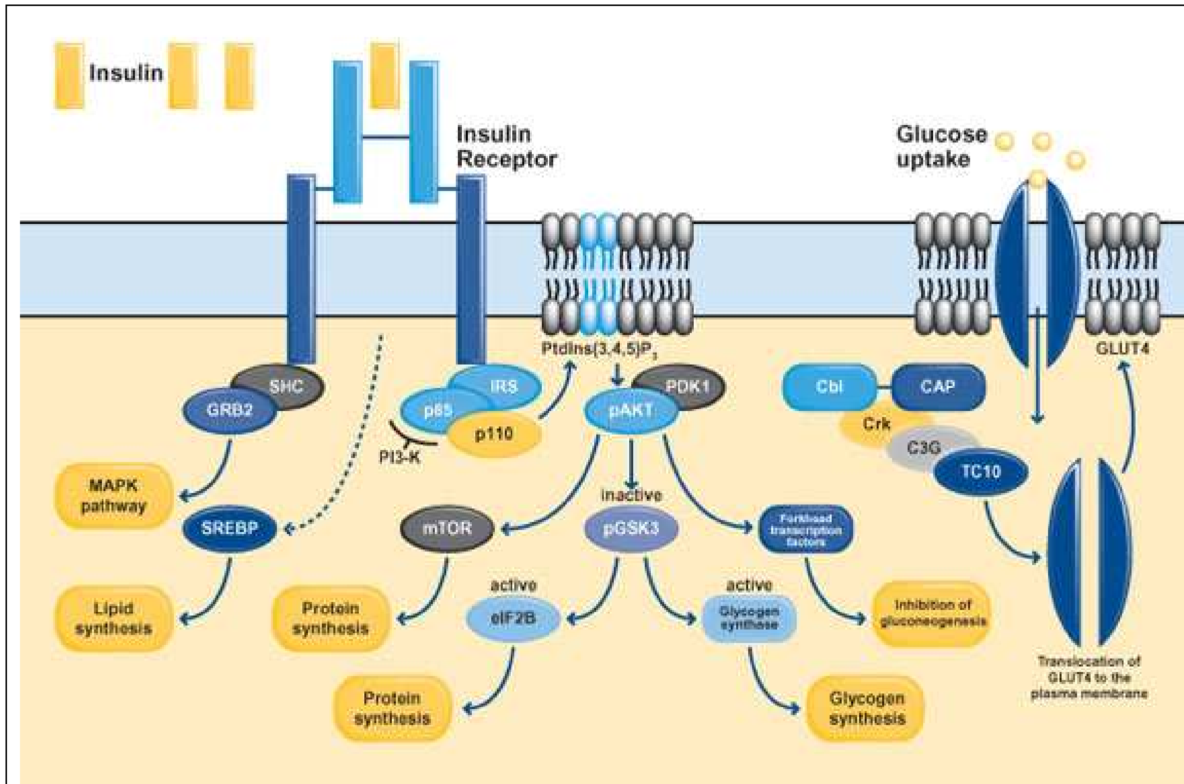


Figure 3: the insulin signaling cascades. In insulin resistance, signaling may be impaired at different levels. [12]

Risk factors to develop resistance against insulin are obesity, a low level of exercise, a bad diet and stress [13]. These risk factors, that are typical for the western world, explain the epidemic growth of diabetes type II because a growing part of mankind is being confronted with them. In fact, 80-85% of the DMII patients are suffering from obesity as well. Obesity is associated with a constant low level of inflammation [5].

Adipocytes in obese people express more cytokines, and certain cytokines like TNF-α and interleukin-6 (IL-6) are capable of attenuating the effect of the insulin receptor pathway. [11]

Upon the initiation of insulin resistance, the Islets of Langerhans will react by producing more insulin, because glucose levels will stay high by the impaired glucose uptake. This state of hyperinsulinemia will keep glucose levels at near-normal for a while and is often called a pre-diabetic state. It has been established that the elevated amount of insulin is a reliable predictor of developing DMII. [8]

The ß-cells in the pancreas have to produce insulin in greatly increased levels to keep normoglycemia (discussed in the next paragraph). Eventually, this constant reactive response of the ß -cells will deprive them, facilitating the disease to progress from a pre-diabetic state into DMII. [8]

1.3 Normoglycemia

In healthy individuals, glucose is metabolized to glycogen by the activity of insulin. This glycogen is stored into the liver, skeletal muscle tissue and other peripheral tissues. When energy is needed, glycogen can be metabolized into glucose by glycogenolysis, and this liberated glucose can be subsequently used to contribute energy.

In the liver the insulin-mediated mechanisms include the inhibition of gluconeogenesis and the synthesis of glycogen from glucose. Gluconeogenesis is the production of glucose from other compounds, like proteins and fatty acids. Insulin thus lowers the blood glucose level in two ways in the liver. Both these mechanisms are important in maintaining a healthy glucose level in the circulation (see figure 4). [6] In skeletal muscle cells and other peripheral tissues, glucose uptake and glycogen production is mediated by insulin as well.

One study showed that in oxidative stress, the potential of smooth muscle cells to synthesize glycogen is deprived. [10] Oxidative stress is found in DMII and the capability to store glucose is thus impaired both by insulin resistance as by the deprivation of glycogen synthesis.

Pathway	Effect of:	
	Insulin	Glucagon/Epinephrine
Fatty acid metabolism		
Fatty acid oxidation	Inhibits	
Fatty acid synthesis	Stimulates	
Lipogenesis	Stimulates	Inhibits
Lipolysis	Inhibits	Stimulates
Carbohydrate metabolism		
Glycogenesis	Stimulates	Inhibits
Glycogenolysis	Inhibits	Stimulates
Gluconeogenesis	Inhibits	Stimulates
Glycolysis	Stimulates	Inhibits
Pentose Phosphate Pathway	Stimulates	
Protein metabolism		
Protein synthesis	Stimulates	
Proteolysis	Inhibits	

Figure 4: effects of insulin and glucagon on metabolic processes. Insulin in general stimulates processes that lead to storage of energy. [14]

In brief, the effect of insulin is lowering the level of glucose in circulation because of an increased potential to store glucose (see figure 4). [6] This leads to quick removal of glucose in the circulation and leads to normal fasting glucose levels, which are between 4-6 mM (see figure 5). [5]

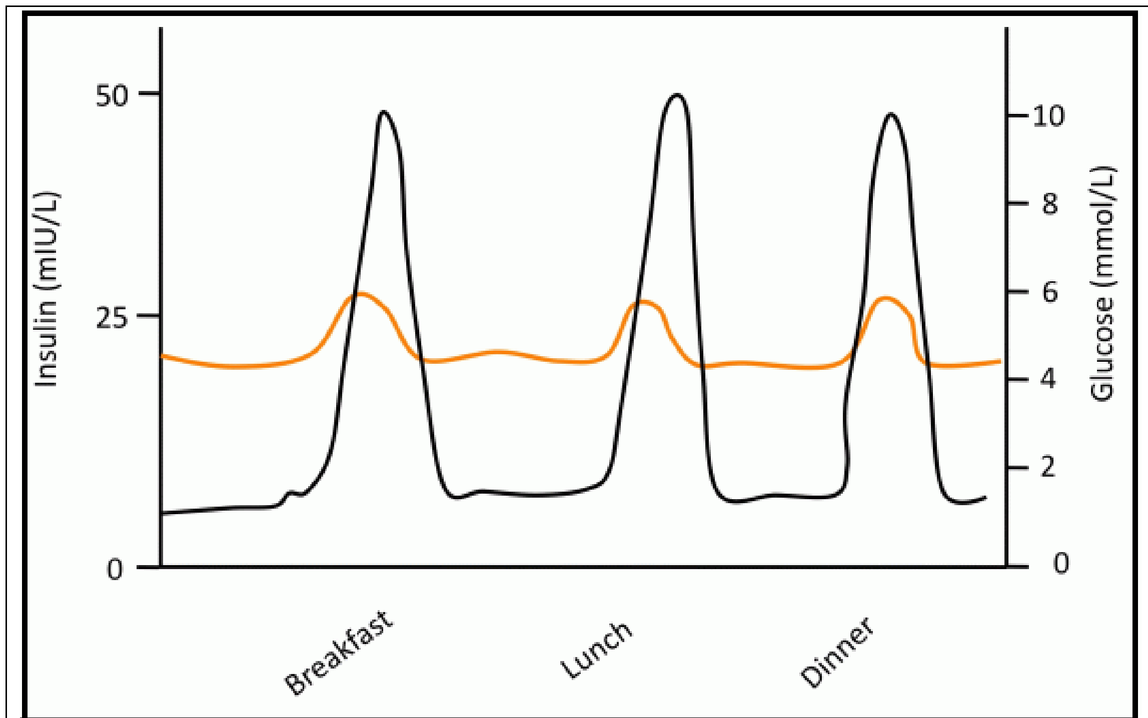


Figure 5: the effect of insulin on glucose levels in healthy individuals. The black line represents insulin, the yellow line glucose. Tight control of glucose levels is achieved by the effect of insulin. [15]

1.4 Hyperglycemia in diabetes

As a result of the deprived uptake of glucose in cells during insulin resistance, (fasting) blood glucose will rise to very high levels, causing a state that is called hyperglycemia. Blood glucose levels in healthy individuals are between 4-6 mM, while diabetic patients can have extreme values up to 30 mM. [16].

Diabetic hyperglycemia is characterized by one of the following observations: fasting blood glucose of ≥7 mM/L, a plasma glucose level of ≥11.1 mMol/L two hours after administration of a 75 g glucose load or symptoms of hyperglycemia in combination with a plasma glucose level of ≥ 11.1 mM/L. A second diagnosis on another day resulting in one of these observations again, confirms DMII. [5]

This high blood glucose-concentration may lead to several severe macrovascular and microvascular effects by a broad set of mechanisms. In fact, 70-80% of the diabetic patients will die of cardiovascular disease (CVD). [6] [16] Microvascular diseases often occur in diabetic patients as well and include retinopathy, neuropathy and nephropathy. Very small vessels thus can get damaged by hyperglycemia as well. Nephropathy may eventually lead to death. [17] [1]

2.1 Hyperglycemia and glycation

Proteins are often modified post-translationally and one of the possible modifications is the addition of sugar groups to proteins, creating glycoproteins. Normally, the production of glycoproteins mainly takes place enzymatically, in a process called glycosylation. Glycoproteins contribute to important structures like the extracellular matrix (ECM) and cell membranes.

In a hyperglycemic state, nonenzymatic reactions of glucose with proteins will occur in a higher rate compared to normoglycemic individuals. The non-enzymatic reaction of these derivatives with proteins is called glycation and occurs by crosslinking of glucose or glucose derived aldehydes with proteins. Saccharides and disaccharides are added with their carbonyl group to the lysine group or NH2-terminus of amino acids, initially forming weak Schiff's bases. This intermediate is still reversible. After a reduction reaction, the less reversible Amadori products are created. These residues will interact slowly with each other in a degradation reaction, in which they become advanced glycation end products (AGE) (see figure 6). [16]

In the glycation reaction, reactive glucose aldehydes will be released by the degradation reaction that occurs by crosslinking in AGE-formation.[18] These aldehydes derived from glucose are up to 20.000 more reactive in glycation reactions than glucose. [19]

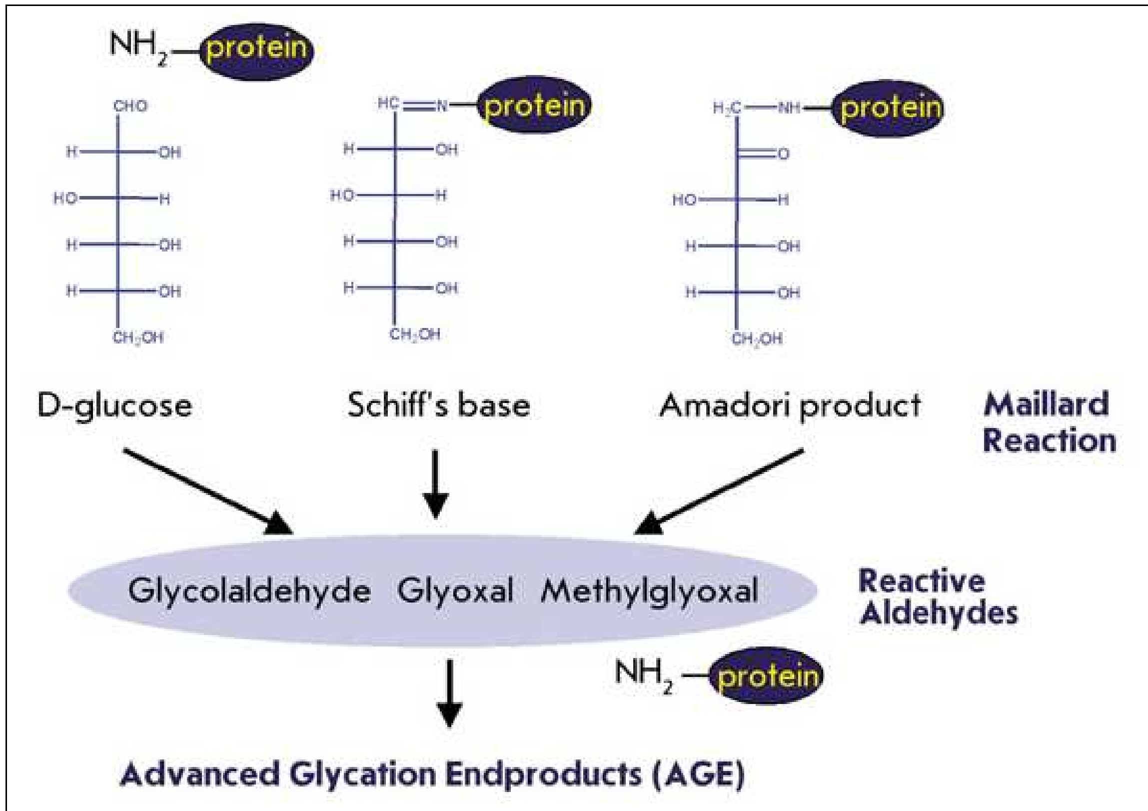


Figure 6: the Maillard reaction in glycation in which proteins get glycated, with the intermediates Schiff's base and Amadori product. Reactive aldehydes released in these reactions can further increase fabrication of harmful AGE. [20]

Therefore, by the initial glycation reaction more components of the glycation process are created (see figure 6). Glucose-derived aldehydes include glyoxal, methylglyoxal, 3-deoxyglucosone and glycolaldehyde. Glucose derived aldehydes are found in higher doses in diabetic persons, despite upregulated cell mechanisms that can break these aldehydes down (see paragraph 2.3). [21]

Hyperglycemia also leads to oxidative stress through activation of the polyol pathway. The polyol pathway is important in retinal, neuronal and nephritic cells and is activated to metabolize glucose that is not required for energy into sorbitol. In hyperglycemia, an excess of sorbitol will be fabricated in these specific cell types, eventually causing oxidative stress to occur. [22]

2.2 Pathology of AGE

AGE levels increase naturally through the process of aging, but in diabetic patients, an excessive amount of AGE is found even in young patients because of hyperglycemia [23]. AGE

accumulates both intracellular and extracellular leading to severe complications (see figure 7).

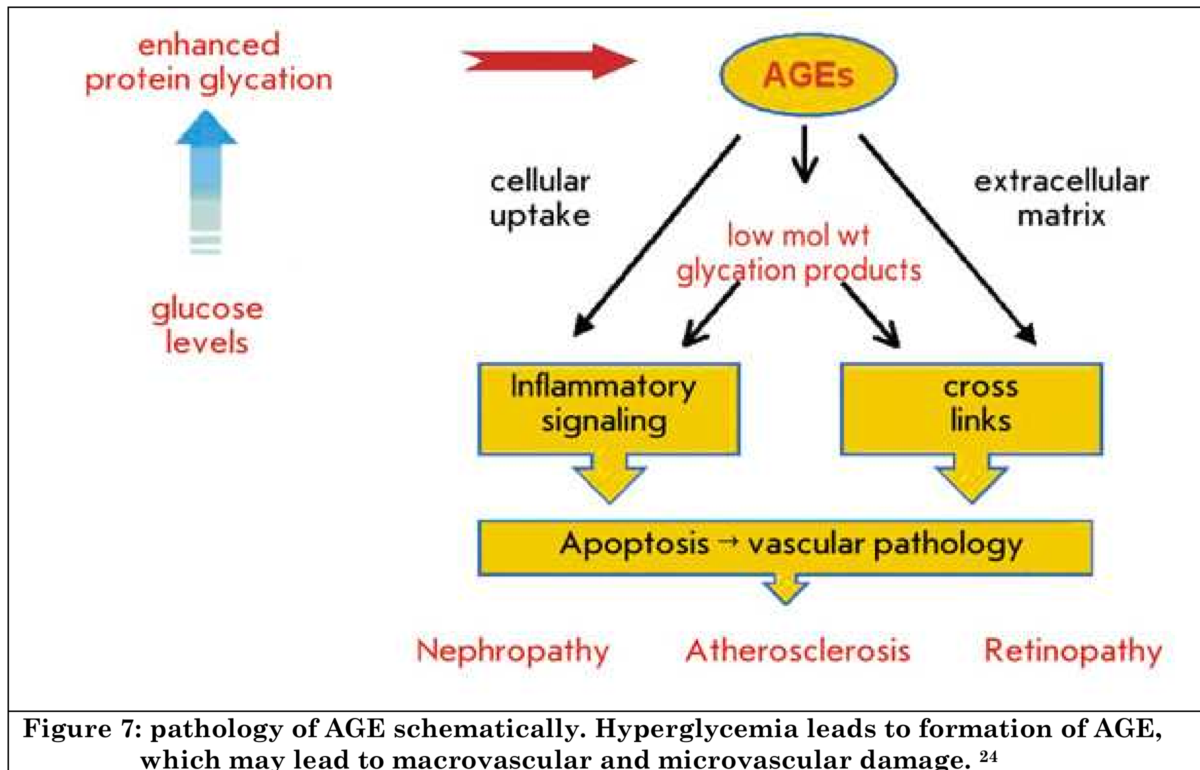


Figure 7: pathology of AGE schematically. Hyperglycemia leads to formation of AGE, which may lead to macrovascular and microvascular damage. [24]

The microvascular symptoms seen in diabetic patients are caused by accumulation and crosslinking of AGE on the basement membranes of endothelial cells of capillaries. This will lead to loss of endothelial integrity and vascular leaking. Diabetes-induced blindness is increasing in incidence in population. [16]

Long-lived proteins, especially found in the extracellular matrix (ECM) are susceptible for AGE-formation as well. This leads to stiffening and decreased scaffolding function of the ECM to construct tissue. [18] It is proposed that glycated hemoglobin (A_{1c}) may predict the risk of developing CVD, even in relatively low levels. [25] One study showed that an increase of 5,5% tot 10% of A_{1c} doubled the chance of CVD in general. [26]

AGE interaction with AGE-receptors (RAGE) leads to inflammation and hypercoagubility, contributing to a pro-cardiac disease phenotype (RAGE will be discussed in paragraph 3.1). In diabetic arterial lesions greatly elevated levels of these AGEs are found. [21]
So both the microvascular and macrovascular symptoms of diabetes are initiated by the formation of AGE. Interestingly, AGE is not only induced by reactions in circulation, it might

be food-derived as well. Heat-processing of foods increases formation of AGE in these products. An unhealthy diet thus may directly add to the pathological reactions of AGE in your system.[16] Intracellularly, AGE accumulation is capable of disturbing breakdown mechanisms, thereby impairing homeostasis in the cell. [23].

2.3 Intracellular breakdown mechanisms

Intracellularly, the system that is responsible for the removal of glycated proteins and reactive aldehydes is the glyoxalase system.[27] This is an enzymatic breakdown system, just like the aldose reductase system, another aldehyde removing mechanism. Aldose reductase is important in the polyol pathway, mainly inducing the production of sorbitol out of excess glucose. The glyoxalase system exists out of two parts; the glyoxalase I and the glyoxalase II system. This system is important in the detoxification of reactive aldehydes like methylglyoxal. [22]

Normally, glycated plasma proteins are broken down by these intracellular mechanisms to prevent them from getting harmful by AGE-formation. The glyoxalase and aldose reductase system are highly activated in diabetic patients because of the abundant amount of reactive glucose-derived aldehydes. [28]

However, even with this upregulated removing systems, most of the cells can not cope with the high levels of these reactive glycation products. The elevated levels of aldehydes lead to an impaired function of some key enzymes in the cell, including glutathione reductase, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. This has deleterious effects on cellular functioning. It is not totally elucidated yet what the exact consequences are but cellular functioning gets impaired with pathological consequences as a result. [21]

The glyoxalase system is upregulated in endothelial cells and monocytes in DMII; however, researchers found that in the diabetic kidney, the glyoxalase system is impaired. Deprivation of the glyoxalase system is the result of the impairment of glutathione, an important antioxidant. [29]

The glucose-derived aldehydes glyoxal, methylglyoxal, 3-deoxyglucosone and glycolaldehyde are also thought to be responsible for the decreased functioning of the proteasome leading to even more intracellular accumulation of damaging constituents.[21] The proteasome is a large protein complex that is present in eukaryotic cells and is responsible for the breakdown of

proteins by proteolytic cleavage. Misfolded and reactive protein parts can be degraded by it, and the complex thus plays a major role in intracellular integrity. [21] As a result of the reduced functioning of the proteasome, more glucose and aldehydes will enter and stay in the cell, in a positive feedback-loop with very damaging effects.

This vicious circle of waste-inducing waste, causing cellular dysfunction, may be an important contributor to the commencement of atherosclerosis in diabetic patients. Endothelial dysfunction is known to add to the risk of developing cardiovascular disease. [21]

3.1 AGE receptor

AGE interacts with a wide array of receptors including oligosaccharyl transferase-48, 80K-H phospoprotein, scavenger receptors type I and II, galectin-3 and AGE receptor (RAGE). RAGE is the most important receptor in the pathology of DMII. [30] Specific reactions of AGEs with RAGE can lead to inflammation, oxidative stress and thrombosis through intracellular mechanisms.

The RAGE receptor is a transmembrane protein of the immunoglobulin family with a cytoplasmatic tail and is diffusely present on endothelial cells, smooth muscle cells of the vasculature and on neurons, innervating muscles of the vasculature. It is important in signal transduction in both acute and chronic metabolic stress. The main signaling pathways of RAGE lead to activation of stress-induced complexes like nuclear factor-kB (NF-kB) and Egr-1, which will be discussed later. [31]

RAGE was first found *in vitro*, when a hyperglycemic environment was created and endothelial cells, monocytes and smooth muscle cells were found to be highly activated. The researchers concluded that AGE participates actively in the pathogenic symptoms of DMII. [32] [31]

3.2 RAGE in diabetes

In diabetic patients, RAGE is strongly upregulated both on endothelial cells and on inflammatory cells. [33] Upregulation of RAGE is found in DMII individuals with hyperglycemia, but in DMII individuals with normoglycemia as well, suggesting that other diabetes-induced mechanisms might be responsible for this upregulation. [31]

Other ligands besides AGE can bind to RAGE; a group of inflammatory derived compounds is able to stimulate RAGE as well. One of them is the group of calgranuline proteins which are released from activated leukocytes. Ligand binding to RAGE of this group will lead to increased chemotaxis of monocytes and thus induces a further inflammatory response. [31] But RAGE also binds to high mobility group box (HMGB-1) and Mac-1. (see figure 6). HMGB-1 activation of RAGE increases cytokine production and neutrophil migration. [31] Mac-1 is a complement receptor on macrophages and expression of it is upregulated in inflammatory circumstances. Mac-1 dimerises with RAGE in order to bind other ligands more firmly. [31] [32] RAGE is also a ligand for integrins on leukocytes like β2-integrinmacrophage-1.

RAGE is thus activated by inflammation as well as an attractant for inflammatory cells. [31] This results in a self-sustaining cycle of inflammation, with a pro-atherogenic phenotype as a result. Studies have shown that homozygous RAGE-null mice had a significantly lowered chance of developing atherosclerosis. [32]

OxLDL is also capable of binding to RAGE, and this specific lipoprotein is found in obese people, which are often diabetic as well. OxLDL is one of the greatest initiators of atherosclerosis, so attraction by RAGE contributes to a pro-atherogenic state. Experimental evidence was found that oxLDL is highly reactive with RAGE compared to normal LDL. [32]

Angiontensin II which is important in the rennin-angiotensin system is capable of upregulating RAGE in diabetic kidneys. Angiotensin II is elevated in diabetic patients and may also cause vasoconstriction leading to hypertension.

The stimulation of endothelial RAGE leads to a cascade stimulating NAD(P)H oxidase which contributes to the formation of reactive oxygen species (ROS) (see figure 8) [34].

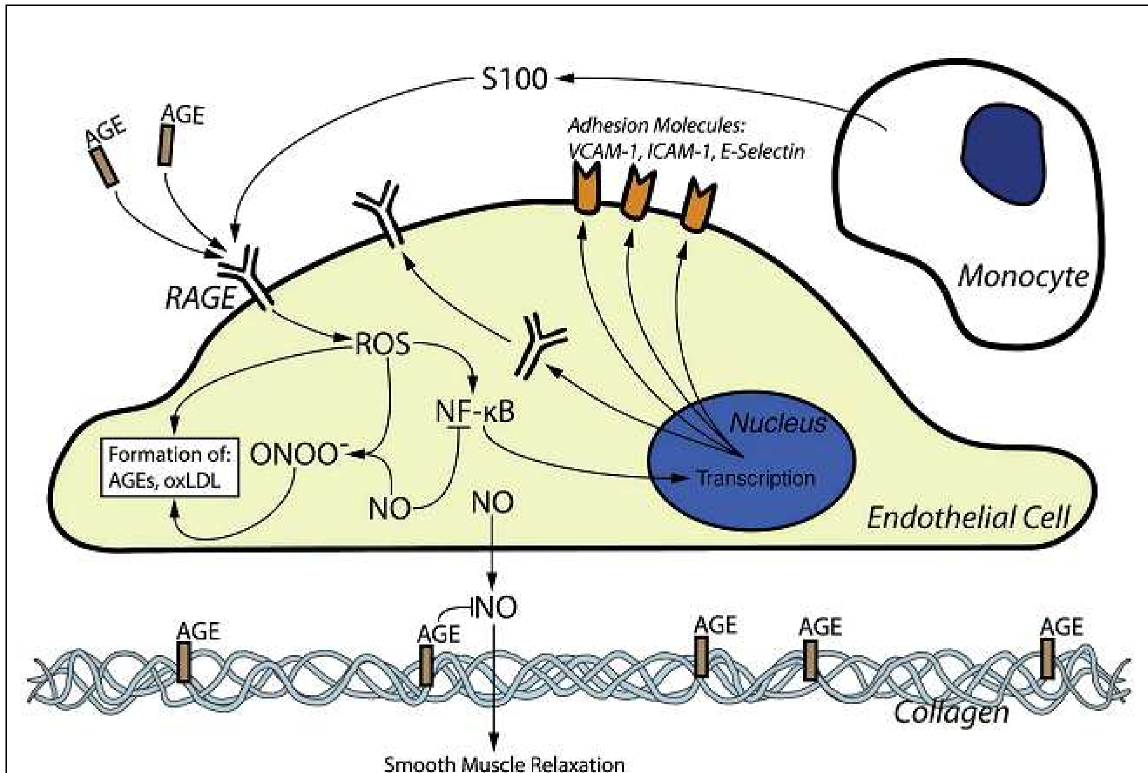


Figure 8: AGE-RAGE interaction leads to formation of ROS and activates NF-kB. Activation and translocation of transcription complex NF-kB to the nucleus leads to upregulation adhesion molecules, increasing the inflammatory response. [35]

ROS are harmful agents contributing to inflammation and cellular damage. ROS are also proposed to be responsible for the onset of insulin resistance (see chapter 1). [36] The main effect of ligand-RAGE binding, however, is the upregulation of NF-kB in the cell and through this complex RAGE is further upregulated in the endothelium. The stimulation of RAGE thus leads to a positive-feedback cycle with expression of even more RAGE through NF-kB activation as a result [32].

RAGE can also be found in a soluble form in blood, and this sRAGE is greatly elevated in DMII II patients. Soluble RAGE activation activates monocytes, adding to an inflammatory response.[37] However, sRAGE is less reactive than endothelial RAGE, thus it might be beneficial in high levels to inactivate AGE. A study in fact showed that sRAGE decreased the chance of atherosclerosis in diabetic mice in a dose-dependent manner. [32]

Some other receptors are known to bind AGE as well, like the class A macrophage scavenger receptors 1 and 2. Although, no intracellular signaling is observed in those receptors and

they thus may be used therapeutically to decrease AGE-RAGE interactions. Less AGE will bind to RAGE if other receptors with no or less harmful effects capture it. [37]

4.1 Inflammation in DMII

Inflammation is a host defense system initiated by foreign and often infectious bodies like bacteria and viruses that enter the system. It can also be initiated by tissue damage caused by external physical or chemical stimulants. Inflammation is a reaction in which your body will try to remove those external compounds by generating cytokines, specific proteins that are able of initiating inflammatory cascades. Inflammatory cascades initiate vasoconstriction and the attraction of monocytes, inflammatory complexes, chemokines and more cytokines in a positive-feedback cycle. This reaction normally sustains until the site of damage is healed and removal of the infectious agent(s) is accomplished. Immunity against bacteria and viruses may be developed, causing a secondary immune response to occur more swiftly. [7]

Inflammation is a very important process in maintaining health, because certain infectious external compounds, like bacteria and viruses are removed by a reaction of the inflammatory system. However, inflammatory responses are also the cause of some diseases, like auto-immune diseases and allergic reactions. In DMI, an autoimmune reaction against the ß-cells in the Islets of Langerhans initiates the symptoms of diabetes. Inflammation can thus damage your body in certain cases as well, by overreacting or reacting needlessly. [7]

Inflammation also occurs chronically in some diseases. Obesity, as mentioned earlier, is one of them. The formation of AGE in diabetes causes damage on endothelial cell walls in an ongoing manner and thus leads to a chronic inflammatory response as well.

4.2 Oxidative stress

AGE-RAGE interaction leads to oxidative stress (see chapter 3.2). Oxidative stress occurs when highly reactive molecular species react with tissues thereby leading to damage. This can be caused by reactive oxygen species (ROS) or reactive nitrogen species (RNS). These reactive species are created by the loss of an electron of a molecule, in which they become a small harmful molecule; a free radical. [8]

Free radicals are lost in normal aerobic reactions in cell metabolism, and the serum is capable of removing them by the activity of antioxidant components (like glutathione).

However, if there are too many free radicals, inflammation caused by tissue damage will occur. Oxidative stress, in short, is an imbalance between the production of free radicals in metabolism and the potential of plasma components to deal with them. [10]

Obesity, hyperglycemia and hyperinsulinemia all have been proposed to be causative for oxidative stress.[38] Obesity is a risk factor for DMII and hyperglycemia and hyperinsulinemia are caused by DMII, so oxidative stress is very likely to occur in a significant rate in DMII patients [10].
Oxidative stress leads to inflammation, via the action of NF-kB. Free radicals are also capable of oxidizing LDL in the plasma, and oxLDL is a major risk factor for developing atherosclerosis. [31]

Oxidative stress also plays a role in the onset of insulin resistance. Under influence of ROS, insulin signaling will get impaired at the Phosphatidylinositol 3-kinase-kinase (PI-3 kinase) level (see figure 3). As mentioned earlier, higher levels of oxLDL were found in insulin resistant patients, revealing a connection between oxidative stress and insulin resistance. [8]
Oxidative stress caused by obesity and a bad diet thus might be the link between DMII and these risk factors. It is plausible to state that the chronic oxidative stress in obesity, caused by hyperactive adipocytes leads to insulin resistance and thus is a great contributor to the onset of DMII in obesity. DMII thus seems to be al logical consequence of obesity.

It has also been found that the transcription of GLUT4-protein decreased when oxidative stress occurred. [10] GLUT4 is normally a glucose transporter in muscle and fat cells that switches on by the action of insulin (see figure 2)[10] .

It is proposed that chronic oxidative stress leads to regression of pancreatic ß-cells, which adds to a decreased effect of insulin in the circulation. The pancreatic ß -cells are one of the cell types that have a very low anti-oxidant defense system and are thus very susceptible to be deprived by oxidative stress. The fact that these cells are susceptible for damage by oxidative stress contributes to the fact that DMII is spreading swiftly. [8]

Oxidative stress may be decreased by a vitamin-rich diet. Vitamins are natural antioxidants and increase the capability of plasma to deal with the free radicals. [36]

4.3 Nuclear factor k-B (NF-kB)

Oxidative stress leads to a decreased bioavailability of NO as well, which is an important agent in vasodilatation. NO is also capable of preventing NF-kB signaling, which will be treated next. [31]

The RAGE-axis stimulates the production of NF-kB in endothelial cells. NF-kB expression can also be stimulated by oxidative stress caused by ROS and many other stimuli. The NF-kB family consists of 5 subtypes and in a hyperglycemic environment, activation of NF-kB1 occurs.

NF-kB1 is a transcription factor complex with many different potential effects including the upregulation of cytokines, leading to pro-inflammatory reactions. NF-kB1 is a complex that is stimulated in most inflammatory responses and is not a specific diabetes-induced signaling molecule. However, the actions of NF-kB1 are very important in the onset of diabetes-related cardiovascular disease. [39]

In a hyperglycemic state the inhibitory subunit of NF-kB1, IkB, is degraded from the complex leading to a translocation of NF-kB1 to the nucleus were it will enhance transcription of a broad array of bioactive compounds. The subunit of NF-kB1 that initiates this action is het p65-subunit.[39]

Some of the important products that are upregulated by the action of NF-kB are vascular cell adhesion molecule-1 (VCAM-1), Inter-Cellular Adhesion Molecule 1 (ICAM-1) (see figure 8), TNF- α, IL-6, vascular endothelial growth factor (VEGF) and tissue factor (TF). These products are all associated with cardiovascular disease. VCAM-1 and ICAM-1 attract monocytes, TNF- α is a cytokine with many pro-inflammatory actions, VEGF is important in neovascularization of atherosclerotic plaques and tissue factor induces coagulation, leading to thrombosis. [37]

Upregulation of Nf-kB also leads to expression of more RAGE on the cell surface. So RAGE is both an activator of NF-kB as well as a product of it. Therefore the process that is induced by AGE-RAGE interaction is a positive-feedback loop with harmful effects.

The activation of NF-kB thus leads to an increased pro-inflammatory, pro-thrombotic and a pro-atherogenic state. [23]

NO is proposed to be an inhibitor of the stimulation of NF-kB, but the formation of NO is inhibited by oxidative stress, which occurs in DMII. [31]

4.4 TNF-α and NO in DMII

One of the cytokines that is upregulated by NF-kB is tumor necrosis factor- α (TNF- α). TNF-α is a proinflammatory cytokine, contributing to the formation of ROS. ROS is one of the major contributors to endothelial dysfunction and atherosclerosis. Because ROS is both the activator (through NF-kB) and the product of TNF- α, this mechanism is a pathological spiral which increases endothelial dysfunction and vasopathology. And this spiral is comparable with TNF- α itself, because this cytokine is both the product and initiator of the activation of NF-kB, as discussed in the previous paragraph. In DMII, clearly many of these positive feedback-loops are found, facilitating the disease to progress slowly. [34]

TNF- α initiates the sphygomyelinase/cerimase pathway, which has an inhibitive effect on nitrix oxid synthase (NOS). NOS stimulates the production of NO in endothelial cells and released NO causes smooth muscle cells to dilate. Less potential to relax the vessel wall will contribute to a more vasoconstrictive state of the vasculature. Upregulation of angiotensin II will also add to the more vasoconstrictive properties of vasculature by inducing vasoconstriction. [40]

Vasoconstriction is very important in atherogenesis because it drives hypertension and blood clotting. Both can contribute to the occlusion of vasculature. NO is also capable of inhibiting the formation of NF-kB and thus a strong anti-inflammatory agent. Furthermore, NO also inhibits the adhesion and activation of platelets on the endothelial wall.[29] A decreased effect of NO in DMII will thus lead to an increased atherogenic phenotype of endothelium.

TNF-α, in conjunction with interleukin-6 (IL-6) and the acute phase protein CRP also initiate the endothelial cell to express more adhesion molecules like ICAM-1, E-selectin and VCAM-1. TNF- α, IL-6 and IL-1 β are also responsible for causing insulin resistance in adipocytes, hepatocytes and skeletal muscle cells. Inflammation can thus lead to decreased insulin sensitivity via the action of TNF-α. [36]

TNF- α also stimulates the production of tissue factor (TF) in vascular smooth muscle cells, fibroblasts surrounding the arteries and monocytes.[34] TF is the main contributor to

coagulation (and thrombosis) and will be treated later more comprehensively (see paragraph 5.4).

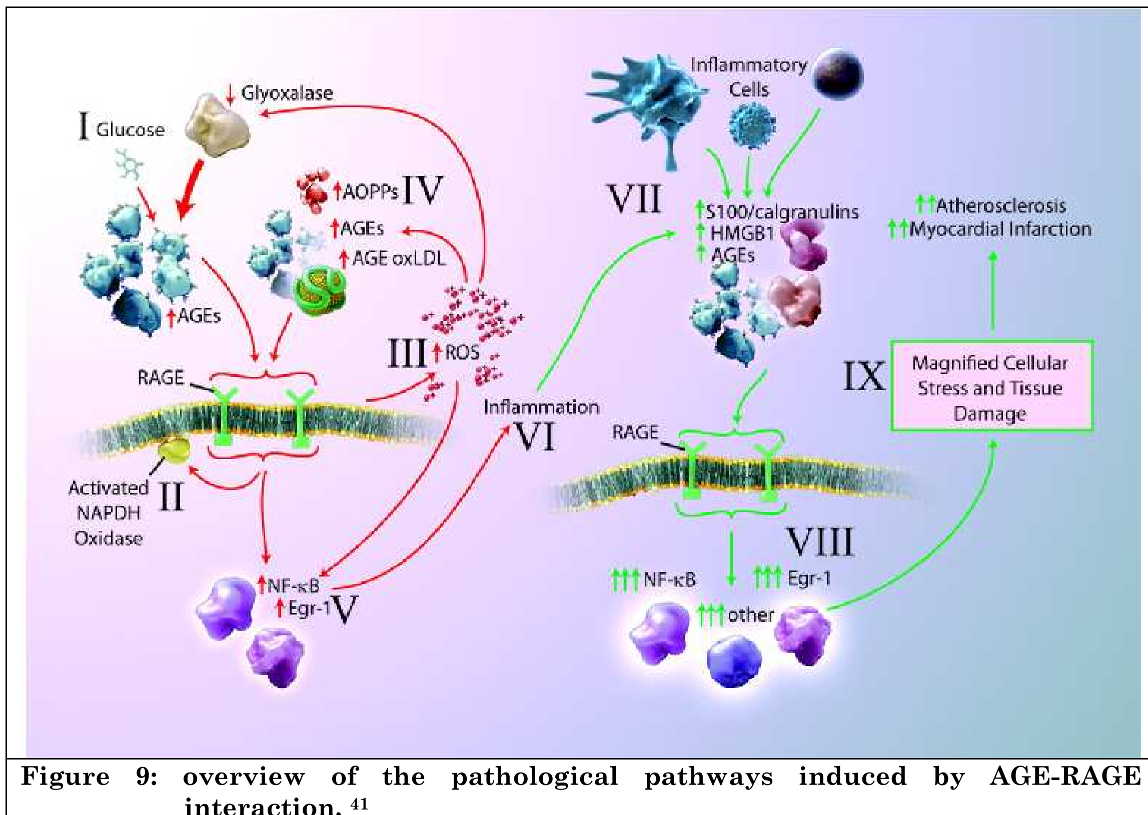


Figure 9: overview of the pathological pathways induced by AGE-RAGE interaction. [41]

In short, DMII leads to a high level of AGE-RAGE interactions, and this leads to a more vasoconstrictive and adhesive state of vessel walls. The decreased potential to relax walls in conjunction with the expression of more adhesive molecules increases the atherogenic properties of vessels (see figure 9). [5]

5.1 Prothrombotic state in diabetes

Haemostasis is an important process in which excessive loss of blood is prevented by platelet aggregation, coagulation and fibrinolysis. Damaged vessel walls are covered by platelets leading to the formation of a temporary blood plug. After this temporary cover, the process of coagulation leads to the formation of a fibrin meshwork. After healing completely, the fibrin network is eventually removed by the process of fibrinolysis (see figure 11). In DMII, vessel walls are damaged because of the formation of AGE and the inflammatory response caused by it. Platelet aggregation and coagulation thus will also play a role in the vessels of DMII patients. [38]

Excessive coagulant reactions and hyperactive platelets can lead to thrombosis. Thrombosis is a pathological process in which clots are formed unnecessarily, potentially causing obstruction of vessels. DMII leads to a pro-thrombotic state through different mechanisms (treated in next paragraphs).

5.2 Extracellular Matrix

The extracellular matrix (ECM) is a scaffold for the formation of complicated structures like vessels and tissues. The ECM is made out of very many different components including elastine, collagen, glycosaminoglycans and proteins. It is a dynamic structure that is both elastic and firming, contributing to the structure of vessels and tissue. When vessel walls are damaged, parts of the ECM stick through the endothelium, causing platelets to adhere. Platelets will grow in number and will temporarily close the wound. Eventually, platelets will disintegrate and release bioactive compounds that initiate the coagulation cascade.

Components of the extracellular matrix can become glycated in DMII. Collagen type I is one of the most abundant compounds of the ECM and is a main target for glycation in diabetic patients because collagen type I has a relatively long half-life time.[42] Later in this paper, an experimental pilot project is discussed in which the effect of glycation of collagen I on platelet adhesion is elucidated (see chapter 7).

Glycation of collagen makes this protein more susceptible of binding LDL. Binding of LDL to collagen in the subendothelial space will stimulate adhesion and inflammation, and is typical for atherosclerosis. [43] Glycation of the ECM also affects the potential to assemble new vessels because the scaffold for angiogenesis stiffens. The increased rigidity decreases the elasticity of ECM, thereby altering its very function. Also, the population of fibroblasts is diminished in DMII causing less potential to induce collagen fibrils to contract through the action of these fibroblasts. [44]

Another effect of glycation of the extracellular matrix is the fact that the potential of degradation of the ECM is strongly impaired. This degradation is needed for ECM remodeling, which is in turn required for its scaffolding function. Glycation of extracellular matrix results in a less adhesion of metalloproteinases (MMP's) which play a major role in the breakdown of ECM components. [23] Stiffening of vascular tissue is a well-known risk factor for developing atherosclerosis. [16] [44]

Glycation of ECM components like fibronectin and laminin underlie the occurrence of neuropathy in diabetic patients. Sensory neurons contain 5 laminin-binding domains and 2 fibronectin-binding domains. Glycation of these components thus has consequences concerning neuronal functioning. Axons will regenerate less easy and neuronal sprouting is inhibited by the more static ECM. [18]

5.3 Platelets

Platelets are important in the beginning of haemostasis. They adhere to collagen fibrils that are exposed through the endothelium when a vessel wall is damaged. Platelets create a temporary cover on the wound and are then activated by thrombin. Under influence of thrombin, the platelets will disintegrate and release serotonin and adenophospate (ADP) accelerating the process of platelet aggregation. However, if platelets are hyperactive and adhere to surfaces that are not damaged, this may lead to thrombosis. Pro-thrombotic alterations of platelets are found in DMII.

An important adhesion factor, von Willebrandfactor (vWF) is seen in higher levels in DMII patients. vWF is fabricated in endothelial cells and promotes platelet adhesion to the vessel wall. Elevated levels of vwF have been associated with a higher risk of macrovascular disease and endothelial dysfunction. vWF originates from endothelial cells, so high levels in circulation may indicate increased endothelial activity caused by inflammation and hyperglycemia. [5]

Membrane proteins of platelets can become glycated in a hyperglycemic environment and these glycated proteins bind to thrombi more easily. [29]. Under experimental conditions an increased potential to clot on prothrombotic surfaces has been found in DMII platelets. [5] In the experimental pilot project discussed later, we will look at a reversed experiment; healthy platelets on a DMII-like surface (see chapter 7).

Insulin resistance has been associated with platelet hyperactivity. [45] Insulin intervenes in the expression of cyclic AMP (cAMP) in platelets which is a major factor in aggregation and secretion of granules. Platelets from individuals suffering from diabetes are more responsive to agonists because of the decreased sensitivity to insulin. As a result, platelets will be activated in a higher rate than normal at a place of vascular damage. The link between DMII and hyperactive platelets is a defective functioning of the insulin receptor substrate-1 (IRS-1).[5]

Thromboxane A2 (TxA2) is also strongly upregulated in platelets of diabetic patients.[5] This TxA2 is a vasoconstrictive and prothrombotic compound. Elevated levels of TxA2 are a result of impairment of glutathione, an important antioxidant. Glutathione is an important part of the glyoxalase system mentioned earlier in paragraph 1.3. [29] Another reason for upregulation of TxA2 might be an altered calcium metabolism in platelets of diabetic individuals. [29]

Calcium metabolism is an important factor in platelet shape and platelet aggregation, and it is altered in DMII patients. A study in elderly men showed that with decreasing levels of insulin sensitivity, levels of serum calcium increased. A higher level of intracellular calcium leads to an impairment of GLUT4 and the insulin receptor. The exact reason for the altered calcium metabolism in DMII is unclear. [46]

Platelets will become hyperactive because of an increased sensitivity to collagen by an upregulation of collagen-receptor glycoprotein VI (GPVI) on the surface of the platelets in DMII. Collagen is the main extracellular matrix component for platelets to attach to. [42]

Glycoprotein Ib is upregulated on platelets in a hyperglycemic environment, and this receptor binds to vWF in the aggregative reaction. [29] Upregulation will lead to a greater adhesion of platelets as a result.

Glycoprotein IIb/IIIa is more abundant at diabetic blood platelets and this glycoprotein is a receptor for fibrinogen. [47] Fibrinogen is a major component of blood clots so blood platelets in diabetic patients will bind easier to the growing clot. This complex also aids in platelet activation. Therefore, overreacting platelets will strongly increase the process of blood coagulation leading to swifter formation of a thrombus. [29]

The hyperactivity of platelets in DMII has been indicated by a study in which elevated levels of sCD40L were found. CD40 ligand (CD40L) is found on the membranes of activated platelets. CD40L stimulates the release of procoagulant, proinflammatory and adhesion molecules and is found in raised levels in cardiovascular disease. After binding to the platelet, a fragment (sCD40L) is released. A higher level of sCD40L thus provides evidence for the hyperactivity of platelets in DMII. [48]

In healthy individual platelets, adhesion of platelets to endothelium is deprived by the effects of NO and prostacyclin (PGI_2). In platelets of diabetic individuals, resistance against these both agents however occurs, leading to a more adhesion-favorable state of the platelets. [5]

5.4 Coagulation

In blood plasma very many different factors that contribute to coagulation are found (see figure 10). The coagulation response requires interplay between a large number of molecules, leading to the formation of a blood clot.

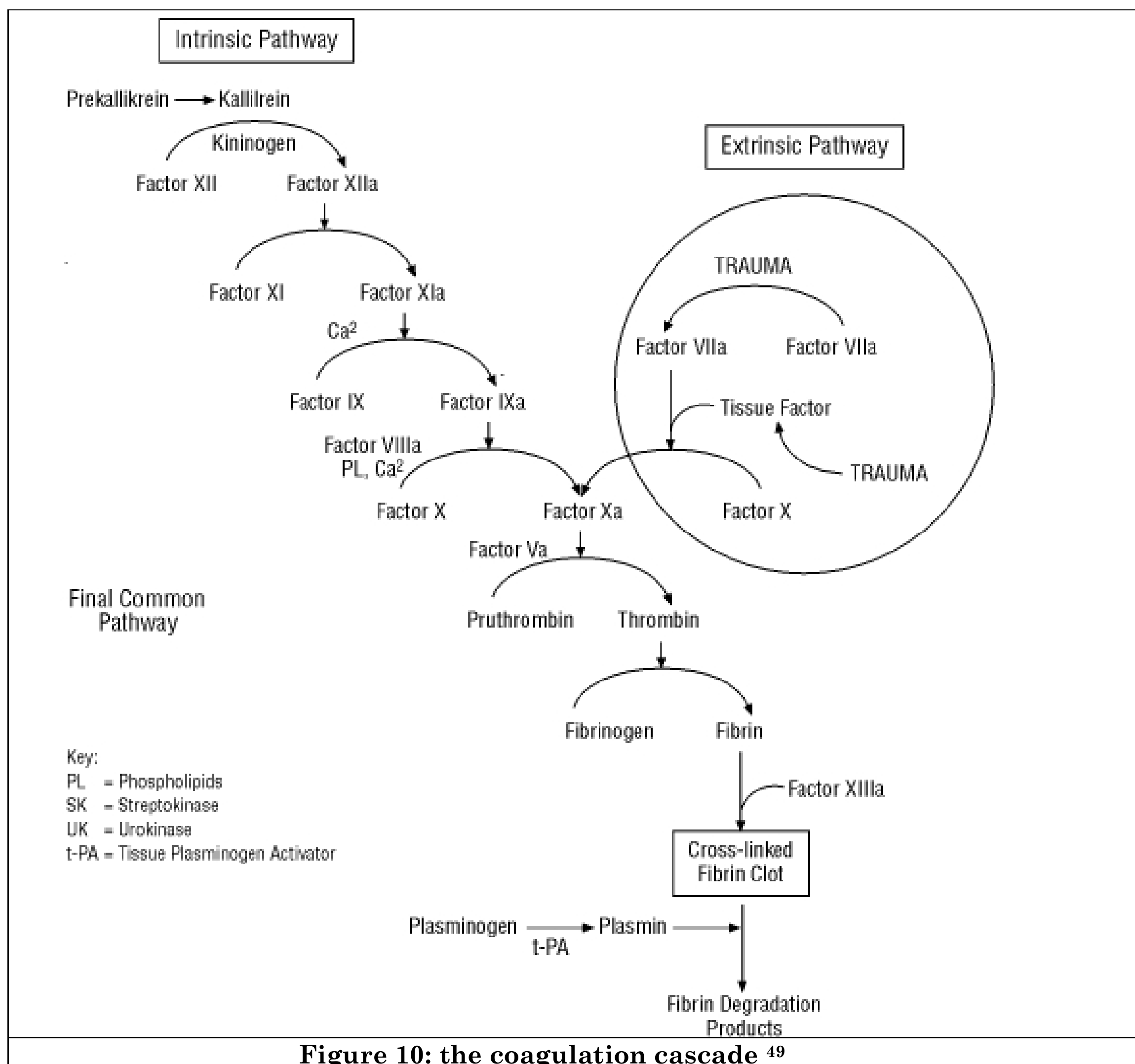


Figure 10: the coagulation cascade [49]

TF is an important factor in the extrinsic pathway of coagulation, by indirectly stimulating the synthesis of thrombin. The extrinsic pathway is the more common than the intrinsic one inside the body. TF induces a cascade that eventually leads to the clotting of plasma components (see figure 10).

In diabetic patients, the expression of TF especially on monocytes is greatly increased. [29] Under physiological conditions monocytes express TF at a very low level, but TF on monocytes is greatly increased as a result of insulin resistance. Insulin inhibits the expression of TF in a dose-dependant manner explaining the higher level of TF in diabetic patients. This dose-dependant relation was shown *in vitro*, but a study in patients confirmed that TF-levels increase when insulin resistance occurs. [5] The level of circulating TF increases 1.6 fold compared to non-diabetic individuals. Monocytes constantly travel through circulation thus a higher expression of TF on these cells is a great risk factor for an increased chance of coagulation.[5]

TF is expressed on platelets as well. In recent studies, it has been shown that platelets, however anucleate, are capable of expressing some proteins by pre-mRNA splicing. In DMII-patients TF-levels on platelets are elevated although in a less manner than TF on monocytes. [5] Expression of TF on platelets also is a very slow process compared to monocytes. In vessel damage experiments in animals, the first TF of platelet origin was measured past 100 seconds. TF is expressed very slowly, but in a very consistent manner. This is why it is proposed to stabilize thrombi [5].

Studies have shown that AGE leads to the expression of TF at the mRNA-level. AGE is thus capable of promoting a pro-thrombotic state. This effect could be attenuated by adding antioxidants. In short, TF is upregulated in diabetes and this leads to a more pro-thrombotic state. [38]

Fibrinogen is an important procoagulant factor at the end of the clotting process, and research showed that fibrinogen is abundantly present in DMII patients compared to healthy subjects, making them more susceptible of thrombosis. [50]

It also has been shown that the biological activity of anti-thrombin III (AT-III) is depressed by hyperglycemia. AT-III is an anticoagulant factor and decreased activity will thus lead to a more prothrombotic state. [29]

Factors VII, IX, XII and kallikrein are also present at elevated levels in blood plasma of DMII patients, and these raised levels add to the state of hypercoagubility in DMII patients as well. [29] The increased level of coagulation reactions in diabetes type II patients is reflected

by elevated levels of thrombin-antithrombin and prothrombin fragment 1+2.[38] Both are indicators of thrombogenic reactions.

5.5 Fibrinolysis

Fibrinolysis is the process of clot breakdown mediated by a couple of bioactive compounds (see figure 11). The main constituent of blood clots is fibrin, and this fibrin is broken down by plasmin, which in turn is made out of plasminogen. Plasminogen is an inactive plasma agent that is fabricated in the liver. Urokinase and tissue plasminogen activator (tPA) stimulate the reaction that turns plasminogen in plasmin. These both agents are inhibited by plasminogen activator inhibitor-1 (PAI-1). [50]

This PAI-1 is expressed in a higher amount in diabetic patients, leading to less potential of fibrinolysis. Especially in DMII patients with microvascular complications higher levels of PAI-1 were found. [50] Less fibrinolysis will stabilize the atherosclerotic plaques even further thus leading to further vascular occlusion. It will also add to the pro-thrombotic environment in DMII.[38]

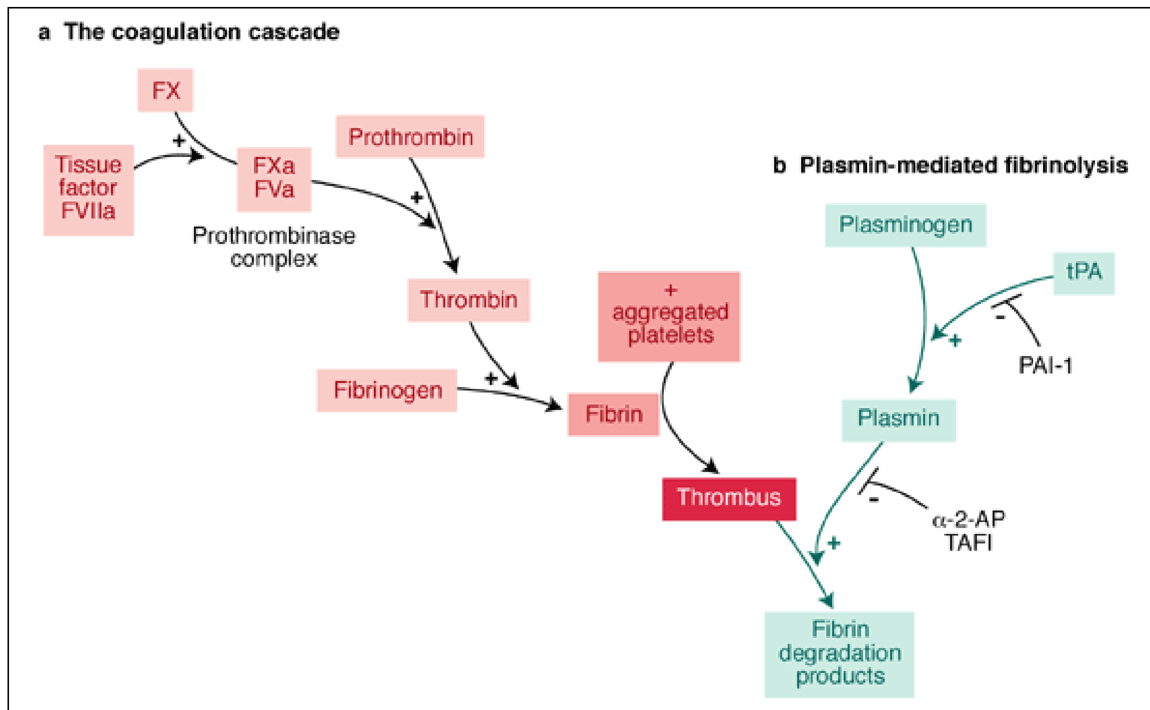


Figure 11: the fibrinolysis cascade. PAI-1 is upregulated in DMII leading to a decreased formation of plasmin, leading to a degraded breakdown of thrombi. A pro-thrombotic phenotype is the result. [51]

It has been shown that hyperinsulinemia, which may also occur in patients with DMII, may work synergetically with hyperglycemia in stimulating the production of PAI-I [38].

Fibrinogen becomes glycated in hyperglycemic individuals as well and this results in a more stable and rigid structure of fibrinogen. This adds to the hypofibrinolysis as well, furthering the formation of atherosclerotic plaques and the pro-thrombotic state. [52]

5.6 Microparticles and diabetes

Micoparticles (MPs) in the circulation are a heterogenous group of budded vesicles from apoptotic cells and platelets. Microparticles constitute a population of very small vesicles between 0.1 and 100 µm and a lot of them are still bearing the biological properties of their mother cell. These cells of origin could be monocytes, platelets and endothelial cells. They can also bud from atherosclerotic plaques [5].

Most of these MPs thus still display proteins and phospholipids of their cell of origin. Microparticles express negatively charged phospholipids as a membrane and this makes them susceptible for the binding of prothrombotic components in the blood. [29]

Microparticles are present in small amounts in healthy individuals, but are abundantly present in pathological circumstances like DMII and atherosclerosis. [53]

Some MPs are shed from platelets and these specific MPs bind very easily to the subendothelial matrix trough glycoprotein IIb/IIIa bridging. The platelet MPs express TF at remarkably high levels, thus adding to hypercoagulability.[5] TF of MP-origin is often called blood-born TF and is detectable in high levels in diabetic individuals. [5] Platelet-derived MPs are found in higher levels in diabetic patients relatively to healthy individuals. [29]

Endothelial microparticles are found in elevated levels in diabetic individuals as well and these microparticles are able to negatively affect the potential to synthesize and store NO in endothelial cells. [53]As mentioned earlier, NO is a very important vasodilative agent in preventing clotting and atherogenesis and in inhibiting the activation of NF-kB. The amount of endothelial-derived microparticles may also reflect the level of endothelial dysfunction and damage because they shed from apoptotic endothelial cells. [53]

5.8 Glycocalyx

The protective layer of the vessel wall, the glycocalyx, is affected by hyperglycemic oxidative stress. [54] The glycocalyx covers the external luminal side of the endothelial cell wall and is

made out of proteoglycans, glycoproteins and blood-derived proteins. It is a barrier between the endothelial wall and the bloodstream and only water and small molecules such as TxA2 and NO can pass freely between these two compartments. It therefore greatly contributes to intercellular integrity and the prevention of leaking of constituents from interstitial tissue and external cellular matrix into the bloodstream and vice versa. Hyaluronan is the most prominent glycoprotein contributing to this integrity. [54]

The glycocalyx prevents strong adhesion of leukocytes and blood platelets to the endothelium thus being an anti-inflammatory and anticoagulant part of the arterial system. Hyperglycemia lowers the potential of the glycocalyx to release NO with less vasodilative capacity of the vessel wall as a result. This will lead to a higher degree of vasoconstriction which improves the risk of thrombosis.

6.1 Cohort studies diabetes-CVD

Many studies in human have been conducted into the relationship between the prevalence of DMII and the incidence of CVD. DMII patients have the same chance of developing CVD as people who had a prior myocardial infarction. Ischemic heart disease (IHD) is the most common cardiovascular death cause in diabetic patients.

Clinical outcomes after cardiovascular injury are worse for diabetic patients compared to other individuals after acute coronary syndromes (ACS). [13] A recent collaborative meta-analysis of 102 studies in a total of 25 countries with 698.782 diabetic people included showed that people with diabetes have a two-fold higher chance of developing cardiovascular disease.[1] None of the people included had earlier macrovascular complications. Risk of myocardial infarction increased two-fold, risk of brain infarction increased over two-fold, and chance of stroke increased with a factor 1.5. In this meta-analysis, other risk factors were excluded.

Diabetic women have a higher chance of developing CVD than men, according to this study, which is odd because normally men have a higher risk of CVD. Another study also concludes that diabetes equalizes the chance between men and women of dying of cardiovascular disease.[55] This study also concluded that there is no difference between the risk of dying of sudden cardiac disease or non-sudden cardiac disease in diabetic patients.

One other result that is particularly odd is the fact that diabetic smokers have a lower risk compared to diabetic non-smokers of developing CVD. The reason for this very strange result requires more investigation.[1] The same researchers also concluded that diabetes was

responsible for 11% of the deaths of myocardial infarction and stroke in the past ten years. In high income countries, this percentage represents around 325000 deaths. [1] This number will increase because of the growing prevalence of DMII worldwide.

Research also elucidated the effect of hyperglycemia on artery walls. Arteries were obtained *post mortem* from diabetic and non-diabetic individuals whom died from cardiovascular disease. The atherosclerotic plaques in diabetic individuals were significantly larger and contained much higher levels of necrotic cores. [32] Also, the cellular structure differed greatly. The diabetic arterial lesions contained highly elevated T-lymphocytes and macrophages compared to non-diabetic individuals. This indicates that inflammatory reactions occur in higher levels in diabetic patients. [32]

In the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study, a diabetic group with tight glycemic control was compared to a diabetic group without tight glycemic control. [43] No decreased risk of developing CVD was found in the tight control group. As a matter of fact, the study was put on a hold when increased numbers of CVD deaths were found in the tight control group. The reason why an increased glycemic control seemingly leads to a higher chance of developing CVD in this study needs further investigation. It was however shown in another study that glycemic control is helpful in preventing the microvascular disease in DMII. [32]
Yet another study showed that an increased glycemic control resulted in a mean lesser thickening of the media intima of vasculature, which is affected in DMII. As a result, chance of cardiovascular death diminished by 57% 11 years after follow-up. [16] Differences between the beneficial effects of glycemic control might be caused by the stadium of the disease. Early intervention of dietary habits seems to be more helpful in preventing cardiovascular disease than intervention in latter stadiums. [29]

Glycemic control may also differ in effect between high and low risk groups of CVD. One study concluded that the high risk cohort benefited from tight glycemic control in contrast to low risk cohorts. [13] In another meta-analysis, glycemic control seemed to lower the chance of developing CVD. In this study it was proposed that glycosylated hemoglobin is a predictor of CVD. [56]

6.2 Therapeutic approach

Many studies have also been conducted in the onset of DMII and its risk factors. The Finnish Diabetes Prevention Study and the US Diabetes Prevention Program showed that lifestyle intervention can help to decrease the incidence of DMII in people with lower baseline glucose resistance. This lower baseline glucose resistance means that these individuals have a lower capacity of processing glucose, and is seen as a prediabetic state. [13] These studies thus proved the point that the onset of DMII is often associated with bad lifestyle habits, like a bad diet and low exercise. [13]

It has been shown that heat-processing of foods increases their levels of AGE. Especially processes like broiling or oven frying greatly contributes to this process. Diabetic patients may be advised to avoid foods that are prepared in these ways. [16]
Evidence has been established in human that an AGE-rich diet increases AGE serum levels about 1,5 fold. Diets with a 5-fold lower level of AGE led to a great deprivation of serum AGE, VCAM-1 and TNF-α. An AGE-poor diet thus may contribute to a less inflammatory response in circulation. [16]

Studies have also been conducted in administering micronutrients to the diet of pre-diabetic individuals, in order to reduce oxidative stress and pro-inflammatory reactions. Vitamins are natural antioxidants and are able of inhibiting insulin resistance at different levels. [36]

As mentioned earlier, glycemic control helps to prevent DMII in an early stadium. However, it seems that latter stadiums of the disease do not benefit from a tight glycemic control in a significant manner. Pharmaceutical ways of curing DMII are therefore a great field of interest. [25]

Antiplatelet therapy by the administration of clopigrel and aspirin has been researched, but no satisfactory results were found. This anti-platelet therapy is able of reducing the chance of atherosclerosis in non-diabetic patients, but not in DMII patients. The reason for this hyporesponsiveness seems to be the fact that membrane proteins of platelets are glycated by hyperglycemia. [29]

The administration of sRAGE also seems to be a possible way of treating DMII, because endothelial RAGE leads to intracellular signaling with inflammation and hypercoagubility as a result. sRAGE circulates in arteries and vessels and is less reactive. However, it is able of stimulating monocytes and thus may lead to inflammation. [26]

Other studies are aimed at targeting the RAGE-axis as well. It seems however very difficult to find the molecules that inhibit the AGE-dependant stimulation of RAGE. Studies are being conducted in finding these specific antagonists. [32]

AGE is known to be a great pathological factor in DMII. In a certain study, adding aminoguanidine, an inhibitor of AGE formation, to the circulation resulted in a less degree of retinopathy and a degradation of AGE found in arterial lesions. [26]

AGE-crosslink breakers are a target for DMII treatment as well. This 'post-glycemic' control has shown to be effective in increasing vascular compliance in elderly in a study. [26]

Experiments *in vivo* have been conducted in restoring the glycocalyx by administering sulodexide to DMII patients. This specific compound is able of increasing the amount of glycosaminoglycans in the glycocalyx. This glycosaminoglycans layer can be degraded up to 50% in DMII patients. [38, 54] The glycocalyx is an early line of defense in endothelial integrity and functioning and therapies that are beneficial for this layer might be a way to attenuate vascular damage caused by hyperglycemia. [54]

6.3 Animal models for DMII

In 2007 the Animal Models of Diabetic Complications Consortium described several ways to create diabetes-induced cardiovascular disease in mice [57]. A way to make mice diabetic is the adding of streptozotocin to them. This agent will destruct the pancreatic ß-cells that are responsible for the expression of insulin, and thus will make them hyperglycemic.

Another way that is often used to create diabetic mice is to make apolipoprotein E-knockout mice. Administration of streptozocin leads to a DMII phenotype and breeding apo-E-null mice creates DMI mice. [32]

Oxidative stress-induced insulin resistance has been shown in muscle tissue of lean mice. This oxidative stress was created by administering glucose oxidase to strips of the muscle tissue of the mice, providing a reaction in which H_2O_2 is created. H_2O_2 is an inductor of oxidative stress. The oxidative stress also inhibited the synthesis of glycogen in these mice, adding to the impaired capability of muscle cells to store glucose in a proper way. [10]

The upregulation RAGE and AGE has been shown in mice models, as well as the impaired relaxation of vessel walls, and the increased protein expression induced by NF-kB. [16]. Research also indicated that DMII mice without hyperglycemia express higher levels of RAGE as well, suggesting that other mechanisms caused by DMII are responsible for RAGE expression. [31]

Increasing levels of AGE in aging non-diabetic rats were shown in a study. These findings stabilize the conclusion that AGE accumulation occurs in non-diabetic patients as well.

Also, an increased sensitivity for insulin has been found in mice when NF-kB was inhibited. [43] An interesting study in DMII mice showed that administration of sRAGE decreased the chance of developing atherosclerotic plaques in a dose-dependant manner. sRAGE thus might be an agent with potential anti-atherogenic effects.[32]

Experiments in mice with different diets have also been conducted to elucidate the role of dietary AGEs on atherosclerosis. It was found that mice with a lower dietary AGE-intake had a lower chance of nephropathy.[16]

In another study by this group, homozygous RAGE-null mice had a significant lowered chance of developing atherosclerosis, adding to the conclusion that RAGE is an important signal transducer in atherogenesis.
The glyoxalase I system, which is important in the removal of glucose derived aldehydes from the cell is upregulated in diabetic mice, according to a study comparing mice and rats. [28] Despite this upregulation, retinopathic complications still occurred in diabetic mice, proving that hyperglycemia exceeds the capability of this system to cope with the reactive aldehydes. Homozgygous RAGE-null mice showed a decreased risk of nephropathy in another research. [40]

An interesting study showed that administering the antioxidants N-acetylcysteine
and taurine to hyperglycemic mice significantly reduced the risk of insulin resistance. This result contributes to the hypothesis of that healthy micronutrients have preventive effects on the onset of DMII. [36]

7. Experimental pilot project

Introduction:

As told in paragraph 5.1, components of the extracellular matrix get glycated as a result of hyperglycemia occurring in diabetes mellitus type II. In this experiment, we will try to mimic a diabetic vessel *in vitro*. We will try to achieve this by glycating collagen type I, and coating it on cover-slips. These cover-slips will be attached to a perfusion chamber. We will let whole blood flow through this perfusion chamber and look at it live with a microscope. In this way, it is possible to compare glycated collagen to normal collagen in reaction with blood.

We will conduct a platelet aggregation experiment with normal Horm collagen and compare this with aggregation with glycated collagen. By measuring light emission we can investigate if platelets aggregate accelerated with glycated collagen. The more light passes through the cuvet, the more adhesion occurs, because clots of platelets will descend to the bottom of the cuvet.

Hypothesis:

Blood will clot easier on glycated collagen in perfusion runs and platelet reactions will occur accelerated with glycated collagen in aggregometry experiments. Glycated collagen leads to accelerated platelet aggregation compared to normal collagen.

Materials and methods:

We added 3.9 g glucose-6-phosphate (G6P) to 4 mL PBS, to make a 500 mM G6P solution. G6P is a reactive glucose specie that interacts faster with other compounds in glycation reactions. This solution must be added to a collagen solution in order to start a glycation reaction. For this collagen solution, we used 300 µg/mL of collagen solution. By shifting the concentration of both compounds in different test-tubes, different degrees of glycated collagen can be obtained.

We made the glucose-6-phospate sterile by pressing it through a sterile syringe filter, in order to exclude bacteria from the reaction.

We made 3 test-tubes; 1 control (normal collagen), 2 = G6P of 250 mM with collagen, 3 = G6Pof 500 mM with collagen. In the control, 500 μL SKS-buffer was added without G6P solution. In the two other test tubes, 50 μL and 100 μL of G6P solution was added to obtain solutions of 250 mM and 500 mM G6P with collagen. We let the 3 tubes incubate for 3 days at 37 degrees Celsius in order to let the collagen get glycated.

The perfusion experiments are done in 2 days and in duplo. This means that 4 cover slips of each collagen compound must be made. Cover slips need to be made to examine reactions of whole blood with glycated collagen with the live-perfusion microscope. For each cover slip, a drop of 40 μL is needed. This means that 160 μL is needed from each concentration of G6P and from the G6P free collagen in order to do the experiment on 2 days. In this way, we will have 340 μL left to do the aggregometry experiment.

After incubation over 3 days autofluorescence was measured in order to quantify the amount of glycated collagen. A peak at 660 nm was found when the solutions were excited at 330 nm. This is an atypical peak for excitation of collagen. A reason for this could be the fact that our concentrations were relatively low compared to other experiments that measured autofluorescence of collagen. Still, a difference between the control and the glycated collagen solutions was found at this peak. The peak of the glycated collagen compounds was higher.

After 3 days, we took the test-tubes out of the incubation oven and prepared them for the perfusion experiment. No bacteria were found in the test-tubes, because they were all transparent. This is a very important fact because bacteria would contaminate the experiment severally. After using the test-tubes, they were put in a refrigerator on -20 degrees Celsius, to prevent bacteria contaminating the tubes after they were opened.

After pipetting the two drops of 40 μL of the three different test-tubes on parafilm, we put washed cover slips on them. These cover slips were washed in HMDS. We made coated cover-slips on two days. The first day we let the cover slips incubate for 1.5 hour with the collagen, the second day for 2.5 hours. This incubation time is important for collagen to bind on the cover slips. After the time of incubation, the cover slips were blocked with HSA/PBS buffer. This is necessary in order to block parts of the cover-slips that were not coated with collagen. It will also wash excess of G6P and other waste away. We let the cover slips in this block-buffer overnight.

After letting the cover-slips in block buffer overnight, we examined the cover slips under a real-time perfusion microscope. We prepared a perfusion chamber to do this. We ordered

blood for both mornings as well. The experiments should be done with the blood in a short amount of time, because results will be best if the blood is still fresh. Otherwise, platelets will get inactive and will not adhere and interact properly anymore. We did the different runs in an atypical order to prevent that freshness of blood and my skill with the microscope would influence results. The proposed run of test-tubes for day 1 was 1,2,3,2,3,1. The second day the order of test-tubes is 1,3,1, 3, 2, 2.

Cover slips can be attached to the perfusion chamber, and a small flow of blood will the pass through the chamber. With the microscope, you can see the blood interaction with the cover slip live. In this way, we are able to compare the different kinds of collagen and their interactions with whole blood. We made movies and snap-shots of the different cover slips and their interactions with blood. The movies were filmed in the middle of the chamber in order to mimic an artery wall the best. The blood flow was 484 µL per minute and this creates a shear rate of 1600 which is the same as blood flow in an artery.

The other experiment that was conduct was an aggregometry of platelet-rich plasma (PRP) with the different kinds of collagen. To obtain this PRP, whole blood was centrifuged for 15 minutes at 1200 rotations per minute. The supernatant was taken from the tubes and this supernatant is the PRP.

With aggregometry you can measure light passing a cuvet. In PRP, little light can pass because PRP is not very transparent. If clotting reactions occur however, clots of platelets with adhesive compounds will descend to the bottom of the cuvet, facilitating more light to pass. Therefore, the more light passes, the more reactions occur. On a computer connected to the aggregometer you can see the level of light passing the cuvet in a graph.

In the aggregometer, one baseline compound is added in a cuvet as a reference to compare the other cuvets to. When measuring reactions in PRP, this reference is blood-plasma.

You can put 4 cuvets in the aggregometer. In the aggregometry experiments, we added different concentrations of the 3 different kinds of collagen to PRP. In this way, we can directly compare the different glycation states of collagen in one graph. We used all 4 places for cuvets in the experiments. In the first spot, PRP with PBS buffer was added as a control. In the second spot, we added 30 µL of normal collagen to 270 µL of PRP. In the third spot, 30 µL of 250mM G6P was added to PRP and in the fourth spot 30 µL of 500mM G6P was added.

We made different concentrations of each of the collagen compounds in order to conduct a titration research. These concentrations were respectively 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, o.5 µg/mL, 0.25 µg/mL and 0.1 µg/mL. We expect that with a decreasing concentration of the different compounds of collagen, greater differences in measured passing light will occur between the different compounds. We made 40 µL of these concentrations from the test-tubes for each concentration, and added 30 µL to 270 µL of PRP to fill the cuvet and start the aggregometry.

To start the aggregometry all the cuvets must be filled with 270 µL of PRP and a small magnet to accelerate clotting reactions. Then we have to baseline all measuring spots by pushing the baseline buttons on the machine. In this way, we create a starting point from which differences in light-emission can be measured. After this, we swiftly added 30 µL PBS to the first cuvet, 30 µL of normal collagen to the second cuvet, 30 µL of 250mM G6P-collagen to the third cuvet and 30 µL of 500mM G6P-collagen to the fourth cuvet.

These different concentrations were made by diluting the solution of the original test-tubes with PBS-buffer. After these experiments we also added a G6P solution to the different concentrations of collagen to correct for the excess of G6P that may be present in the test-tubes. We did not dialyze the G6P out of the test-tubes at the beginning of the experiments so this loose G6P may interact in clotting reactions as well. We added 500mM G6P to the control group, 250 mM to the 250mM G6P-group and buffer to the 500 mM group. In this way, the same concentrations of loose G6P were created in each test tube.

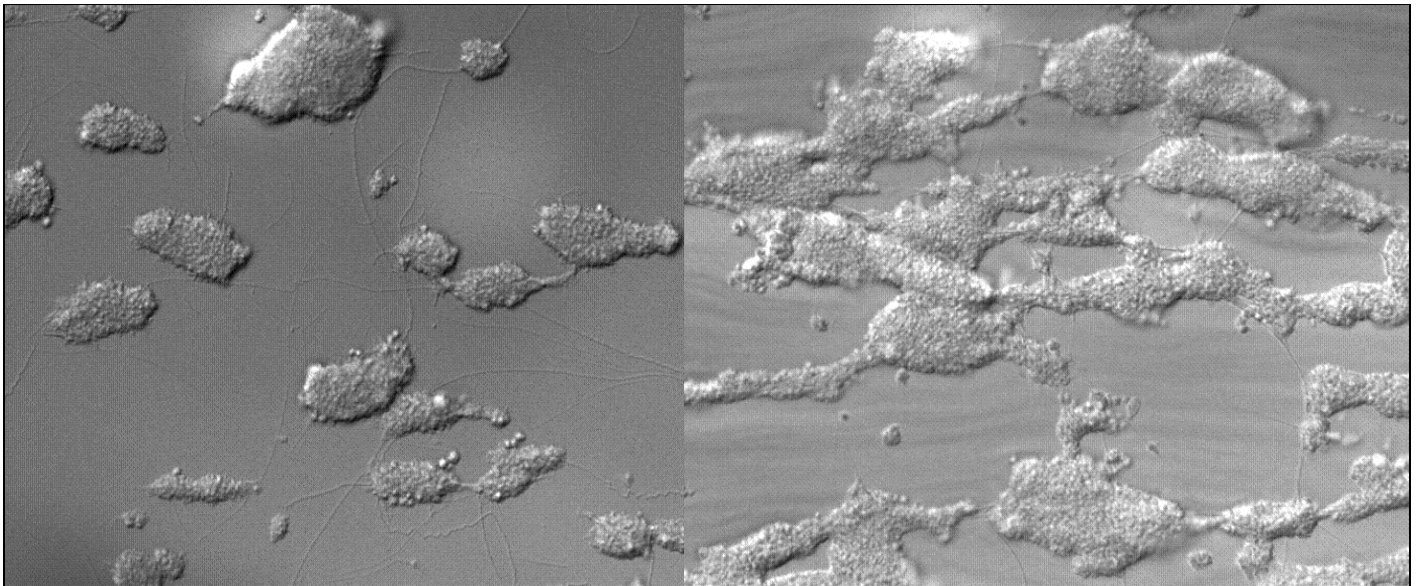
In this correction experiment, we made solutions of collagen of 0.5 µg/mL and 0.25 µg/mL. In order to do this, we added 2 µg/mL collagen to 18 µL of PBS and then added 20 µL G6P or buffer to the test-tubes. We added 1 µg/mL collagen to 19 µL of PBS and 20 µL of G6P for the 0.25 µg/mL solution. In this way, we obtained 40 µL of solution of which 30 µL was added to 270 µL of PRP.

Real-time perfusion results:

On the first day of the perfusion experiment, no differences between the different amounts of glycated collagen were found. There were very few clotting reactions in all three kinds of collagen. The collagen was washed away with the blood flow, leading to the conclusion that collagen was not bound firmly enough to the cover slips. Glycated collagen might have to incubate longer on cover-slips in order to bind properly. We also washed all cover-slips with HSA/PBS-blocking buffer two times that morning, which might have washed some of the

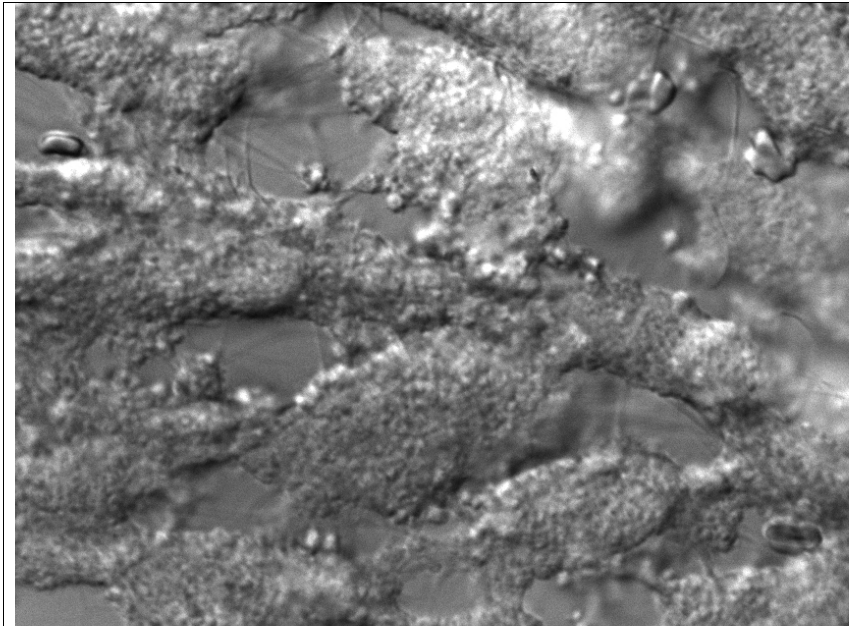
collagen of. We did 4 runs this day, because one tube of blood was already clotting and was not applicable to use for the experiment.

On the second day we did perfusion runs with the different cover-slips again. Collagen incubated longer on this cover-slips and this resulted in great differences of clotting reactions on the cover slips. Our first run was the control group of normal collagen, and in this run platelets clotted normally on the collagen (picture 1). After this control group, we did a perfusion with a cover-slip that was coated with 500mM G6P. On this cover-slip, great accumulations of bound platelets on collagen were found (picture 2).



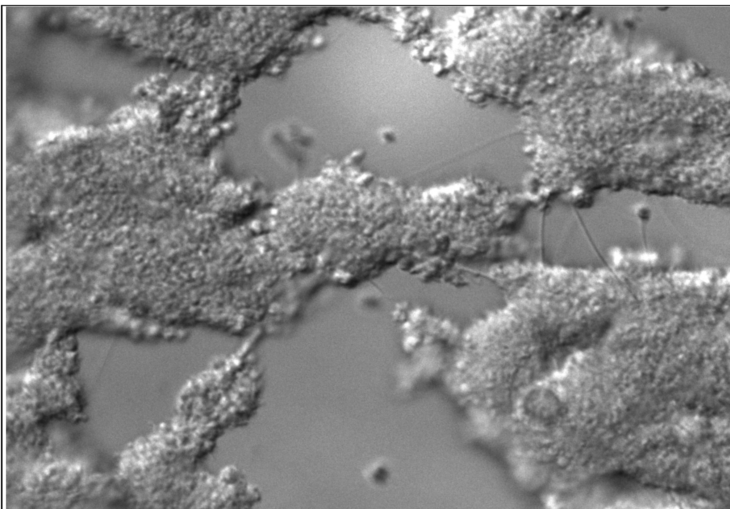
Picture 1: platelets adhering on normal, non-glycated, collagen	**Picture 2: platelets adhering on 500 mM G6P glycated collagen.**

In the pictures 1 and 2, you can see differences between the control group (left) and the 500 mM G6P bound collagen (right). Picture 1 was taken from run 1 (control), and picture 2 from run 2 (500 mM G6P).

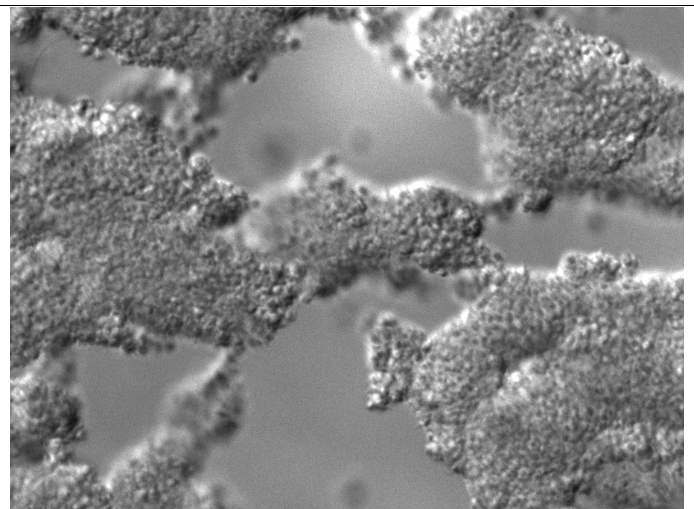


Picture 3: run with 500 mM G6P. This part of the chamber is completely occluded.

In picture 3, nearly the whole perfusion chamber is occluded. This picture was made in run 3, which was a run with 500mM G6P again. The pictures also show that the platelets were not only bound at the coating level of collagen, but grew in height as well. We changed the scope of the microscope to make snap-shots of different distances from the cover-slip. This made clear that platelets bind on different levels of the clots (see pictures 4 and 5).

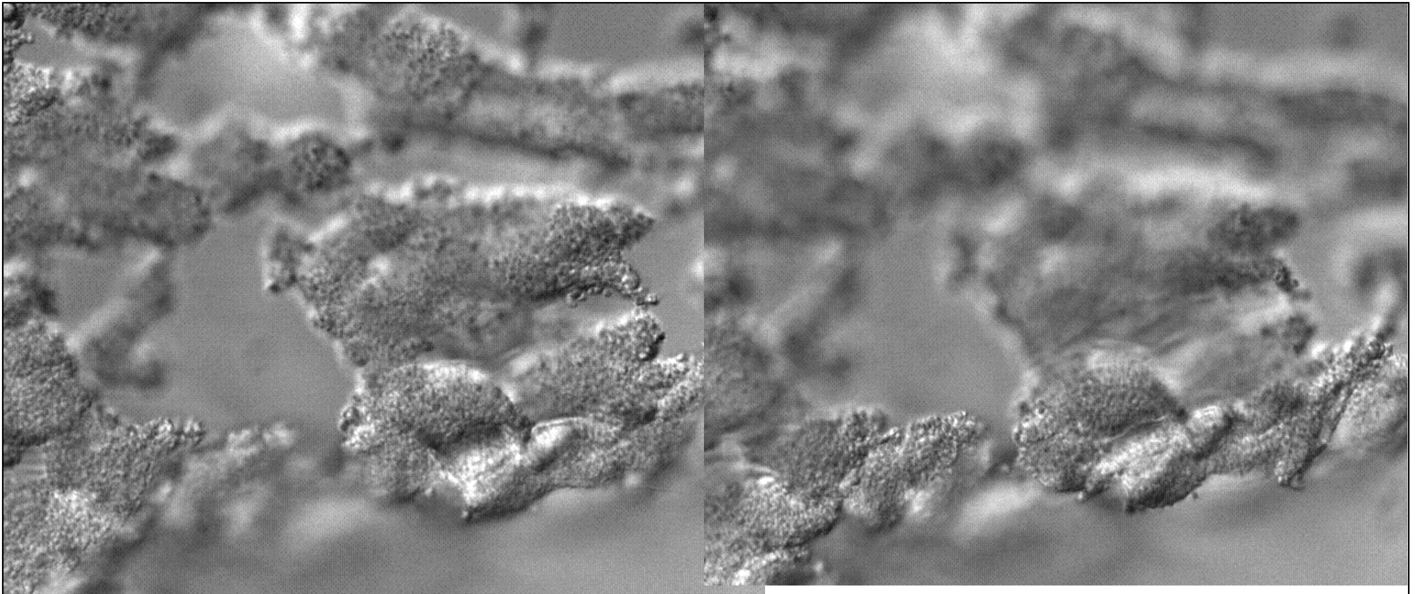


Picture 4: run with 500 mM G6P-glycated collagen. The fibrils you can see between the groups of adhered platelets are collagen.



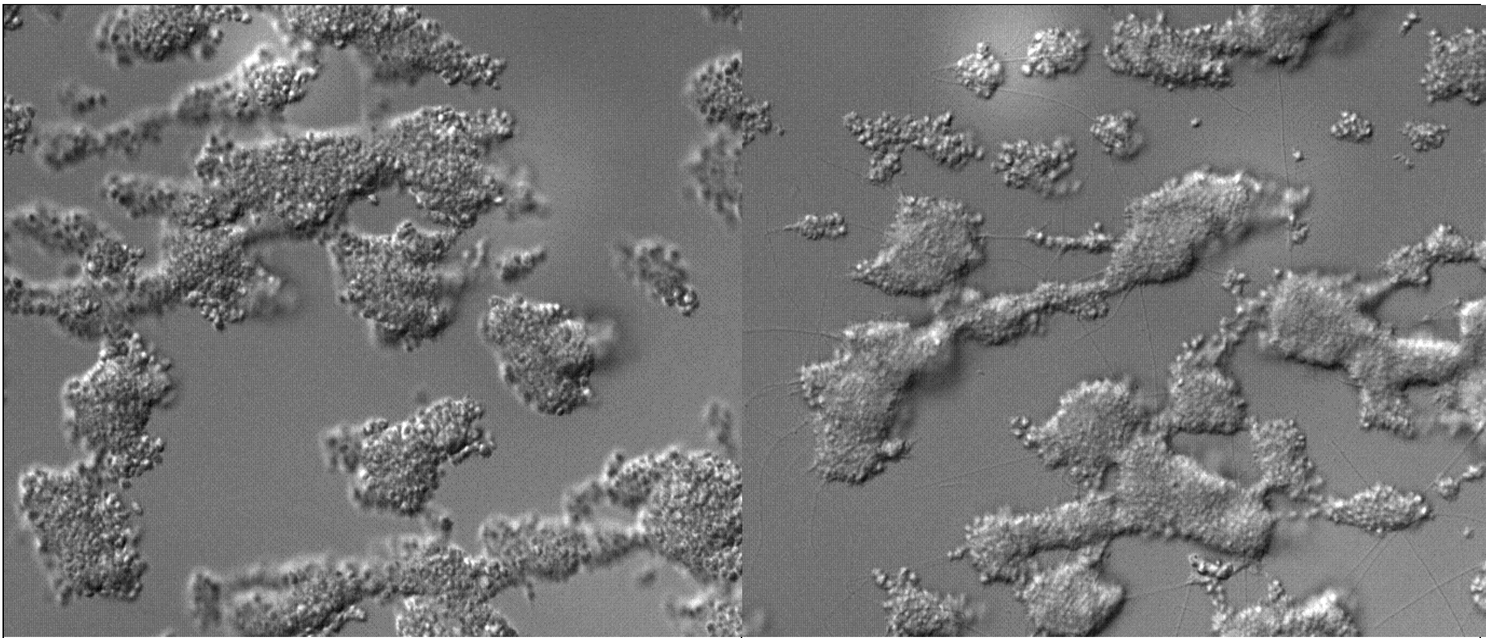
Picture 5: same run as picture 4, with a different scope.

On picture 4 you can see the collagen fibrils which are the stripes between the accumulations of platelets. The platelets bound here are platelets that adhere primarily to the coated collagen. In picture 5 a shot of greater distance was made, making collagen invisible and you can see a lot of platelets adhering as well. Picture 4 and 5 were both made at the same spot from greater distance as well, and you can see that platelets bind vertically on each other as well. All these pictures were made in run 3, with the 500 mM G6P-collagen solution.



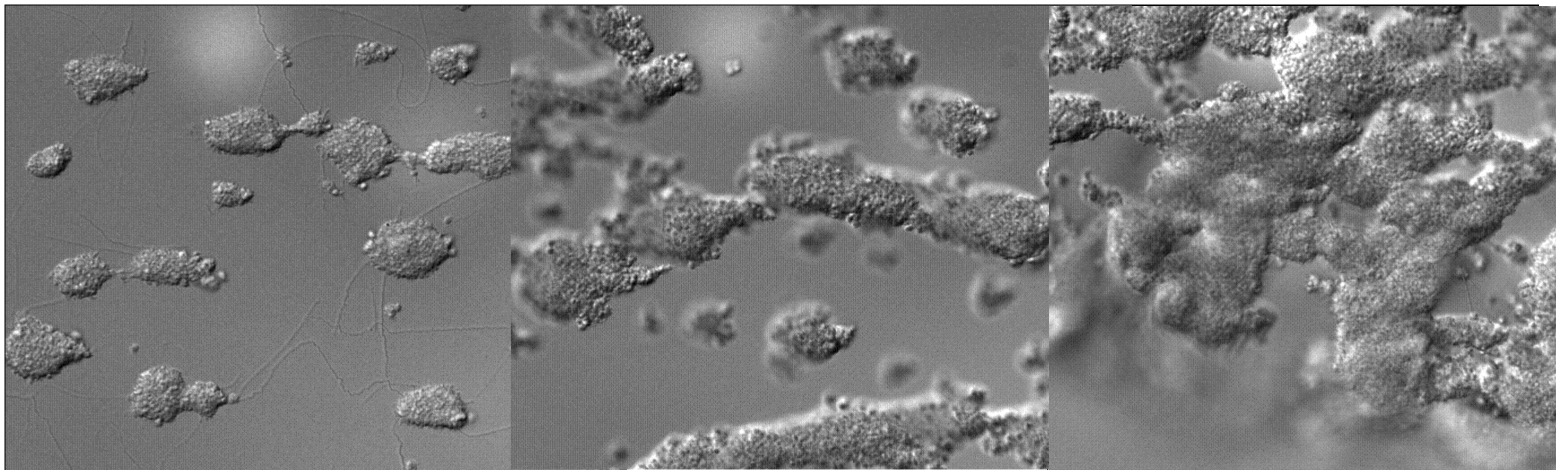
Picture 6: run with 500 mM G6P-glycated collagen	**Picture 7: run with 500 mM G6P-glycated collagen at same spot as picture 6 with a different scope.**

The last two runs were done with the cover-slips of group 2, the collagen with 250 mM of G6P. Snap-shots of the clotting reactions on these cover slips were made as well. Pictures 7 and 8 are two of them.



Picture 7: run with 250 mM G6P-glycated collagen	Picture 8: run with 250 mM G6P-glycated collagen.

Clearly, more adhesive reactions occur in the 250 mM G6P group as well. However, it seems that more aggregation occurred in the 500 mM G6P group, which may elucidate a dose-dependant role of glycated collagen in platelet aggregation. Below you can see three snap-shots of respectively the control group, the 250 mM G6P group and the 500 mM G6P group.



Picture 9, 10 and 11: normal collagen, 250 mM G6P-glycated collagen and 500 mM G6P-glycated collagen respectively.

In figure 12 and 13 you can the area covered by platelets is sketched versus the time of the experiment. You can see clearly that in the 500 mM G6P-glycated collagen run, a much greater area is covered than in the normal collagen run.

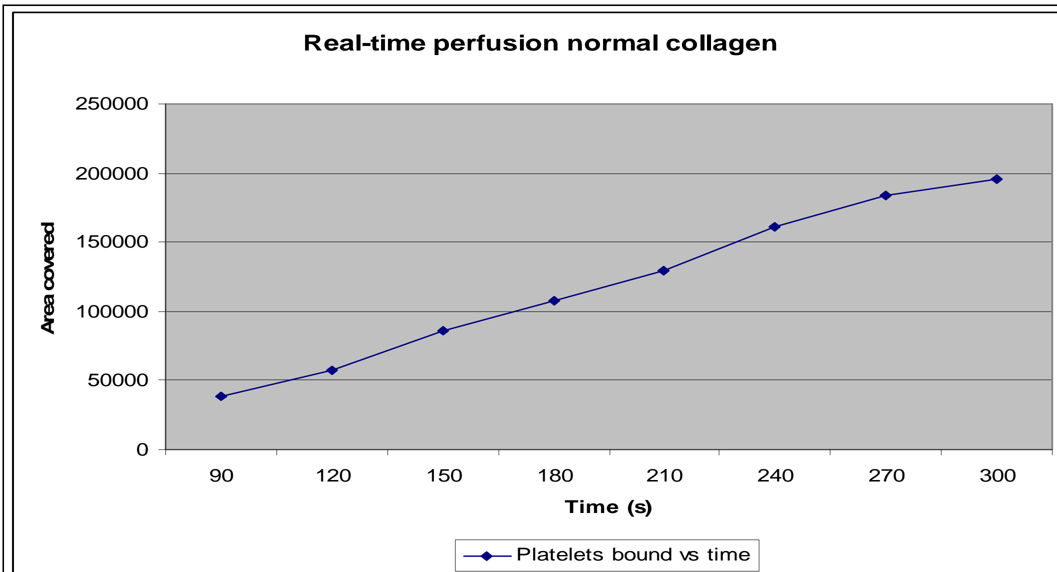


Figure 12: graph of the area covered by platelets in time on normal collagen.

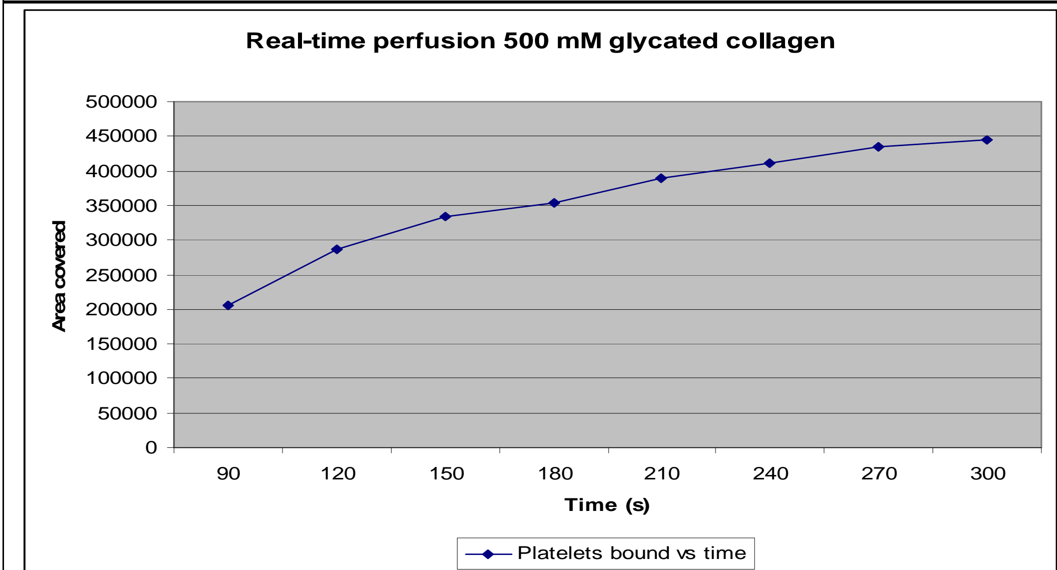


Figure 13: area covered by platelets in time on 500 mM G6P glycated collagen. The area covered is much greater in comparison to normal collagen.

The two runs with 250 mM G6P had very different result regarding the measurements of the covered area. One of the runs had results near normal collagen and the other run had results above the 500 mM G6P glycated collagen. These runs were conducted after all the other runs so the freshness of the blood used for the perfusion may have played a role in these contrary results. Another possibility is that something went wrong in the program that is used to examine the snap-shots. The data are not suitable to use in this paper and in a future experiment, the runs of 250 mM G6P glycated collagen should be done earlier in order to find a possible dose-dependant role of glycation of collagen. The snap-shots taken might reveal such a role.

In general, an increasing level of adhesion is found with an increasing level of glycated collagen (regarding the snap-shots). This may contribute to the conclusion that glycated collagen contributes to atherosclerosis under shear stress in arteries. It may point at a crucial role for vWF, which is important in platelet adhesion under shear stress.

Aggregometry results

In the aggregometry experiment, no differences were found between the 5 μg collagen/mL, 2.5 μg collagen/mL and 1.25 μg collagen/mL concentrations of the different test-tubes with collagen. However, when the concentrations were diluted to 0.5 μg collagen/mL, the 500 mM G6P collagen test-tube showed less aggregation than the other test-tubes. Less light passed the cuvet, and this same result was found when the solutions were diluted to 0.25 μg collagen/mL. This result is reproducible and shows that the titration set-up of the experiment is effective in finding differences between the degrees of glycated collagen. At 0.1 μg collagen/mL all types of collagen did not show aggregation reactions anymore. You can find a titration series of the aggregometry with glycated collagen on the next pages.

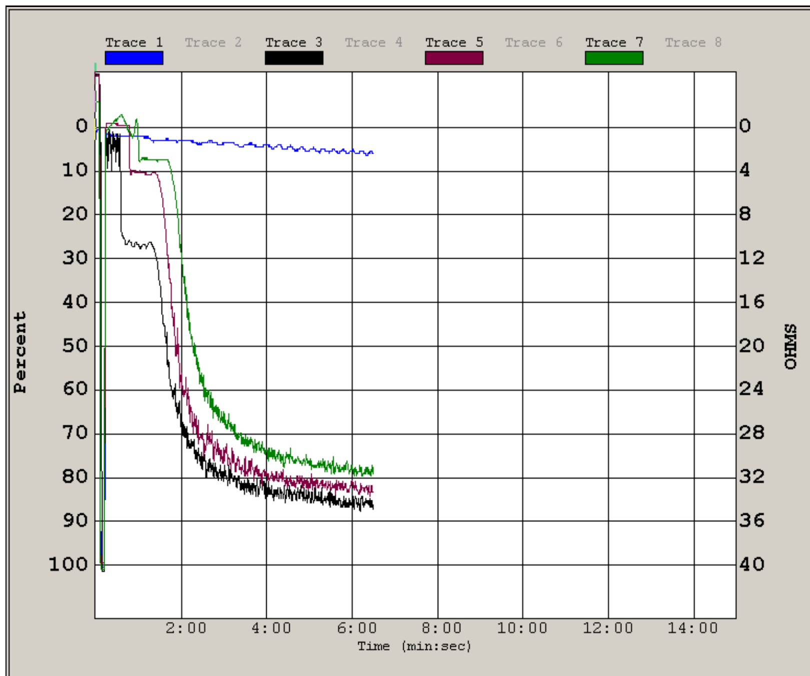


Figure 13: percentage of light passing(Y) in time(X), concentrations of all types of collagen is 1.25 µg collagen/mL PBS.
---: 500 mM G6P glycated collagen.
---: 250 mM G6P glycated collagen.
---: normal collagen

In figure 13, you can see the three different sorts of collagen and the percentage of light passing (Y-axis) in time (X-axis). No relevant differences are found at 1.25 µg collagen/mL. The differences seen between the three different kinds of collagen are not significant.

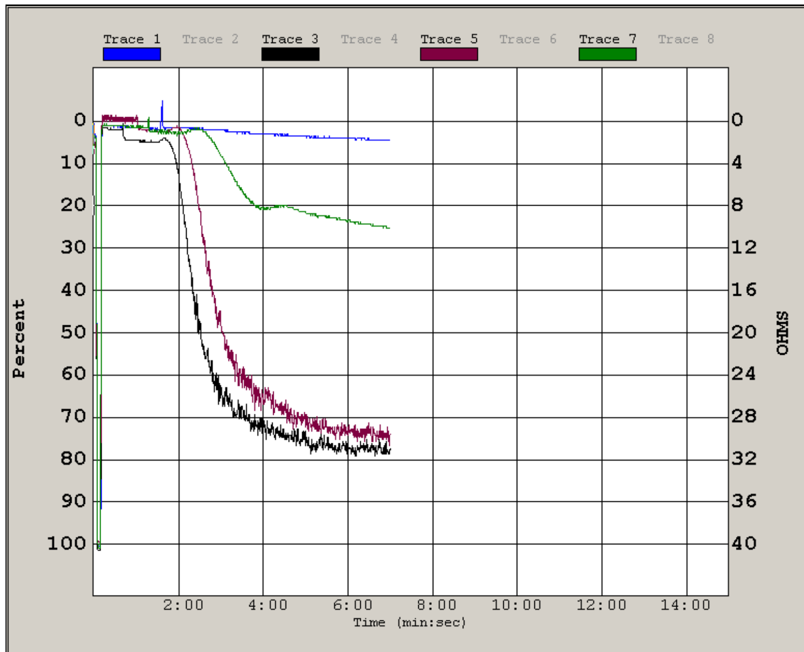


Figure 14: percentage of light passing(Y) in time(X), concentrations of all types of collagen is 0.5 microliter collagen/mL PBS.
---: 500 mM G6P glycated collagen.
---: 250 mM G6P glycated collagen.
--- : normal collagen

In figure 14, the concentrations of the three types of collagen in buffer were 0.5 µg collagen/mL. Less aggregation now clearly occurs in the 500 mM glycated collagen group. This result is reproducible. It seems contrary to what we expected; in the perfusion experiment, a higher degree of glycated collagen led to a higher degree of aggregation.

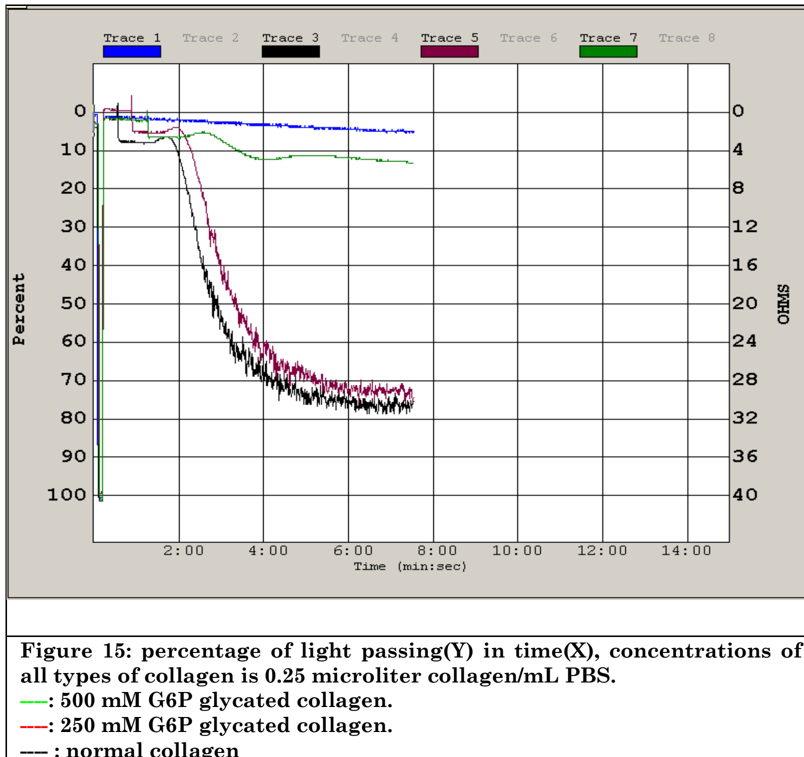


Figure 15: percentage of light passing(Y) in time(X), concentrations of all types of collagen is 0.25 microliter collagen/mL PBS.
—: 500 mM G6P glycated collagen.
—: 250 mM G6P glycated collagen.
— : normal collagen

In figure 15, the concentrations of collagen were 0.25 μg collagen/mL. The difference is very clear; less aggregation occurs in 500 mM glycated collagen in a titration sequence. The effect amplifies when the concentration of collagen dilutes more. At a dilution of 0.1 μg collagen/mL, no aggregation was measured in any of the collagen samples, so there is a limit to the amplifying effect of diluting.

This is an unexpected result because it seems adversary with the earlier perfusion experiment. Under shear stress, more platelets adhered to the 500 mM G6P collagen surface than to the other collagen surfaces. In the aggregometry experiment however, less aggregation occurs in the 500 mM G6P solutions from a concentration beneath or equal to 0.5 μg collagen/mL.

When we conducted the experiment with correction for G6P, no aggregation was found in any of the collagen types. A reason for this result may be the fact that these correction experiments were done after the runs with uncorrected collagen. The PRP was less fresh at this point and the platelets may have aggregated in the test-tubes already, causing all the light to pass in aggregometry. Otherwise, free G6P has an inhibitive effect on platelet adhesion and this does not seem to be plausible. In future experiments, the correction experiment should be conducted immediately after obtaining the PRP in order to find out if

the 500mM G6P collagen solution still reacts less in coagulation reactions. Or the free G6P should be dialyzed out of the test-tubes with collagen after the glycation reaction in order to exclude it from the aggregometry.

Discussion pilot project

In the second day of the experiments, in duplo experiments of cover-slips with different amounts of glycated collagen clearly showed that more platelet aggregation occurs when the degree of glycated collagen increases. On base of these results, our hypothesis that more aggregation occurs on glycated collagen is right. It seems that glycated collagen reacts dose-dependently with platelets, considering the snap-shots taken from the different types of collagen in a perfusion experiment. However, to elucidate a dose-dependant role of glycated collagen, future experiments with different levels of glycation should be done in a titration-like manner. Other degrees of glycated collagen between the control group, 250 mM G6P-glycated collagen and 500 mM G6P-glycated collagen could be observed as well.

Our second hypothesis that more platelets will bind to glycated collagen in an aggregometry experiment was not underpinned by our results. In fact, the opposite seems to be true from our uncorrected runs with the aggregometer. This may point at a crucial role for vWF in reactions of glycated collagen with platelets.

Von Willebrandfactor is important in platelet-aggregation under shear stress, and glycated collagen thus may bind vWF more easily than normal collagen, considering the perfusion runs. Our results from the aggregometry support this hypothesis, because when no shear stress is exposed to the glycated collagen, fewer reactions occur with 500 mM G6P. This adversary result is very interesting, because glycated collagen seems to inhibit aggregation when no shear stress is present.

Further research should be conducted into this topic, because only few experiments were done and no proper correction for G6P has been done in the aggregometry. Better perfusion runs with the 250 mM G6P-glycated collagen should be conducted.

An ELISA-experiment with vWF attaching to normal collagen and glycated collagen might lead to a more definite conclusion on the role of vWF in coagulation with glycated collagen. This experimental pilot project deserves further follow-up in order to obtain a better understanding in the effect of hyperglycemia on cardiovascular disease.

8. Discussion

Diabetes mellitus type II is a disease with an epidemic increasing incidence in the world. The consequences of it are disastrous: over 10% of deaths caused worldwide by myocardial infarction and stroke in the past 10 years were in diabetic patients.[43] 70-80% of the diabetic patients will die from cardiovascular disease.[5] But other severe complications adding to loss of quality of life, like retinopathy, nephropathy and neuropathy are caused by DMII as well. These microvascular diseases may eventually lead to blindness and neurological disorders.

The risk factors for developing the disease are typical for the western world; obesity, a bad diet, a low level of exercise and stress. These factors lead to oxidative stress with a lot of disadvantages, including insulin resistance. Insulin resistance in its place is associated with hyperglycemia, hypercoagulability and atherosclerosis.

There are many steps between insulin resistance at the onset of DMII and the symptoms of DMII that eventually lead to cardiovascular disease. Advanced glycation end products (AGE) are formed in circulation because of hyperglycemia, and AGE reacts with RAGE thereby expressing a lot of complexes, cytokines and chemokines that eventually lead to inflammation.

Hyperglycemia will influence the probability of thrombosis in circulation as well, by inducing hyperactive platelets and upregulating coagulation factors. A pro-thrombotic phenotype is thus created by different mechanisms.

It is possible to interfere at different levels to suppress DMII. Therapies with AGE-crosslink breakers are being studied, antiplatelet therapy to inhibit platelet aggregation is a strategy and anti-inflammatory therapies might help to alleviate symptoms of DMII.

However, the best way to handle the upcoming epidemic in DMII seems lifestyle intervention. In this way, prevention of the disease may be accomplished and this is an easier and cheaper strategy to face future problems. Diet plays a major role in the onset of DMII. A diet with low levels of vitamins results in less antioxidant capacity of the plasma, increasing the chance of oxidative stress. The hazardous AGEs can also be food-derived so an adapted diet may also help to diminish this effect.

Obesity is one of the greatest risk factors of developing DMII and explains why the disease is gaining ground in the western world. Obesity is a risk factor for cardiovascular disease itself, but in conjunction with DMII, risk is increased even further. Solving the obesity epidemic might thus also be the way to solve the DMII epidemic with its undoubtedly disastrous consequences.

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