ΠΑΝΕΠΙΣΤΗΜΙΟ ΙΩΑΝΝΙΝΩΝ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

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ΣΟΦΙΑ ΑΝΤΩΝΙΟΥ

ΙΑΤΡΟΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΟΚΤΩΒΡΙΟΣ 2022

«Η έγκριση της διδακτορικής διατριβής από την Ιατρική Σχολή του Πανεπιστημίου Ιωαννίνων δεν υποδηλώνει αποδοχή των γνωμών του συγγραφέα Ν. 5343/32, άρθρο 202, παράγραφος 2 (νομική καταχώρηση του Ιατρικού Τμήματος)».

Ευχαριστίες

Το πολυετές ταξίδι της διατριβής φτάνει με την ολοκλήρωση της συγγραφής στο τέλος του. Πρόκειται για έναν δύσκολο και συνάμα συναρπαστικό προσωπικό αγώνα και έργο, που ωστόσο χωρίς την ουσιαστική συνδρομή μιας σπουδαίας ομάδας ανθρώπων η ολοκλήρωση του δε θα ήταν δυνατή και συνεπώς τους οφείλω την ειλικρινή μου ευγνωμοσύνη.

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Στους γονείς μου, Γερασιμούλα και Κωνσταντίνο

Στον αδερφό μου, Βλάσιο

CONTENTS

Abbreviations

ABI: Ankle-Brachial-Index

ACE: Angiotensin-converting enzyme

ADMA : asymmetrical dimethylarginine

α-GI: Alpha-glucosidase inhibitors;

AIx : augmentation index

AS: arterial stiffness

ARBs: Angiotensin II receptor blockers

ARIC: Atherosclerosis Risk In Communities

bFGF: basic fibroblast growth factor

BMI: Body mass index;

c AMP: cyclic adenosine 3′,5′-monophosphate

CHD: coronary heart disease

CACS: coronary artery calcification score

CVD: cardiovascular disease

cGMP: guanosine 3',5'-cyclic monophosphate

cIMT: carotid intima media thickness

cfPWV: carotid-femoral pulse wave velocity

C1: large artery elasticity index

C2: small artery elasticity index

DPP4: dipeptidyl peptidase 4.

ED: endothelial dysfunction

eNOS: endothelial NO synthase

GP1b: Glycoprotein Ib Platelet Subunit Alpha

FMD: flow mediated dilatation

HbA1c: glycated haemoglobin

HDL: high-density lipoprotein

HSPGs: heparan sulfate proteoglycans

IMT: Intima–media thickness

IGT: impaired glucose tolerance

IR: Insulin Resistance

LDL: Low-density lipoprotein;

NAFLD : nonalcoholic fatty liver disease

NO: Nitric oxide

NGT: normal glucose tolerance

MetS: Metabolic syndrome

PAD: Peripheral arterial disease

PAI-1: Plasminogen activator inhibitor-1

PAF: platelet activating factor

PGI2: prostacyclin

RAAS: Renin angiotensin aldosterone system.

RCT: Randomized controlled trial;

SD: Standard deviation

T2DM: type 2 Diabetes mellitus

TRG: triglycerides.

VEGF: vascular endothelial growth factor

vWF: Von Willebrand factor

uPA: urokinase plasminogen activator

Chapter 1: Diabetes and cardiovascular risk

Diabetes mellitus Type 2 (T2DM) is a recognized risk factor for cardiovascular morbidity and mortality. Patients with T2DM have a two to 4-fold increased risk for coronary heart disease and stroke and similar increase in mortality as well. Compared to healthy individuals they have a reduction of life expectancy of 4-8 years (Gu 1998).

The statement that Diabetes is a cardiovascular risk equivalent was based on a Finnish Study, which suggested that diabetic patients without previous myocardial infarction have as high a risk of myocardial infarction as non-diabetic patients with previous myocardial infarction. Therefore they be should be equally aggressively treated (Haffner SM 1998). Since diabetic subjects seemed to appear with a worse prognosis after cardiovascular event (CV), the 2001 NCEP-ATP III recommended they should be regarded as high risk patients (NCEP Panel, 2001).

Later, this argument has been questioned by Bulugahapitiya et al, who performed a metanalysis of 13 studies with more than 45000 participants back in 2009. The duration of follow-up was 5-25 years and the age range of participants was 25-84 years. The risk for developing CVD events for diabetic subjects without prior myocardial infarction was 43% lower compared to non-diabetics with previous event. This pointed out the necessity for appropriate risk stratification rather than "blanket approach", as the authors suggested (Bulugahapitiya 2009).

Age is indeed the strongest non- modifiable cardiovascular risk factor, since with increasing age the risk for CVD increases as well, for both men and women. A large population study suggested that there is an age of transition from lower to higher risk and that seems to be 48 years in men and 54 years in women. This should imply that for patients above those limits greater effort must be done to avoid future events (Booth 2006). Gender seems to play an important role as well: although men with diabetes seem to be found more likely with higher incidence of new myocardial infarction, in women the mortality rate is greater in that case (Huxley 2006). The role of hypertension has also been extensively described: for each reduction of blood pressure by 10 mmHg significant reduction in CVD events, stroke, coronary heart disease has been found (Emdin 2015). As a result, ADA had set a goal of systolic blood pressure of 40 mmHg and diastolic of 90 mmHg in diabetic subjects with hypertension. Cholesterol-levels seem to be one of the most reversible risk factors for CVD in T2DM, with statin treatment reducing almost linearly the relative risk per each mmol/l reduction of LDL (Cholesterol Treatment Trialists 2008).

So it is becoming more evident that the CVD risk among diabetics is highly heterogenous. The proper categorization is of utter importance, since it contributes to the recognition of those who might benefit the most from intensive cardiovascular prevention. This was later on reflected in the published guidelines from ADA und ESC, according which age, diabetes duration of longer than 10 years or presence of microalbuminuria/ renal disease considerably contribute to increased risk for future events, and therefore in their presence diabetes should be rather regarded as CVD equivalent (Standards of medical care in diabetes 2016, Piepoli 2016).

Diabetes duration seems to play a key role in terms of CVD risk and mortality. Both early and late onset of diabetes are associated with increased risk for CVD events and mortality, but only early onset (meaning > 10 years of diabetes duration) appears to be a coronary heart disease (CHD) equivalent (Wannamethee 2011). On the other hand, diabetes duration is defined as the time since diagnosis of diabetes. It is, clear, though, that persons are in fact already predisposed to significant cardiovascular risk even at the pre-diabetic stage (Haffner 1990). Moreover, they often remain asymptomatic and when they develop clinically evident CVD, their prognosis and outcomes are worse compared to individuals with CVD but without DM2 (Raza 2015, Arnold 2014), as already mentioned. This underlines the heterogenity in diabetes-related- CVD risk.

And what about the role or degree of hyperglycemia?

The deleterious effects of hyperglycemia develop even before the onset of diabetes. Norhammar et al showed that abnormal glucose tolerance was a strong and independent predictor of subsequent cardiovascular complications and death. In this study, many patients in the post-infarction period were found to have undiagnosed diabetes and impaired glucose tolerance, suggesting that fasting and post-challenge hyperglycaemia in the early phase of an acute myocardial infarction could be used as early markers of high-risk among individuals (Norhammar 2002). Glucose fluctuations and hyperglycemia lead to endothelial dysfunction, which is known to be the first step of atherogenesis (Brownlee 2001, Ross 1999).

Hyperglycemia has been considered the main factor related to impaired intracellular signaling dysfunction, with subsequent production of increased toxic metabolites (e.g. advanced glycated products) that ultimately lead to microvascular dysfunction and disease (i.e. retinopathy, neuropathy, nephropathy) (Barrett 2017).

The degree and duration of hyperglycemia is strongly associated with both microvascular and macrovascular complications in T2DM. Despite evidence linking hyperglycemia with CVD several large randomized trials failed to show a cardiovascular risk benefit from strict glycemic control.

Large trials that included patients with T2DM were designed to clarify whether glycemic control with blood glucose levels near to normal would reduce CVD risk. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study (Gerstein HC 2008), Veterans Affairs Diabetes Trial (VADT) (Duckworth 2009) and the Action in Diabetes and Vascular Disease:

Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) (Patel 2008). None of these trials showed significant reduction in cardiovascular events between those assigned to receive intensive treatment compared to those receiving standard treatments.

There have been many speculations in order to explain these rather disappointing results. The first one was the incidence of hypoglycemia, which could attribute to the observed mortality in the intensive treatment group. In fact ACCORD was terminated because of severe hypoglycemia. A post hoc Analysis of this trial, though, showed that there was a decrease in mortality in the intensified group (Riddle 2010). Moreover patients participating in this study were suffering from ohter conditions that affect awareness of hypoglycemia and increase the risk of severe indicendence such as renal failure, congestive heart failure etc (Calles Escandon 2010) or even adverse effects of insulin or sulphonylureas (Skyler 2009).

The UK Prospective Diabetes Study, though, included newly diagnosed type 2 diabetic patients also failed to show a significant reduction of CVD events in the intensive treatment arm. On the other hand, the extended follow up 10 year-post-trial observational period resulted in significant reduction of diabetes related mortality and myocardial infarction of about 17 % and 15 % respectively. These led to suggestion that there is a legacy effect - known as metabolic memory, a possible protective role of good glycemic control at an early stage of the disease (Holman 2008).

Taking these into consideration it is becoming more evident that diabetes duration and probably medication used to achieve glycemic control might play an important role regarding risk stratification.

Since diabetes is reaching epidemic proportions, the use of cost effective tools in order to screen patients at high risk is crucial.

Endothelial dysfunction is an early event in the progression of atherosclerosis. Over the past decades several non-invasive markers of subclinical atherosclerosis have been developed, with anticipation that their use could be a cost effective alternative in order to screen high risk patients. These markers include impaired vasodilation, carotid intima media thickness (cIMT), arterial stiffness (AS) and reduced arterial elasticity.

To this end we decided to use these markers in order to assess endothelial dysfunction in T2DM and examine the effect of glycemic control and diabetes duration, in both T2DM with and without cardiovascular disease.

Chapter 2: Non-invasive markers of subclinical atherosclerosis – endothelial function

Atherosclerosis has always been a major cause of morbidity and mortality in developed countries. Traditional risk factors such as dydlipidemia, hypertension, diabetes mellitus and smoking interact to initiate and promote atherosclerotic disease, which in turn leads to cardiovascular disease (CVD). Several prediction models have been suggested to assess CVD risk, with special interest on how these can be used widely in large population and hopefully lead to preventive strategies.

Over the past decades important knowledge has been acquired regarding comprehension of the pathological pathway from subclinical to clinically evident atherosclerosis. The need for a non-invasive and individual approach, so as to study or even quantify the subclinical atherosclerosis, lead to development of several markers. These are thought to be useful tools for the assessment of cardiovascular risk, probably add additional information to known risk algorithms and further predict risk for CVD in individuals without symptomatic CVD or diabetes mellitus.

In this chapter we will discuss the most important markers of subclinical atherosclerosis.

2.1 Carotid intima-media thickness (Intima Media Thickness, cIMT)

Intima–media thickness (IMT) is a measurement of the thickness of tunica intima and tunica media, the innermost two layers of the wall of an artery. There are two ways to assess it: either by external ultrasound or occasionally by internal, invasive ultrasound catheters.

The use of B-mode ultrasound for IMT assessment (Intima Media Thickness, IMT) is a non-invasive, sensitive and reproducible technique that can be used to identify and quantify subclinical vascular disease and detect carotid plaques. It is safe and easy to use. Since its introduction in 1990, cIMT it has been widely used as a surrogate marker for atherosclerosis.

In February 2008, the American Society of Echocardiography ASE (ASE) published a large study, the “Carotid Intima Media thickness Task Force” that provided recommendations for the

use of common carotid artery ultrasound in the study and detection of subclinical cardiovascular disease (Stein 2008).

IMT measurements are obtained with the patient in the supine position with the neck slightly hyperextended and the head rotated to the opposite side. A soft substrate should be used during the test in favour of patient´s comfort. High frequency ultrasound probes are used (usually frequency of >7 MHz). IMT can be measured using external ultrasound in large arteries relatively close to the skin, with depiction of the three layers, tunica intima, tunica media and adventitia (Figure 1-1).

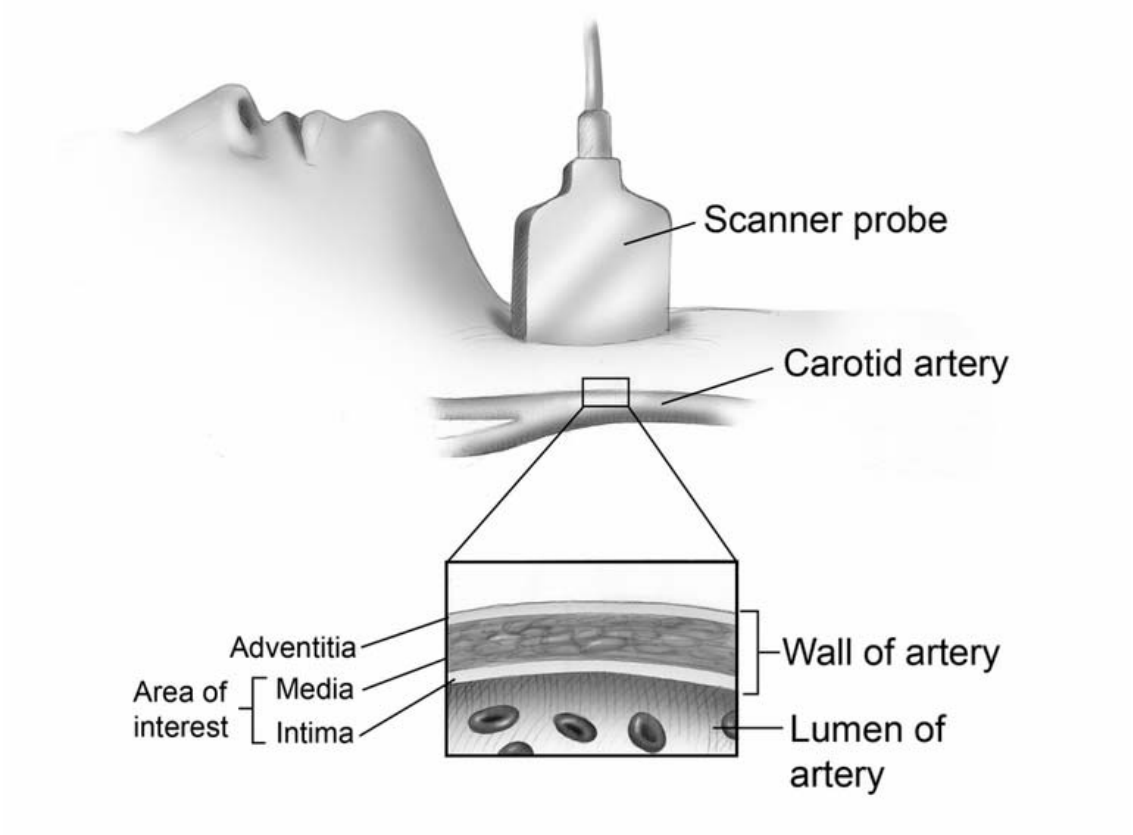

Figure 1-1. Schematic representation of the ultrasound recording of carotis artery (reproduced from: Kim et al, **2016**)

Measurements are obtained in the distal CCA, 1-2 cm from the flow divider, in the far wall, using automated edge detection software (Figure 1-2). It has most commonly been measured from B-mode images. M-mode guided images of the distal CCA may be obtained as an alternative. Whatever the method, because of the small diameter of the intima-media layer, the thickness should be measured using computer assistance with electronic calipers or semi-automated edge-detection algorithms. Due to alterations of the lumen diameter and associated variations of IMT during cardiac cycles, IMT should preferably be measured during end-diastole (considering minimal end-diastolic and maximal peak-systolic diameters). For this purpose, an ECG is recorded during the measurement.

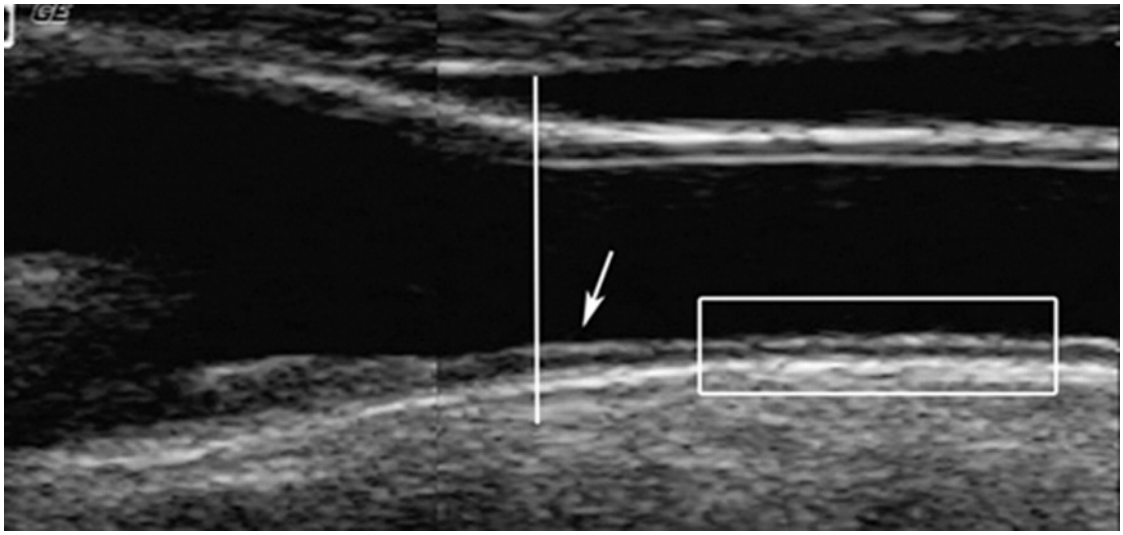

Figure 1-2. Ultrasonographic presentation of intima-media thickness using semi-automated software (reproduced from *Polak et al, 2011*).

The measurements should be performed by an experienced operator based on internationally validated criteria based published literature (Touboul 2004, Spence 2006). Images should be obtained from both vessels (left and right). Plaque should be reported separately and is defined as follows:

1. Focal structure encroaching in the lumen >0.5 mm, or
2. 50% of the surrounding intima-media thickness, or

3. Plaque thickness >1.5 mm

Generally, the presence of plaques is rare in the body of a common carotid artery, as these are found either in the carotid bulb or in the internal carotid artery and are therefore synonymous with extensive disease.

The results of the measurement are usually given in IMT mm. It increases with age and on average is larger in men than in women (Ebrahim 1999). Modest racial differences in IMT have been also reported. Therefore, a standardized table of IMT measurements accounting for age, gender, must be used to determine the true value of single IMT measurements (see Table 1-1).

Table 1-1. IMT normal range regarding sex and age group. **RT CCA IMT: right common carotis artery intima media thickness, ** Lt: left.*

Sex	**Age (years)**	**RT CCA IMT* (mm)**	**LT CCA IMT** (mm)**
Men	<30	0.39-0.48	0.42-0.49
	31-40	0.42 - 0.50	0.44 - 0.57
	41-50	0.46 - 0.57	0.50 - 0.61
	>50	0.46 - 0.62	0.53 - 0.70
Women	<30	0.39 - 0.43	0.30 - 0.47
	31-40	0.42 - 0.49	0.44 - 0.51
	41-50	0.44 - 0.53	0.46 - 0.57
	>50	0.50 - 0.59	0.52 - 0.64

According to the recommendations of the National Cholesterol Education Program Adult treatment Panel (NCEP), any value of CIMT above the 75th percentile for age and sex can be considered an additional risk factor (NCEP 2002).

The advantages of this method for the study of cardiovascular risk are:

a) It is a simple, radiation- free and well tolerated technique, which can be repeated without risk or discomfort for the patient.

b) As it is non-invasive, it does not pose a risk of complications e.g. spasm of a vessel.

c) The required equipment is relatively inexpensive, while ultrasound technology is now familiar to the majority of doctors and researchers, further facilitating the training and learning of the method.

The main disadvantages of this method are that they include:

(a) Absence of a defined study and implementation protocol, which could lead to underestimation or inaccurate estimation of the thickening- progress of the common carotid endothelium after pharmaceutical or other interventions.

b) The isolated ultrasound scanning of the common carotid artery poses a serious realistic risk of underestimation of extensive atherosclerotic disease, with the presence of plaques in upper parts of the carotid "tree".

This method has also considerable limitations. Occasionally, imaging a patient is difficult because of the size or contour of the neck. Secondly, calcium deposits in the wall of the carotid artery may make it difficult to evaluate the vessel. Last but certainly not least, cIMT is only an indirect assessment of the possible atherosclerotic burden in the coronary arteries.

As already mentioned, a key risk factor affecting the thickness of the wall of the common carotid artery is age. In healthy subjects, studies have calculated an increase in the wall of 0.015 mm per year, a process that is accelerated with the presence of risk factors, as for people with known coronary heart disease (CHD) this annual increase ranges from 0.017 mm to 0.026 mm per year (Bots 2003).

Interesting data from the ARIC (Atherosclerosis Risk In Communities study), a large population study including people aged 45-65 years, suggested that the mean value for c IMT was by 0.07 mm increased for each age group of diabetic patients compared to non-diabetics, after adjustment for other cardiovascular risk factors, such as age, race, the presence of T2DM, hypertension and tobacco use (Nambi 2010). In this direction, Brohall et al showed that diabetic patients have cIMT values 0.13 mm higher compared to non- diabetics (Brohall 2006).

The use of the common carotid wall is a now recognized predictor for the occurrence of stroke and cardiovascular disease and has been used in numerous studies to detect patients at high risk of developing these complications.

Regarding the predictive value of the incidence of macrovascular complications, it is worth noticing that according to the above-mentioned ARIC study, in diabetics who had already suffered acute MI (acute myocardial infarction) in the past, the mean value of c IMT was significantly higher compared to non-diabetics (cIMT 0.83 mm vs 0.78 mm respectively, $p < 0.05$), with an additional annual progressive value of 0.07 mm for each diabetic age group. Similarly, the cardiovascular health study, in which patients were studied for a follow-up period of 6.2 years, showed an increase in the incidence of MI with an increase in c IMT in patients > 65 years of age, without prior evident cardiovascular disease, independently of other risk factors such as age, sex, arterial hypertension, diabetes mellitus etc. (Brohall 2006).

A meta-analysis from Lorenz and his colleagues demonstrated that a difference of 0.1 mm in cIMT is accompanied by an increase in the risk of stroke from 13 to 18%, with a significant increase also for the risk of myocardial infarction (O´Leary 1999). These data are suggestive of the important role of cIMT as surrogate marker and indicate the synergistic action of other risk factors, such as T2DM, in its progression.

At this point it should be noted that there is a difference between IMT as a surrogate marker of atherosclerosis and IMT progression. Although carotid IMT is predictive of future cardiovascular events, the usefulness of measuring change in carotid IMT over time was debated, as meta-analyses initially have not found that change in carotid IMT was predictive of cardiovascular events (Lorenz 2007). Therefore, the use of change in carotid IMT as a surrogate endpoint of

drug efficacy in clinical trials or management of cardiovascular disease was rather debated (Lorenz 2012).

2.1.1 Intervention trials and IMT

Due to the association with cardiovascular events, cIMT was used in many studies of therapeutic interventions as a primary endpoint. Like already mentioned, the relatively low cost of this method and its availability, make it an attractive option for large population trials.

Several data have been published in recent years, supporting the beneficial effect of statins on IMT, such as the METEOR Trial, according to which the administration of 40 mg rosuvastatin vs placebo led to a delay in IMT progression over a follow up period of two years (-0.0014mm/year vs 0.0131 mm/year, $p<0.0001$) (Crouse 2007). The ARBITER 6-HALTS trial, which examined the effects of combination hypolipidemic therapies (addition of ezetimibe or niacin on statin treatment showed changes in IMT of -0.0007±0.0035 mm and -0.0142±0.041mm respectively ($p=0.003$) (Villines 2010). The ASAP-TRIAL on the other hand, compared atorvastatin vs simvastatin (80 mg vs 40 mg daily). After treatment with atorvastatin for 2 years, IMT decreased (-0.031 mm [95% CI -0.007 to -0.055]; $p=0.0017$), whereas in the simvastatin group it increased (0.036 [0.014-0.058]; $p=0.0005$). The change in thickness differed significantly between the two groups ($p=0.0001$). Atorvastatin showed greater reductions in cholesterol concentrations than did simvastatin in terms of LDL-Cholesterol, concluding that that aggressive atorvastatin treatment was accompanied by regression of carotid intima media thickness in patients with familial hypercholesterolaemia (Smilde 2000).

The effect of antihypertensive treatment on c IMT has been extensively studied, as well (Zannad 2000, Ariff 2006): calcium antagonists have been shown to even a reduce cIMT: According to the ELVERA trial, amlodipine has caused a decrease in IMT after one year of administration to newly diagnosed hypertensive, older patients, while lisinopril by 0.065 mm, with the greatest benefit occurring after one year of follow-up (Terpstra 2004).

The relation of IMT and glucose control as well as antidiabetic treatment will be further discussed in the next chapters.

2.2. Flow mediated dilatation (FMD)

Another non-invasive method for the assessment of endothelial dysfunction is the flow-dependent vasodilation also known as flow mediated dilatation (FMD).

This is a method originally described by the research team of the Cardiothoracic Unit of the Children's Hospital in London, led by DS Celermajer (Raitakari 2000), who developed an ultrasound- method to assess the reaction of the brachial artery to an ischemic stimulus (Figure 1-3).

The participant/patient should rest in a supine position in a comfortable bed. A fasting period of at least 4 hours (ideally 8 hours), without prior consumption of coffee or nicotine is suggested, as these might affect the artery´s reaction to induced ischemia. They should also abstain from practicing exercise for 12 hours. Another yet important detail is that premenopausal women should be examined during the same phase of menstrual cycle to limit the impact of endogenous estrogens and progesterone (Hashimoto 1995, Adkisson 2010). This test should be performed in a quiet environment, with stable temperature so as to avoid external stress- induced vasoconstriction. The examined arm (usually right side) should be extended laterally at about 80° of shoulder abduction, while an occlusion cuff should be placed in the upper third of forearm, proximally to the elbow. The placement of a cuff closer to the wrist does not cause an equally adequate ischemic stimulus, resulting in lower FMD values, while in other locations it is associated with higher discomfort of patients undergoing this examination (Mannion 1998, Vogel 2000) which is why this study protocol has prevailed.

During the whole examination a 3-lead ECG should be recorded to define end-diastole. A longitudinal scan of the brachial artery should be at baseline obtained by using B-Mode ultrasound; ideally an image between 2 and 10 cm above the antecubital fossa. Depending on

experience and operator, the probe can be secured by using a holder. Satisfactory images are those depicting the endothelial layers of the brachial artery and especially the intima layer at both sides of the artery. Prior to cuff inflation, diameter and blood velocity-flow should be recorded for at least 30 seconds.

Then the forearm occlusion cuff should be rapidly inflated to supra-systolic pressures for 5 minutes. The patient/participant should be informed that a slight discomfort might occur especially after 4 minutes of inflation. After deflation, further images should be acquired including blood flow initially after 5 seconds and then every 30 seconds for a total of 2 minutes.

Thus, 'FMD' is defined as the % change in the diameter of the vessel:

FMD = [Peak diameter after reactive hyperemia - baseline diameter)/ baseline diameter] x 100 %

FMD represents the maximal change in brachial artery diameter after release of the forearm cuff compared to baseline diameter. A special software is needed for precise calculations of the arterial diameter, including at least 10 cardiac cycles.

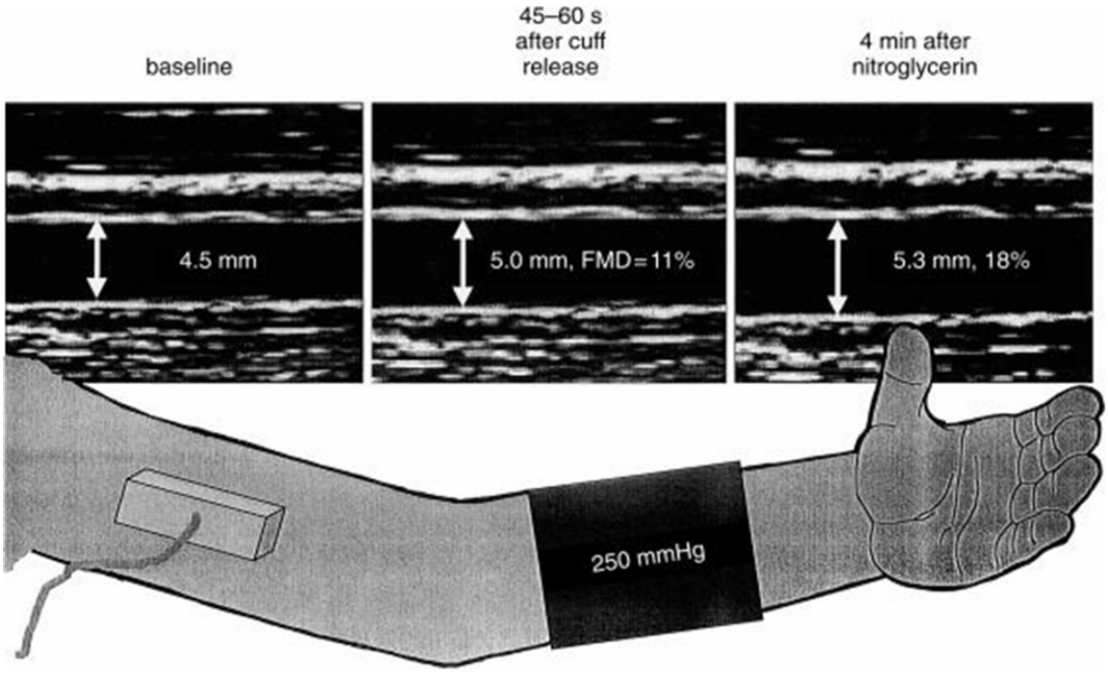

Figure 1-3. Flow mediated dilatation-study protocol including ischaemia-induced hyperemia as well as dilatation after exogenous administration of nitroglycerin. (reproduced from *Corretti et al, 2002).*

The mechanism behind this examination is the release of local NO from endothelium-derived NO synthase as a response to reactive hyperemia and shear stress, which in turn induces smooth muscle relaxation and dilatation of the artery (Joannides 1995). This is exactly why 5 Minutes of cuff inflation are suggested, since the reaction in mainly due to NO release, while after longer periods other vasodilators are responsible (Kooijman 2008).

The second part of the examination includes assessment of non - endothelium dependent vasodilatation (NMD). After 10-15 minutes 0,4 mcg nitroglycerin in form of sublingual spray are administered-if not contraindicated. Administered nitrites cause the release of NO into circulation and the increase of cyclic guanosine monophosphate (cGMP) from smooth muscle fibers, causing vasodilation independent of the endothelium. Four minutes after administration of nitrites, when the artery has reached the maximum of vasodilation, NMD can be similarly calculated:

NMD = [diameter after administration of nitrites - baseline diameter/baseline diameter] x 100

If use of nitroglycerin is contraindicated (for instance in case of hypotension, bradycardia, glaucoma, etc) this part of the examination should be refrained.

The examination is generally well tolerated. For the operators a relatively short learning period is required, although approximately 100 supervised scans are suggested to guarantee accurate measurements (Corretti 2002).

As expected, endothelial integrity is necessary for a normal reaction. Healthy vessels respond with a 5- to 6-fold increase in their diameter. Factors affecting the endothelial integrity lead to reduced reaction to the induced stimulus; paradox vasoconstriction might also rarely occur.

A meta-analysis reported that in healthy subjects FMD ranges from 0.20 to 19.2%, in diabetics values from 0.75 to 12% can be observed, while in people with coronary heart disease the values range from -1.3 to 14% (Bots 2005).

Celemajer and his colleagues reported that there is a decrease in endothelium-dependent vasodilation in the presence of cardiovascular risk factors such as smoking, dyslipidaemia, advanced age, diabetes mellitus.

Ever since, this method has been used to examine the effect of pharmaceutical agents or other interventions on endothelium, aiming to develop strategies that might delay the progression to clinical evident atherosclerosis.

Taking into account the broad availability of this method in the absence of radiation, it has been applied to a large number of patients, including children and young adults.

2.2.1 FMD and various factors

The effect of cholesterol on endothelial function was first reported in children with familial hypercholesterolemia, as an association of FMD with LDL levels was observed (Jongh 2002).

Various studies have been designed to report the effect of diet or physical activity, oestrogen administration, and other pharmaceutical factors such as antihypertensive, hypolipidemic and antidiabetic.

Smoking appears to affect flow-dependent vasodilation in a dose-dependent manner (Neunteufl 2002, Myhan 1997). Zhang ZW et al showed that nicotine results in reduced NO production and elevated oxidated stress by significantly reducing the activity of eNOS. The exposure to smoke also increased the levels of asymmetrical dimethylarginine (ADMA), a well-known risk factor for cardiovascular disease (Zhang 2006).

Improved FMD was also observed in obese children after a systematic diet and exercise (Nylor 2016), regardless of changes in insulin sensitivity.

Dyslipidemia is another risk factor affecting FMD. Many studies showed that statins have beneficial effects on atherosclerotic risk factors and markers such as flow mediated dilatation (FMD). Lowering cholesterol levels appears to improve endothelial dysfunction (Tamai 1997,), but this benefit disappears after treatment discontinuation (Stroes 1995). Vogel et al showed an increase in FMD after treatment with 10 mg simvastatin in male subjects (Vogel 1996), while Simons et al showed improvement of endothelial function after 32 weeks for both atorvastatin and simvastatin, both in combination with cholestyramine (Simons 1998). FMD at baseline

correlated with high density lipoprotein (LDL). The benefits of statins are attributed to the activation of nitric oxide synthesis (NOS), phosphoinositide 3-kinase (PI3) and mechanisms related to endothelial cell apoptosis and reduction of inflammation. By increasing NO production, statins may interfere with atherosclerotic lesion development, stabilize plaque and inhibit platelet aggregation; this means that they prevent atherosclerotic disease by pleiotropic effects (Laufs 2003).

Similar data are reported after use of fibrates, since patients with T2DM were presented with an improvement in FMD by 1.48% after treatment with fenofibrate for 40 weeks ($p = 0.01$) (Playford 2002).

The effect of antihypertensive treatment has also been studied. The use of angiotensin-converting enzyme inhibitor (ACEi) enalapril in T2DM was not accompanied with changes in FMD after 12-24 weeks, probably reflecting the complex nature of vascular diabetes disease, affecting both endothelial as well as smooth muscle function (Mullen 1998). A low dose of quinapril or enalapril for 8-12 weeks in the post myocardial infarction period appears to improve FMD, by decreasing inflammatory markers, such as tumor necrosis factor alpha (TNFa) and high sensitivity C-reactive protein (hsCRP) (Kovacs 2006).

Data are also available for beta-blockers. Nebivolol exerts beneficial effects on FMD in patients with coronary heart disease compared to atenolol or bisoprolol, an effect that appears to be independent of the reduction of systolic blood pressure in newly diagnosed hypertensive patients (Simova II 2009). A more recent review from 2015 reported that beta-blockers- especially third generation such as nebivolol- improve the endothelial function compared to placebo. Moreover, they appear to have similar effect on endothelial function as Angiotensin II receptor blockers (ARB), calcium-channel blockers (CCB) or diuretics - but are inferior to ACEi (Peller 2015).

Diabetes mellitus and impaired glucose tolerance are associated with reduced FMD. The underlying mechanism is the reduced bioavailability of NO, the activation of protein kinase C, the increase of end-glycation products, hyperglycaemia and insulin resistance that cause oxidative stress (Tesfamariam 1992, Fard 2000). An important observation is that in T2DM hyperglycemia occurs mostly postprandial. Kawano et al aimed to study FMD responses in patients with normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and diabetes

mellitus type 2 (T2DM). They found that FMD was rapidly suppressed in response to oral glucose loading-probably through increased production of oxygen-derived free radicals. This response was gradually decreased from NGT to IGT and finally T2DM. These findings strongly suggest that prolonged and repeated post-prandial hyperglycemia plays an important role in the development of atherosclerosis (Kawano 1999) (Figure 1-4).

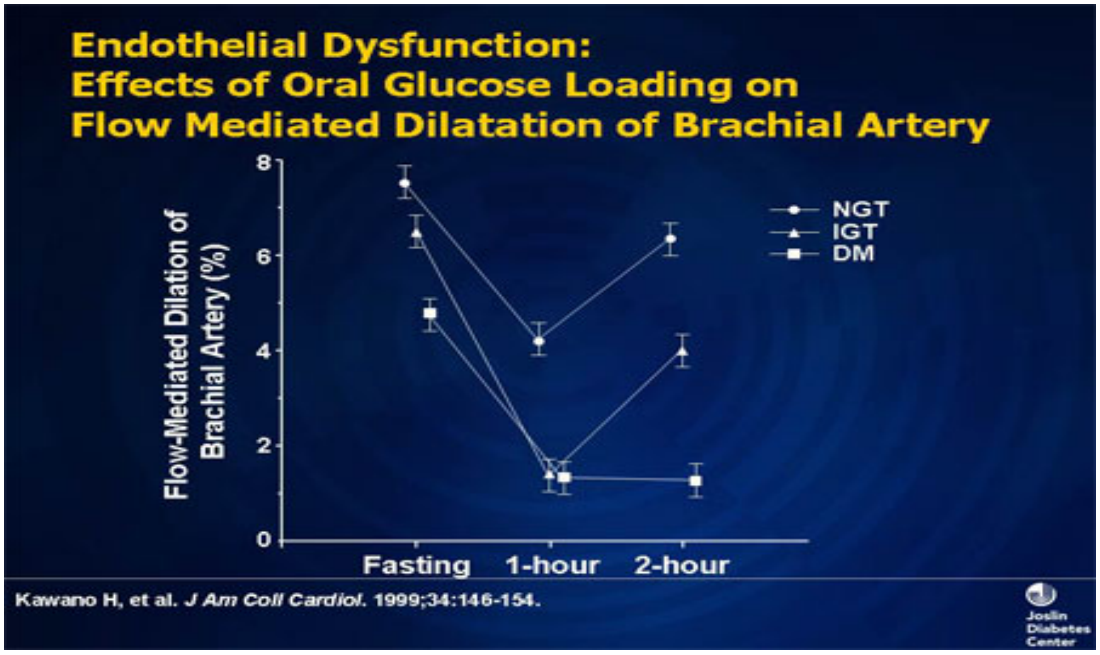

Figure 1-4. FMD gradually reduced from NGT, IGT and FMD (reproduced from *Kawano et al, 1999*).

The magnitude of T2DM in FMD and the effect of antidiabetic medication have been extensively studied. Nakamura et al aimed to assess FMD in 66 patients with T2DM, with poor glycemic control already on metformin, sulfonyluria or pioglitazone monotherapy. The addition of sitagliptin or acarbose for 12 weeks led to an improvement of FMD, while in the sitagliptin group increased circulating CD34, a marker of endothelial progenitor cells, was observed (Nakamura 2014). Another study from Italy recruited patients with metabolic syndrome (MetSyn), assigned to receive 1000 mg metformin for a period of 3 months. The authors demonstrated an improvement in FMD compared to placebo, as well as improvement in insulin resistance(IR); IR plays a key role in MetSyn and appears to be associated with impaired

endothelial function as well (Vitale 2005). Again, the effect of glycemic control will be discussed in following chapter.

It seems that FMD is impaired in the presence of classic risk factors for atherosclerosis such as arterial hypertension, diabetes mellitus and dyslipidemia and has therefore been used as a predictor for cardiovascular events. The change in FMD can be useful to evaluate the effect of various interventions on endothelium.

2.3 Arterial Stiffness

Arterial stiffness expresses the reduced flexibility and elasticity of blood vessels, which occur normally with increasing age or reflect the destructive effect of metabolic factors as well, such as T2DM (Domanski 2001, Boutouyrie 1992). Large elastic artery stiffness and wave reflection are independent predictors of future cardiovascular events and all-cause mortality (Benetos 2002, Vlachopoulos 2010). Carotid-femoral pulse wave velocity (cfPWV) and augmentation index (AIx) are non-invasive markers of arterial stiffness and wave reflection strength (Vlachopoulos 2010).

Large artery stiffness assessment has been introduced from Sphygmocor technology (CvMS; AtCor Medical, Sydney, NSW, Australia) back in 1998 (Figure 1-5). It is one of the earliest devices used to non-invasively assess PWV based on applanation tonometry.

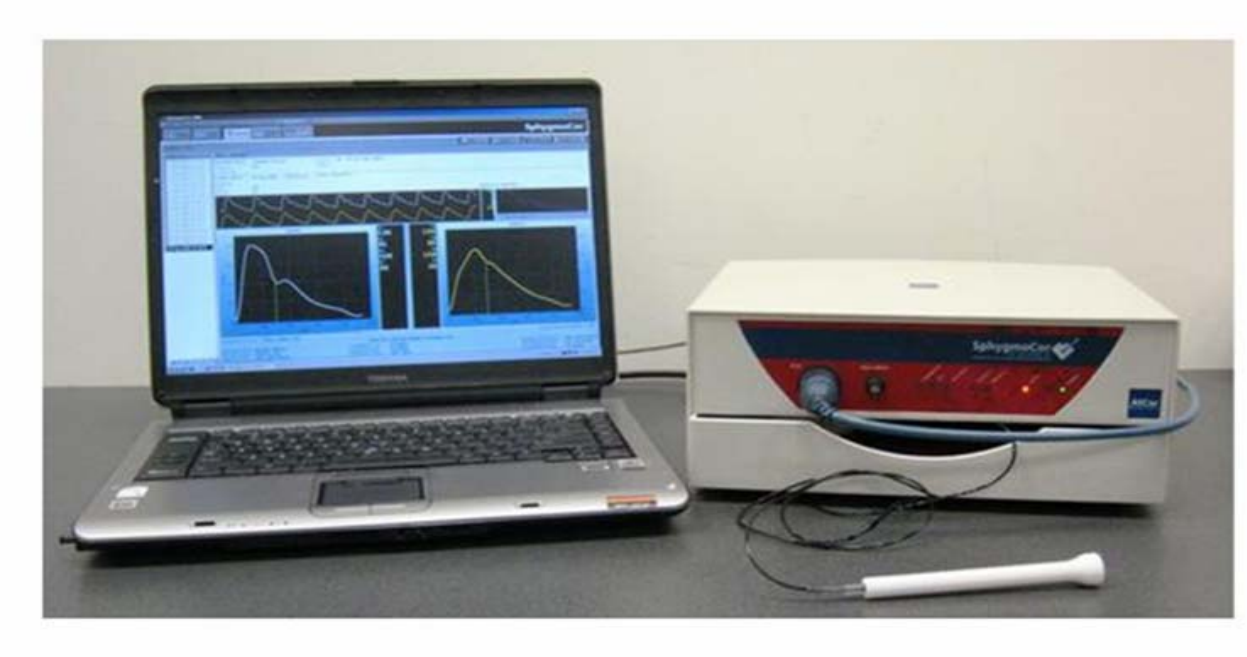

Figure 1-5. The Sphygmocor system for the assessment of PWV and Aix.

The patient lies in bed in supine position and a pressure cuff is placed on the upper arm, while the pen-like tonometer is placed over large elastic arteries to record the arterial waveforms. The guiding points used are the suprasternal notch, the femoral, carotid and radial artery. The operator records electronically a measured blood pressure (systolic and diastolic), the patient's height and weight and the measured distances (common carotid artery to suprasternal notch and then from carotid to femoral artery) in cm. By measuring these linear distances, a reliable estimation of the vascular path is made. Comparison with MRI techniques revealed that this method leads to a slight- though acceptable- underestimation of the length of about 0.05 ± 0.04 m (Huybrechts 2011). Another yet important information is that the abdominal aorta tends to become more spiral with age, meaning that the actual pulse wave velocity may be as well slightly underestimated (Wenn 1990).

A special software calculates the trasmission time, by using the R-wave of an electrocardiogram as reference frame and the given distance from the stable anatomical positions (Figure 1-6). Then it applies a validated process that transforms the recorded waveforms from these arteries and pressure measured at the arm to the waveform and pressure at the heart. This is important because the pressure at the heart is more powerful than the pressure in the arm for predicting the risk of macrovascular complications (Hickson 2010).

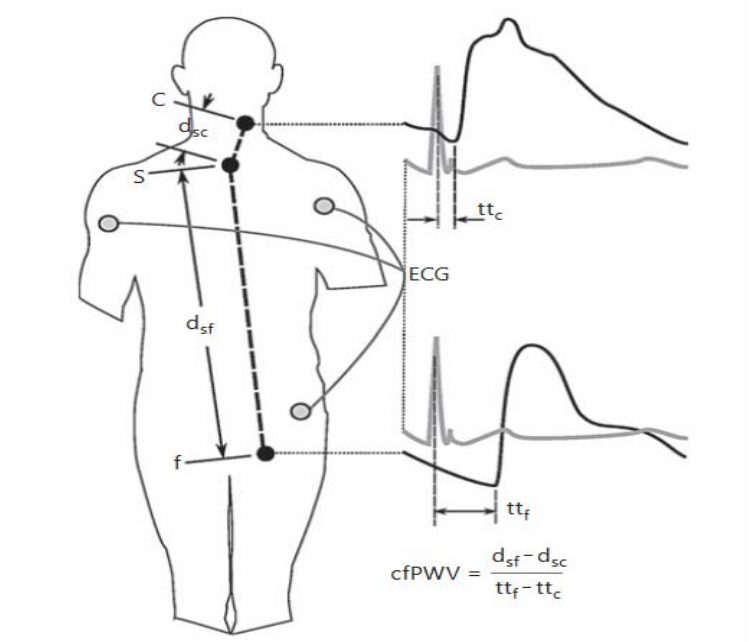

Figure 1-6. SphygmoCor carotid-femoral pulse wave velocity (cf-PWV) measurement method with applanation tonometry of the carotid (c) and femoral (f) sites. Pulse transit times (tt) are calculated from the electrocardio-gram (ECG) R-wave to the foot of the applanated waves. Distances (d) are measured from the supra-sternal notch (s) to the sites of applanation tonometry (reproduced from *Butlim M et al, 2016*) (53).

PWV represents the speed at which the pulse wave travels along the arterial tree (here from carotid to femoral arteries) at a given length (Δm) and at a given time (Δt) and is calculated as following:

$$PWV = \Delta m / \Delta t \text{ (in meters per second, m/s)}$$

It is related to the intrinsic stiffness of the aorta and determines the pressure load on the heart both through the compliance of the aorta itself and the transmission of the reflected pressure wave.

As expected, the properties of the arterial wall- meaning arterial thickness and the lumen diameter -are the major determinants of PWV. A mathematical equation from Moens-Korteweg has formalized this concept by calculating $PWV=\sqrt{Eh/2\rho R}$, or by the Bramwell-Hill equation, $PWV= \sqrt{\Delta P \cdot V/\Delta V \cdot \rho}$, where E is Young's modulus of the arterial wall; h is wall thickness; R is arterial radius; ρ is blood density; and ΔV and ΔP are changes in volume and pressure, respectively (Butlin 2016).

It is an examination that is easy to perform and painless. Alternatively, direct measurements are also possible by using either coronary computed tomography or phase contrast magnetic resonance imaging (MRI) (Huybrechts 2011). However, these are expensive, non-portable and especially for CT hazardous in terms of radiation exposure.

The Augmentation Index (AIx) is calculated with the same method using radial artery pressure waveform. It is related to the extent and speed of both central and peripheral wave reflection and commonly accepted as e measure of the enhancement of central aortic pressure by a reflected pulse wave (Figure 1-7). It is defined as the ratio of late systolic pressure (P2) to early systolic pressure (P1):

Augmentation Index: P2 / P1

There is linear relationship between AIx and heart rate. This is likely to be due to alterations in the timing of the reflected pressure wave, produced by changes in the absolute duration of systole. As a result, in our study Aix was normalized for a heart rate of 75 bpm (Aix@75) as previously described (Wilkinson 2000). This adjustment is important in the interpretation of PWV values as well, as data supports that by a normal blood pressure an increase of heart rate by 40 bpm led to increase of PWV by 1 m/s (Lantelme 2002).

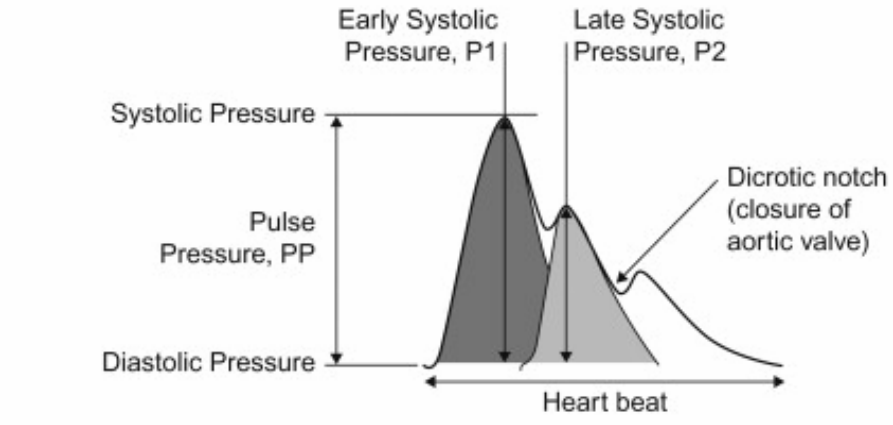

Figure 7. Peripheral augmentation index (AI) is defined as the ratio of late systolic pressure (P2) to early systolic pressure (P1): Pai = P2/P1 (Ref: http://www.pulsecor.com/augmentation-index.html).

Limitations of this method are:

1. difficulties in obtaining a high-quality radial or femoral pulse in some subjects with lower blood pressure or obesity
2. operator dependency of the peripheral signal acquisition
3. aortic aneurysma or stenosis might influence the measurements leading to overestimation (Munakata 2016)
4. it cannot be performed in case of implantable pacemaker /defibrillator or cardiac arrhythmias such as atrial fibrillation

Another considerable limitation is the lack of a normal range of PWV. A healthy adult has normal values for PWV 4-6 m/s but can reach levels of 8-10 m/s in peripheral, less susceptible muscle arteries (Taylor 1965, London 2004).

Due to different methodologies and studied populations, as well as the strong dependence on age and blood pressure, there are important differences in absolute PWV values. The European society of Cardiology and European Society of Hypertesion (ESC/ESH) suggested a threshold of 12m/s (Mancia 2007) in hypertension studies based on available data; still not taking into account multiple factors other than age and bloop pressure affecting this value.

As seen in Figure 1-8, there are different reference values for PWV according to age group and blood pressure group:

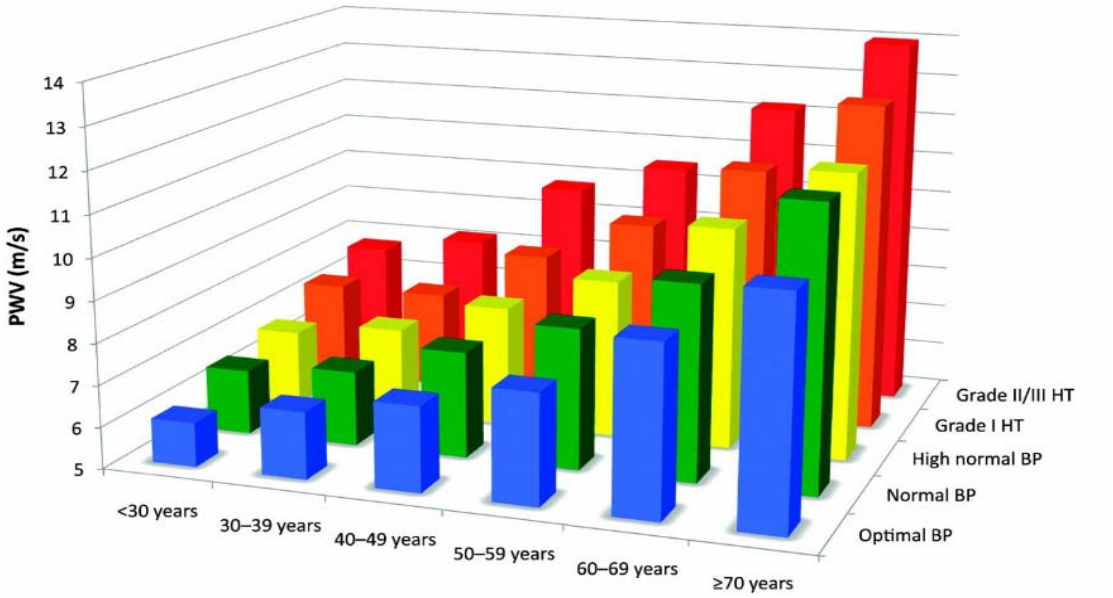

Figure 1-8. PWV in different age and HT (Hypertension) groups (reproduced Reference Values for Arterial Stiffness' Collaboration 2010). Hypertension Grade I: SBP 140-159 mmHg and/or DBP 90-99 mmHg. Grade II: SBP 160-179 mmHg and/or DBP 100-109 mmHg. Grade III - SBP ≥180 mmHg and/or DBP ≥110 mmHg).

Decreasing elasticity with accumulation of vascular interstitial collagen, diffuse thickening and eventually endothelial dysfunction occur with increasing age. These changes are accelerated in the presence of hypertension and other diseases (Najjar 2005). The elasticity is important in order to provide low resistance in the blood flow so as to sufficiently supply the organs and limbs; during diastole it ensures that the pressure wave of the conduit arteries from periphery returns to the heart and supplies sufficiently the coronary arteries. The integrity of this circle is important and explains the prognostic value of arterial stiffness in cardiovascular disease.

In patients with hypertension without a history of cardiovascular disease PWV is a prognostic factor for cardiovascular events, regardless of classic risk factors. PWV values > 13 m /s are associated with increased cardiovascular mortality in hypertension (Blacher 1999). In a large study with 483 participants over a follow-up period of six-years, the annual rates of progression in PWV in treated hypertensives were significantly higher than in normotensive persons. The presence of high blood pressure, heart rate and serum creatinine levels were the major determinants of this progression of aortic stiffness in treated hypertensives (Benetos 2002).

The presence of diabetes has also been associated with increased PWV levels. In a first study by Taniwaki H. et al higher PWV was observed in young patients with T2DM (Taniwaki). The authors concluded that age, diabetic state, and cigarette smoking were independently common risk factors for the increase in PWV and carotid IMT, that was also studied. Interestingly age, hyperlipidemia, and duration of diabetes were strongly associated with the carotid IMT ($r = 0.232$, $P < 0.0001$), while for PWV age and duration of diabetes were stronger determinants ($r= 0.334$, $P < 0.0001$).

The prognostic value of PWV in T2DM was subject to further studies. Hatsuda et al compared 70 diabetics with known cardiovascular disease to 525 diabetics without and concluded that for every 1 m / s increase in PWV there was a 14.5% increase in the risk of cardiovascular disease (Hatsuda 2006). Even newly diagnosed T2DM and patients with impaired glucose tolerance were found to have increased arterial stiffness (Li 2012). Since that was not case for those with impaired fasting glucose tolerance, it is probably the postprandial hyperglycemia- the main feature in T2DM- responsible for these vascular alterations. Diabetes mellitus and hypertension seem to have a synergistic effect in the progression of arterial stiffness (Cameron 2003, Bruno 2012).

Chronic inflammation has also been associated with elevated levels of catabolic metalloproteinases (MMP), mainly MMP-9, which are involved in the production of collagen and elastin. This possibly explains the increased arterial stiffness in chronic inflammatory diseases, such as rheumatoid arthritis (Ye 2000).

In addition to diabetes and hypertension, the effect of hyperlipidemia on arterial sclerosis has been studied. Decreased vascular elasticity and a negative correlation with LDL cholesterol levels (Lehmann 1992) have been reported, especially in patients with familial hypercholesterolemia (Giannattasio 1996). Dart et al concluded that aortic distensibility might indicate coronary heart disease in symptom-free patients with modest hypercholesterolaemia, suggesting an indication for the need for a more aggressive treatment (Dart 1991).

Last but not least, smoking appears to worsen PWV. Failla et al showed that in smokers without known cardiovascular disease, smoking caused an acute decrease in medium and large vessel

susceptibility (Failla 1997), while a similar increase in PWV was reported among normotensive and non-hypertensive smokers (Levenson 1987). Regarding the chronic consumption of tobacco, despite the acute decrease in the susceptibility to common carotid and brachial artery, with a parallel increase in blood pressure (Stefanadis 1997, Brunel 1992) no significant differences were seen between chronic smokers and non-smokers (Berlin 1990, Kool 1993).

Finally, changes in arterial stiffness have been reported in patients with heart failure (Ramsey 1995, Giannattasio 1995) but also in patients with end-stage renal disease (London 1990). Increased stiffness in end-stage renal failure is an independent prognostic factor for total mortality and cardiovascular disease (Blacher 1999).

Arterial stiffness is associated with most known risk factors, with an independent prognostic value for overall mortality and cardiovascular mortality. The coexistence of diabetes mellitus and hypertension appears to have a synergistic effect on the progression of arterial sclerosis (Tomiyama 2006, Muir 2009). The effect of pharmaceutical interventions to improve glycemic control on PWV will be discussed in extend in the upcoming chapters.

Available data suggest that PWV and Aix- both been assessed by Sphygmocor (Figures 1-9, 1-10) -are useful tools to screen patients at high-risk.

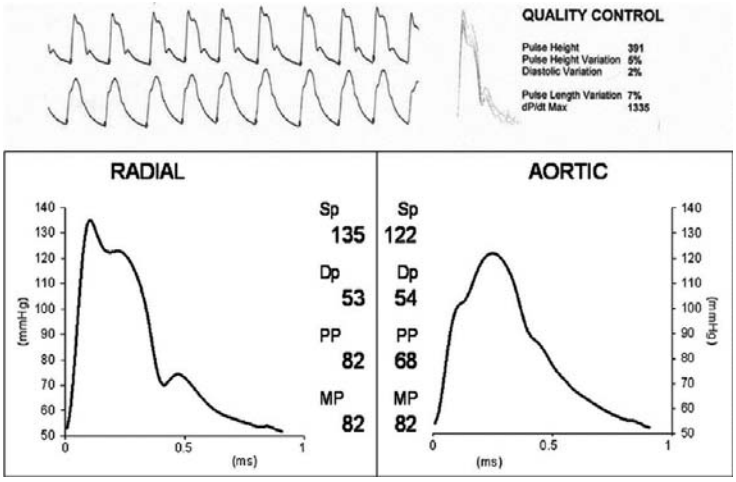

Figure 1-9. Waveforms from radial artery and aorta for the calculation of Augmentation Index (Aix). Figure from our department.

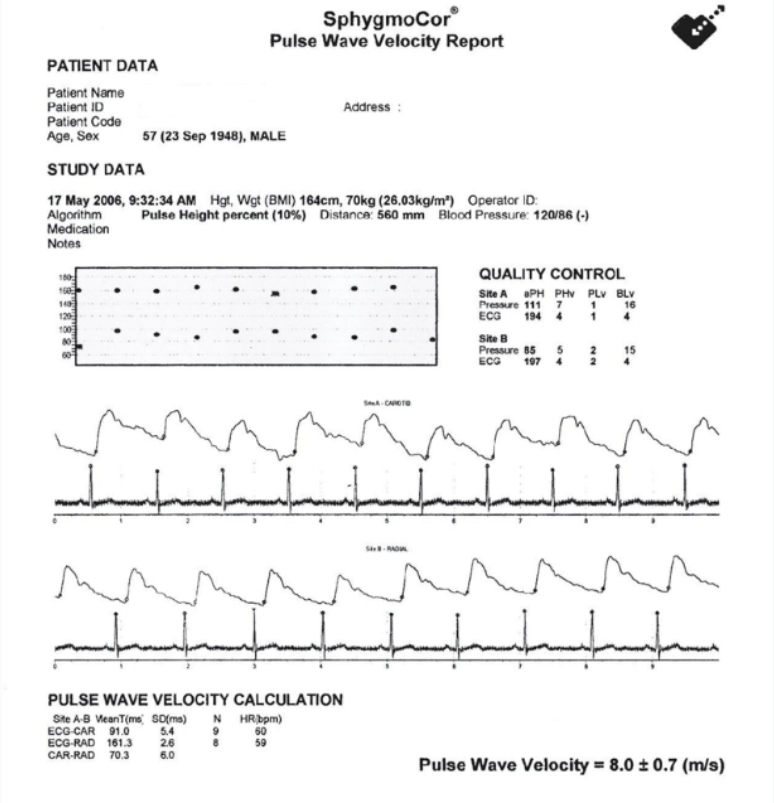

Figure 1-10. Evaluation report after PWV calculation. The operator has to insert patient characteristics namely weight, height, the given distances as well as reports of systolic and diastolic blood pressure. By using applanation tonometry, waveforms are recorded and with the help of special software PWV is automatically calculated. Figure from our department.

2.4 Ankle-Brachial-Index (ABI)

Peripheral arterial disease (PAD), like cerebrovascular and cardiovascular disease, is an arterial disease caused by atherosclerosis. Its incidence increases with increasing age and in the presence of risk factors such as arterial hypertension, hyperlipidaemia, diabetes mellitus and smoking (Muir 2009). Diagnosis is usually made by patient's history. The involved physician must carefully evaluate patient's symptoms typical of intermittent claudication, also known as vascular claudication, skin ulcers, bluish or cold skin, or abnormal nail and hair growth in the affected limb. Half of the patients with PAD, though, remain asymptomatic and diagnosis requires further testing, such as ultrasound examination (Violi 2012).

It is estimated that more that 202 million people worldwide suffer from the disease (Fowkes 2013). Common complications are systemic infections and lower limb amputations, while it is associated with high rates of myocardial infarction and stroke. If PAD is suspected, then ankle-brachial index (ABI) should be measured. This simple test was introduced back in 1960 and is based on the idea that the pressure at lower limbs is slightly higher than at the elbow in normal subjects. It is calculated by dividing the systolic blood pressure at the ankle by the systolic blood pressure in the arm. Values between 0.90-1.30 are considered normal.

For PAD prediction following classification is suggested (Rosei 2010):

- 1.30: incompressible vessels
- 1.0-1.2 Normal
- 0.91-0.99: borderline normal
- 0.70-0.90: mildly abnormal
- 0.40-0.69: moderately abnormal
- < 0.40: severely abnormal

Generally, it is thought that an ABI < 0.9 has 95% sensitivity and 99 % specificity for detection of angiographically confirmed PAD (Fowkes 1998).

Initially the patient must lie in supine position for 5 minutes in stable condition prior to measurement. One hand-held Doppler device is needed, a classical sphygmomanometer and a larger pressure cuff. By measuring the systolic blood pressure at brachial artery and dorsalis pedis or posterior tibial artery (the higher of two ankle pressures should be used) the ABI can be obtained (Figure 1-11) (Ouriel 1982, Xiaoyun 2013).

The ABI study has some considerable limitations. First, a medial arterial calcification results in non-compressible arteries. This fact causes false elevation (ABI > 1.4) or false normal values. This is a common feature in patients with diabetes mellitus, kidney failure or heavy smokers

(Aboyans 2008). Therefore, in these patients ABI <0.9 or >1.3 requires further investigation. Secondly, in patients with aorto-iliac disease ABI can be normal or borderline (Faglia 2011).

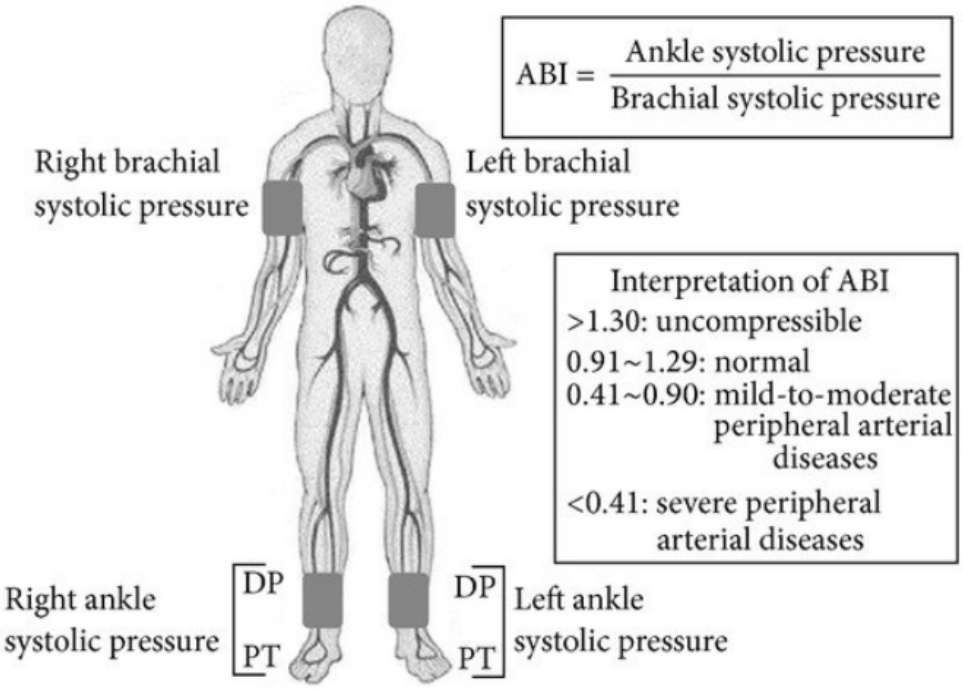

Figure 1-11. Assessment of ABI (reproduced from Li et al, 2013)

Data suggest that 41% of patients with peripheral arterial disease (PAD) have diabetes (Novo S). The UK Prospective Diabetes Study (UKPDS) study with over 5000 participants showed that for each increase in glycosylated haemoglobin (HbA1c) by 1 % the incidence for PAD increases by 28 % (Adler 2002). The general data on the incidence of the disease are also indicative: the disease affects 4 % of the general population and 9.5-13.6 % of patients with T2DM (Selvin 2004). This shows that PAD and Diabetes are closely related. Diabetes is a risk factor for both PAD and PAD-associated mortality, emphasizing the critical need to detect and monitor PAD in diabetic patients (Leibson 2004). According to Adult treatment panel III of National Cholesterol Education Program, PAD should be regarded as being equivalent to DM as a CHD risk factor (NCEP 2001).

The PAD in T2DM has certain characteristics. Unlike smokers, occlusive disease is widespread, and the anatomical localization is mainly distal and extends to the femoro-popliteal and infra-popliteal axes (van der Feen 2002). Occlusion occurs more often than stenosis (Faglia 2002). Another yet important observation is that arterial wall calcification is frequently present compared to non-diabetics. It also correlates with the presence of neuropathy, which in turn complicates the PAD, since typical claudicatio symptoms are absent. The clinical implication is that and ABI value of less than 1.3 should never be a reassurance and it should be indicator of calcification as well as poor cardiovascular prognosis (Young 1988).

Several studies have questioned the prognostic value of ABI in T2DM. In patients with intermediate cardiovascular risk and without neuropathy, Williams and colleagues showed that an ABI < 0.90 had 100% sensitivity and 88% specificity, with similar results in non-diabetics (Williams 2005). Alnaeb and colleagues later reached a similar conclusion in the same group of diabetics (Alnaeb 2007). It has been reported that for ABI levels ≤ 0.9 there is a 67% increase in the risk of cardiac death (Norman 2006), with much higher death rates in a follow-up study after 14 years than in those with normal ABI (Ogren 2005). The Strong Heart Study, in an analysis of 4393 veteran diabetics, concluded that ABI >1.4 is an independent risk factor for cardiovascular events, reflecting diffuse atherosclerotic disease with vascular calcifications (Resnick 2004).

In the general population, every symptomatic patient or patient with specific risk factors should undergo ABI test. The American Diabetes Association recommends that all diabetic patients at the age of 50 years and younger patients who have other PAD risk factors should be screened. Normal test should be repeated within 5 years. The availability of small-size, low-cost continuous Doppler devices enable the extended use of this method for the purpose of screening for PAD, including in outpatient settings.

2.5 Large and small artery elasticity index

Another marker of subclinical atherosclerosis includes the assessment of Large (C1) and small artery elasticity index (C2), an independent measurement of proximal capacitive compliance (C1) and distal or oscillatory-reflective compliance (C2).

Both C1 and C2 are reduced with age and the C2 compliance has been identified as a sensitive marker of structural and functional abnormalities of the vasculature associated with hypertension, diabetes, smoking and heart failure (MacVeigh x 2).

For the assessment the special HDI-Pulsewave CR-2000 system (Figure 1-12) is commonly used (Prisant 2002). It consists of a wrist stabilizer (that enables an easier assessment of the radial artery), a piezoelectric pressure sensor mounted in a holder, an oscillometric blood pressure cuff, and a special system algorithm. After stabilising the participant's right wrist, the sensor is placed over radial artery to achieve maximal pulsation. The oscillometric cuff is then placed on the left arm so as to measure a systolic and diastolic blood pressure (SBP and DBP) and pressures were sampled from the right radial artery at a rate of 200 per second for a total of 30 seconds. The software uses an algorithm to calculate C1 and C2 from a modified Windkessel model after the operator notices the height, weight, blood pressure, and age of the participant and radial artery pulse waveform is recorded (Finkelstein 1992). This method is easy to learn, has a relatively low cost and good repeatability. Like PWV and Aix, it is well tolerated with no hazardous radiation.

Figure 1-12. The HDI-Pulse wave CR-2000 system.

Chapter 3: Endothelium as an endocrine Organ

The importance of endothelium was first described by Furchgon and Zawadzki, that contributed to its recognition as a major regulator of vascular hemostasis (Furchgon 1980). Endothelial cells, forming the inner lining of blood vessels, are strategically located between circulating blood and the vascular smooth muscle. This dividing surface between the blood vessels and the circulation represents about 1% of the total body mass and an estimated area of about 700 m^2 (Cryer 1983).

It has been characterized as a complex endocrine organ with an important role in vascular homeostasis. Endothelial cells exert their autocrine, paracrine, and endocrine activity through membrane receptors that "perceive" hemodynamic changes and in response release vasoconstrictory or vasodilatory peptides. This normal function is disrupted by various risk factors, leading to endothelial dysfunction and subsequent atherosclerosis.

3.1 Hemostasis Regulation

Hemostasis is complex homeostatic mechanism, based on the interaction between blood cells (mainly platelets), coagulation factors and the vascular endothelium. Under normal conditions, there is a harmonious balance between the thrombotic and anticoagulant properties of the endothelium aiming to maintain vascular patency by inhibiting blood clotting and platelet aggregation. By engaging several vascular and extravascular receptors it contributes to seal off the damage inflicted to the vasculature and the surrounding tissue (Henri 2013). This normal function is disturbed in the presence of inflammation on the surface of the endothelium, caused by several conditions such chronic hyperglycemia in diabetes.

The endothelium produces in response to various stimuli a variety of factors involved in blood clotting and thrombus production, such as:

1. Nitric oxide (NO), which causes vasodilation.

2. Von Willebrand factor (vWF) a blood glycoprotein produced by the surface of endothelial cells- which is also a carrier protein for factor VIII (Factor VIII).

3. Plasminogen activator inhibitor-1 (PAI-1) (also known as endothelial plasminogen activator inhibitor), whose main function is the inhibition of urokinase plasminogen activator (uPA), an enzyme responsible for the cleavage of plasminogen to form plasmin.

4. Prostacyclin (PGI2), an important vasodilator, which also inhibits platelet aggregation.

5. Heparan sulfate proteoglycans (HSPGs), proteoglycans produced from epithelial cells, involved in inhibiting coagulation while also exerting antiplatelet activity.

6. Thrombomodulin, which in conjunction with thrombin activates the coagulation inhibitor Protein C.

7. The tissue plasminogen activator (t-PA), which activates the degradation of fibrin.

8. Tissue factor (TF), present in subendothelial cells and leukocytes, which enables cells to initiate the blood coagulation cascades

The production of prostacyclin and nitric oxide (NO), which are important anticoagulants, causes vasodilation by increasing the production of cyclic adenosine 3′,5′-monophosphate (c AMP) and guanosine 3',5'-cyclic monophosphate (cGMP) respectively in the cytoplasm of platelets, preventing their adhesion and agglomeration (Mendelsohn 1990).

Following endothelial injury, vWF is activated. It serves as a molecular bridge between the glycoprotein Ib receptor (Glycoprotein Ib Platelet Subunit Alpha, GP1b), which is also located on the surface of platelets, and the vascular wall, thus promoting their accumulation at the sites of vascular damage (Khazaei 2008). It also acts as a vascular transporter of coagulation factor VIII, helping to stabilize circulating blood levels and improving its activity. On the other hand, thrombomodulin, which is also present on the surface of endothelial cells, catalyzes the activation of protein C by thrombin and inhibits platelet activation and fibrin production (Gross 2000), thereby inhibiting coagulation.

Endothelial cells also produce platelet activating factor (PAF), an important active mediator of inflammation, with a variety of actions such as leukocyte attraction and increased vascular permeability (Benveniste).

The endothelium plays an important role in the production and regulation of fibrinolysis, by maintaining the balance of opposing mechanisms. It releases t-PA, which in turn degrades fibrin and thus dissolves clots (Hekman 1987). On the other hand, inflammation of the endothelial surface triggers the production of endothelial plasminogen activator inhibitor (PAI, also known as serpine). It belongs to the family of proteases. Elevated levels of PAI are observed in various thrombogenic conditions, such as cancer, obesity and metabolic syndrome. Its increased production accelerates the progression of atherosclerosis (Stassen 2004). Table 3-1 summarizes the most important pro-thrombotic and anticoagulant factors of the endothelium, as well as some of their actions.

Table 3-1. Endothelium-derived anti- and prothrombotic factors and most important mechanism of action

Substance-Factor	**Category**	**Mechanism**
Proteoglycans-Heparinoid	Antithrombotic	Inhibit platelet adhesion
Protein C	Antithrombotic	inactivates factor Va and VIIIa
Thrombomodulin	Antithrombotic	binds circulating thrombin, protein C activation
Protein S	Antithrombotic	co-factor to Protein C for inactivation of Factors Va and VIIIa.
Nitric oxide and prostacyclin	Antithrombotic	inhibits platelet aggregation and activation
t-PA (tissue plasminogen factor)	Antithrombotic	increases fibrinolytic activity
V WF (von Willenbrand)	Prothrombotic	promotes thrombosis

3.2 Maintenance of vascular tone

One of the most important roles of the endothelium is the regulation of vascular tone, due to vasoactive substances that are produced in response to various mechanical and non-mechanical stimuli.

Nitric oxide (NO) is an important cellular molecule first described by Furchgott and Zawadzki, as already mentioned above (Furchgon 1980). It helps modulate vascular tone and is involved in angiogenesis and neural development. There are a number of enzymes catalyzing the production of nitric oxide (NO) from L-arginine (Palmer 1988). There are three isoforms of this enzyme: (a) the isoform eNOS, which is responsible for the production of NO in the vascular system; (b) the neuronal isoform, which produces NO to then act as a neuronal transmitter; (c) the isoform iNOS (inducible NOS), produced after exposure to pro-inflammatory agents, such as tumor necrosis factor-a (TNFα), that cause endothelial injury resulting in macrophage activation (Prast 2001, Michel 1997, Kinlay 1997). These isoforms are named after the cells in which they were discovered, although myocardial cells, skeletal muscle cells, and hippocampus are also known sites of production.

The inactive form of eNOS binds to a cell membrane protein called caveolin. When intracellular calcium levels increase, eNOS detaches from caveolin and is activated. It then converts L-arginine to NO, as shown in the figure below (Figure 3-1) (Kinlay 2001, Gross 1995).

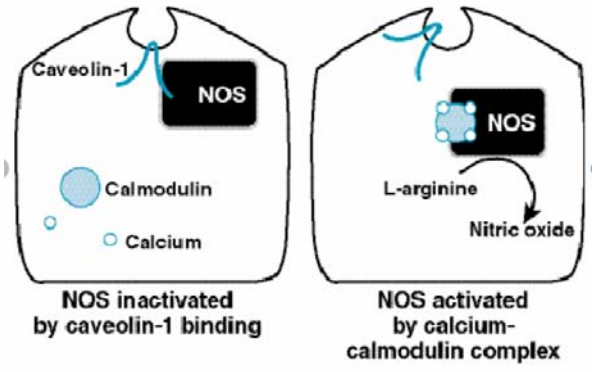

Figure 3-1. Nitric oxide (NO) is held in an inactive state by caveolin, Phosphorylation of NO synthase (NOS) ba caveolin-1binding of the calcium-calmodulin complex to NOS, increases its activity and the production of NO from L-arginine.

Substances that act as NO agonists, such as bradykinin, acetylcholine (Ach), adenosine diphosphate, triphosphate (ATP, ADP) and thrombin, cause eNOS cleavage from caveolin (Figure 3-2).

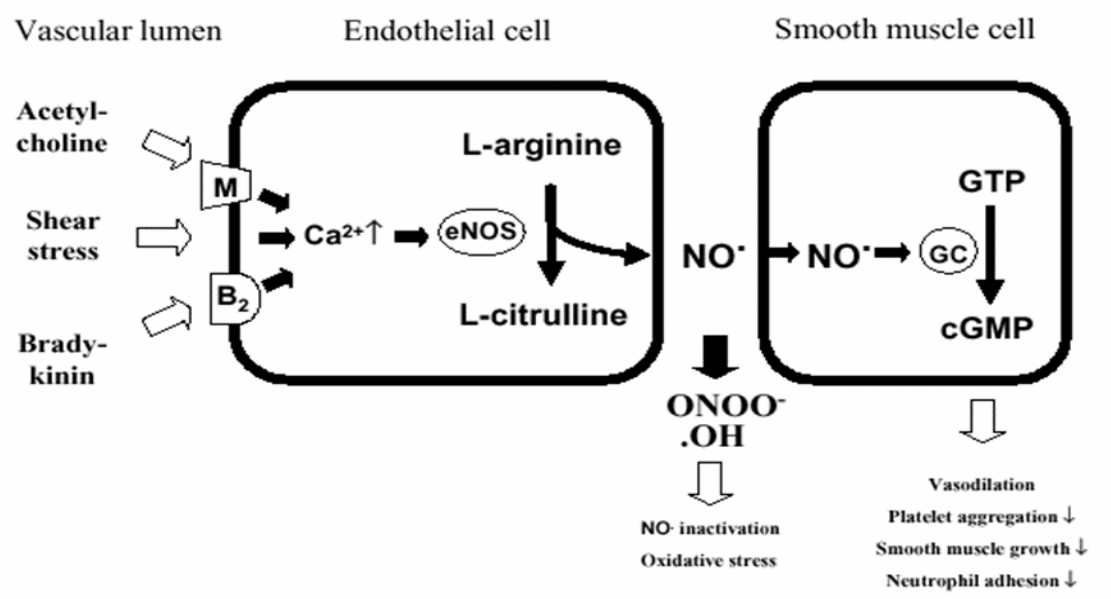

Figure 3-2. Schematic representation of nitric oxide production by L-Arginine on response to acetylcholine, bradykinin and shear stress on the vessel surface. Abbreviations: ***bradykinin B2 receptor; cGMP, cyclic guanosine 3',5'-monophosphate; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclase; GTP, guanosine5'-triphosphate; M, muscarinic receptor; NO, nitric oxide; .OH, hydroxyl radical; ONOO-,peroxynitrite vasodilation*** (reproduced from Lassila et al, 2001)

Then NO diffuses from the endothelial cells to the smooth muscle cells where it interacts with the enzyme guanylate cyclase. The levels of c GMP increase, with subsequent reduction in intracellular calcium levels and finally relaxation of smooth muscle fibers and dilatation (13). Increased blood flow velocity causes increased shear stress on the surface of the vessels, inducing phosphorylation of NO synthesis, resulting in vasodilation. On the other hand, substances such as arginine analogues inhibit NO production (Celermajer 1997).

As expected, NO production and inhibition mechanisms are constantly active, allowing NO to maintain vascular tone. In cases of endothelial damage, the administration of exogenous nitrites can induce non-endothelial-dependent vasodilation, with the same release mechanism.

In addition to NO, an important role in the regulation of vascular tone is held by the synergistic action of two prostanoids, prostacyclin (PGI2) and thromboxane (TXA2) (Bunting 1983). Their synthesis is regulated by the enzyme cyclooxygenase COX 1 (Cyclooxygenase-1), which is continuously produced by endothelial cells, as well as COX2 (Cyclooxygenase-2), derived from injured endothelium, after exposure to inflammatory cytokines (FitzGerald 1997). COX2 in turn converts arachidonic acid to prostaglandin H2 (Prostaglandin 2, PGH2), to take its final active form from the enzyme prostacyclin synthase. By binding to specific receptors, it activates the synthesis of c AMP and ultimately relaxation of smooth muscle fibers, just like NO. Interestingly, in the case of NO inhibition, PGI2 is produced at an increased rate, thus causing vasodilation, suggesting a complementary role in cases of reduced NO (Beverelli 1997).

Unlike prostacyclin, which inhibits platelet aggregation and vasodilation, thromboxane promotes thrombus formation and vasoconstriction (Thomas 1998). The enzyme COX1 converts arachidonic acid to prostaglandin H2 (PGH2), which is eventually converted to thromboxane by the enzyme thromboxane synthase, exerting its final actions through receptors on thrombocytes and smooth muscle. A balance composition of these two substances is very important for the maintenance of vascular homeostasis, with great pharmacological interest. For instance, the administration of selective COX2 inhibitors reduces the inflammatory response, without affecting the thrombus activation cascade (Masferrer 1994).

Last but not least, endothelin is a very potent vasoconstrictor peptide derived from endothelial cells as a response to shear stress and inflammatory cytokines (Ynagisawa 1988, Alonso 2003). Interestingly, the administration of selective endothelin receptor antagonists appears to have a beneficial effect endothelial dysfunction in animal models and is of special interest (Kowala 1995).

3.3 Endothelial permeability

The endothelium is, among other things, a natural barrier between bloodstream and the underlying tissues (Irie 1991). This barrier is not, however, impenetrable. The elements that make the endothelium permeable are related to cellular connections, synthesis and electrostatic charge of cell membranes, as well as specific membrane protein receptors.

Intercellular connections, which play the most important role, are divided into three categories (Toborek 1999):

1. tight junctions, that provide additional mechanical strength
2. gap junctions, that which allow the exchange of ions and molecules between adjacent cells and finally
3. Adherens junctions, with critical role in the preservation of normal endothelial structure and permeability, controlling cell migration, growth and differentiation (Staddon 1997, Lampugnani 1997, Dejana 1997).

Several adhesion molecules are produced in response to the aforementioned stimuli and have an equally important part in the leukocyte aggregation and the inflammatory response. The most important of these are:

• Intercellular adhesion molecule (ICAM-1)

• Vascular cell adhesion molecule (VCAM-1)

• Platelet adhesion molecule (Platelet-endothelial Cell Adhesion Molecule-1, PECAM-1) (Lum 1994, Carlos 1994, Springer 1995).

As the cascade of leukocyte aggregation begins, leukocytes roll along the endothelium within the first two hours after the onset of inflammation and are activated by chemokines of the endothelial surface. After their adhesion, they pass through the endothelial barriers after their interaction with specific endothelial cells, while the relaxation of intercellular connections

loosens permeability and facilitates cell transmigration (Springer 1995, Muller 2003) (Figure 3-3).

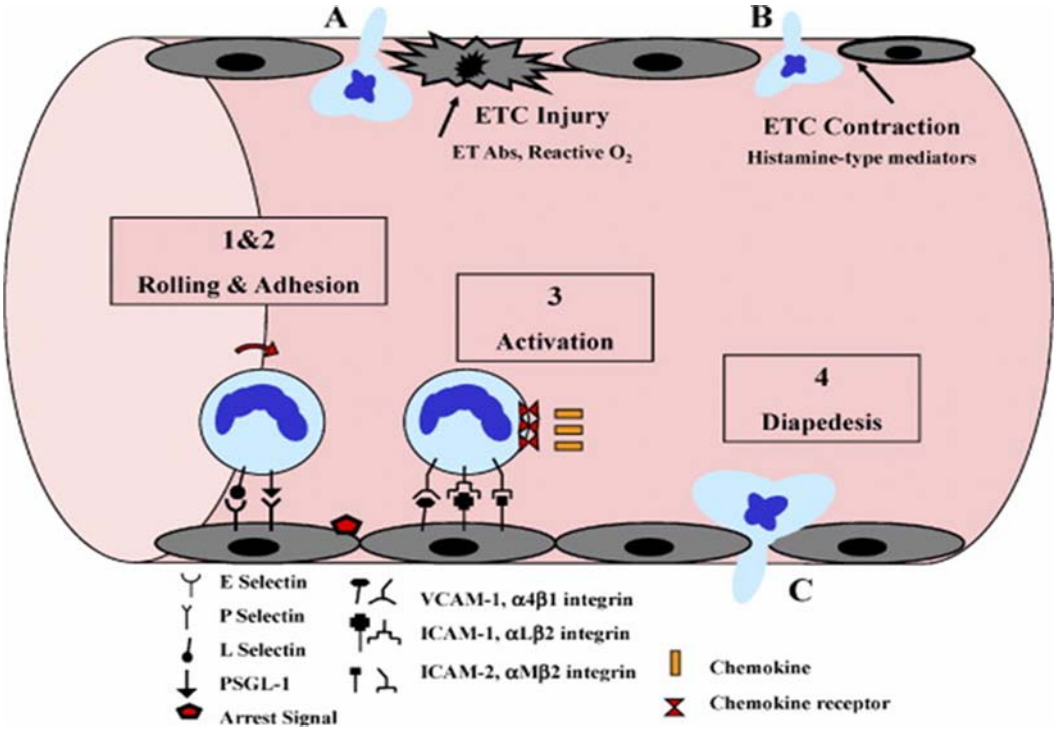

Figure 3-3. Leucocyte migration following endothelial inflammation. (A and B) Produced antibodies and reactive oxygen species lead to junction defects. Endothelial cells roll into endothelium; adhesion is mediated through selectin, while chemokines increase the expression of adhesion molecules (VCAM-1, ICAM-1 and ICAM-2) and activate integrins. Through this interaction of chemokines and integrins the link between leukocytes and endothelial cells is tightened and allows leukocyte transmigration. PSGL-1: P-selectin glycoprotein ligand-1; VCAM-1: vascular endothelial cell adhesionmolecule-1; ICAM-1 and ICAM-2: intercellular adhesion molecule 1 and 2; Abs: antibodies (reproduced from Khazaei et al, 2008).

The endothelium is affected by inflammation and morphological changes, which lead to increased permeability and naturally release of vasodilators, such as nitric oxide (NO) and prostaglandins (PGI2).

3.4 Angiogenesis

Angiogenesis is the growth of new blood vessels from existing ones. It occurs normally during development and reproduction but is also present in pathological conditions such as inflammation, rheumatoid arthritis, diabetic kidney disease and cancer (Lingen 2001, Szekanecz 1998). The degradation of the vascular basement membrane and remodeling of the extracellural matrix is required (Liekens 2001, Stamenkovic 2003).

Several proteins that promote angiogenesis have been identified the most important of which is the basic fibroblast growth factor (bFGF) and the vascular endothelial growth factor (vascular endothelial growth factor, VEGF).

Angiogenesis begins as appropriate stimulus, such as inflammation and hypoxia, cause vasodilation of the existing capillary with subsequent release of growth factors and cytokines. These agents bind to specific receptors on the surface of endothelial cells, inducing the production of plasminogen activator and metalloproteinases (MMPs). The conversion of plasminogen to plasmin further activates MMPs, which degrade the cell membrane, allowing cells to migrate and proliferate to form new vessels. MMPs not only release pro-angiogenic factors but also facilitate cell migration (Szekanecz 2001, Folkman 1987, Dejana 1996).

Chapter 4: The endothelium in various diseases

The vascular endothelium has been recognized as an important organ for the regulation of vascular tone and structure. Endothelial cells express endothelial NO synthase (eNOS), which in turn produces NO from l-arginine, as already mentioned. The production and release of NO has an important cardio-protective role: it causes smooth muscle cells relaxation with subsequent vessel dilatation but prevents the inflammatory cascade as well, that begins with leukocyte and platelet adhesion and aggregation leading to atherosclerosis (Luscher 1990, Taddei 2003). Endothelial dysfunction (ED) should be thought as a pro-coagulant and pro-inflammatory state that favors atherosclerosis, best characterized by an imbalance of vasodilatory and contracting mechanisms (Ross 1999).

All traditional cardiovascular risk factors such as diabetes, hypertension, dyslipidemia, and tobacco toxins are associated with endothelial dysfunction (ED).

4.1 Endothelium and arterial hypertension

Hypertension is an important risk factor for atherosclerosis and endothelial destruction. Whether ED is the result of hypertension or the other way round has been debated. Some authors suggested that endothelial dysfunction is the result of hypertension. Hypertension causes structural alterations in microcirculatory beds vessels, such as remodeling, which is exactly responsible for the increased vascular resistance observed in in hypertension (Jacobsen 2011). Hypertension is also linked to deficient levels of NO and increased vascular production of ROS- the main characteristic of ED (Kiowski 1999). So a logical hypothesis should be that if ED is caused by hypertension, antihypertensive treatment could reverse the progression of atherosclerosis. Panza et al, though, showed no improvement in endothelial dysfunction after antihypertensive therapy, suggesting that ED could probably precede the development of hypertension (Panya 1993).

On the other hand, reduction in NO synthesis-due to oxidative stress- leads to arterial vasoconstriction and hypertension. The chronic administration of NOS inhibitors causes sustained hypertension: NO infacilitates sodium excretion, so that systemic inhibition of NOS promotes salt and water retention (Melikian 2009). Another interesting notion is that

endothelium in hypertension might be heterogeneously affected across vascular bed. In animal models, the endothelial dysfunction in coronary and renal arteries was not affected by higher blood pressure; other experimental works suggested that the aorta, mesenteric and carotid arteries were presented with impairments of endothelium-dependent relaxation (Dohi 1990, Calver 1993, Luscher 1986).

Since there are various conflicted data in terms of antihypertensive treatment, specific drug effects that could influence these results cannot be ignored. Schiffrin et al suggested that the administration of β-blockers does not seem to have a significant effect in terms of ED (Schiffrin 2002); others showed that some β-blockers have endothelial protective effects by increasing NO (Tzemos 2001).

Similarly, the administration of angiotensin converting enzyme inhibitors or Angiotensin receptor antagonists (AT1) was associated with an improvement in endothelial dysfunction (Schiffrin 2000). ARBs seem to improve peripheral endothelial function, although not for a long time. ACEs on the other hand improved both coronary and peripheral endothelial dysfunction in high risk patients for CAD, although the coexistence of other risk factors and genetic variables could affect their magnitude (Ferroni 2006). Despite conflicting data and different effect on target organs, endothelial dysfunction seems to be associated with hypertension and the effects of various antihypertensive drugs on ED has been tested (Mancia 2013).

4.2 Endothelium and dyslipidemia

Endothelial dysfunction is also present in dyslipidemia.

Both increased levels of inflammatory agents, such as C-active protein (Pasceri 2000) and the presence of oxidized LDL, for example, result in a reduction in eNOS and therefore NO availability (Vidal 1998). In addition, lipids induce expression of endothelin-1, which in turn triggers further inflammation via inflammatory factors, such as interleukins 8 and 6 (IL-8, IL-6) and TNF (Cunningham 1997). Eventually, endothelial dysfunction itself makes atherosclerotic plaques more unstable.

4.3 Endothelium and smoking

Smoking works synergistically with other risk factors, increasing the risk of coronary heart disease. There is ample evidence of nicotine-dependent vasodilation in a variety of arteries, such as the brachial artery and carotid artery in humans. This effect appears to be independent of atherosclerotic lesions (McBride 1992, Poredos 1999, Highman 1996).

Tobacco use is also associated with thrombosis, increased risk of bleeding and vasoconstriction, which in turn causes further ischemia, explaining the endothelial dysfunction seen in smokers. This is the result of the effect of toxins present in tobacco, with subsequent release of free radicals in the bloodstream. Nicotine consumption accelerates the apoptosis of endothelial cells, with further exposure of the weakened endothelium, therefore causing inflammation, thrombosis and finally promoting atherogenesis (Ota 1997, Wang 2001, Cerami 1997).

4.4 Endothelial dysfunction and diabetes

Endothelial dysfunction is also present in diabetes mellitus. Hyperglycemia, which is a key feature of this disease, modifies the endothelium by causing oxidative stress, activating protein kinase C, and subsequent formation of advanced glycation end-products. This result in impairement of NO production (Laight 2000, Hink 2001, Weisbrod 1993).

Moreover, hyperglycemia has been found to have a direct effect on guanylate cyclase, regardless of the effect on eNOS (Tuck 2003), while oxidative stress reduces NO bioavailability and promotes its degradation as well (Tesfamariam 1989), with parallel change in the synthesis of antithrombotic prostaglandins (Lagaud 2001). All these changes have a synergistic effect on vascular tone, the increase of which is the first important step in the development of endothelial dysfunction.

This is evident in coronary arteries: animal studies have shown this imbalance of reduced endothelial-dependent vasodilation and excessive response to stimuli, which induce increased

vascular and muscle tone, resulting in decreased blood flow to the heart (Frisbee 2002, Jiang 1991).

The exact role and effect of diabetes on endothelial dysfunction will be discussed in extense.

4.5 Endothelial dysfunction and estrogens.

In postmenopausal women, intracoronary infusion of estrogen led to acetylcholine-induced vasodilation (Herrigton 1994). In women with early atherosclerosis, however, acetylcholine acts as a vasoconstrictor. In this group of patients, estrogen replacement therapy converts vasoconstriction to vasodilation (Bell 1995). Interestingly, reduction of angina has been reported in animal models after sublingual administration of estrogen, which appears to increase blood flow and reduce resistance in the brachial artery, without causing changes in mean blood pressure (Kleinert 1998). The release of NO has also been suggested as a possible mechanism, as estrogens regulate the synthesis of e NOS and induce a decrease in endothelin -1 levels (Armour 1998, Best 1998). Moreover, a study in ovariectomized rats, which resulted in decreased estrogen levels, showed a decrease in endothelial-dependent vasodilation and increased endothelial cell apoptosis. These results may indicate a potential benefit of estrogen administration to the vascular bed (Rossouw 2002).

Despite these findings, however, larger studies in humans have not shown any reduction in cardiovascular events. As a result, estrogen replacement therapy is no longer indicated for cardiovascular risk reduction (Hulley 1998, Widlanksy 2003).

Chapter 5. Effect of glycemic control on markers of subclinical atherosclerosis in patients with type 2 diabetes mellitus.

In this chapter the existing literature on the effect of glycemic control on each of these markers separately will be discussed.

Cardiovascular disease (CVD) is the predominant cause of death in type 2 diabetes mellitus (T2DM), as already mentioned. Generalized vascular disease is found even in asymptomatic patients with T2DM, who appear to have worse cardiovascular prognosis compared to healthy individuals. Increased cardiovascular risk cannot fully be attributed to the presence of traditional risk factors, such as dyslipidemia, hypertension, smoking or hyperglycemia, in these patients. Although existing evidence suggests the presence of a strong association between duration and degree of hyperglycemia and vascular disease (Haas 2018), large trials have failed to show cardiovascular benefit of strict glycemic control in T2DM, especially those with longer disease duration (ACCORD Study Group 2008, Montori 2008).

Endothelial dysfunction is an early event in the progression of atherosclerosis (Calles Escandon 2001). Subclinical atherosclerosis, which is increased in T2DM patients, includes impaired vasodilation, increased coronary artery calcification (CAC), carotid intima media thickness (cIMT), arterial stiffness (AS) and reduced arterial elasticity. Each of these alterations is represented by a marker of subclinical atherosclerosis, serving as a cost-effective alternative to classic complex cardiac testing.

This is of great importance, given that the current diagnostic strategy is based on targeting traditional risk factors or using scoring systems that might either be insufficient to identify high-risk patients or present limited value in asymptomatic populations who lack these risk factors and yet suffer from CVD complications (Brindle 2006, Law 2004). Therefore, imaging-guided risk assessment for detection of subclinical atherosclerosis might not only improve the compliance of those at high risk but also help reclassify lower risk patients who might benefit from targeted or more aggressive treatment. The Framingham risk score, an established tool for asymptomatic patients, appears to be less predictive for diabetics as opposed to the general population (Gore 2009). Therefore, increasing interest has led to the development of other screening tests for this

population. As a subclinical marker of CVD, CAC scoring is known to predict cardiac events and has been a valuable tool for coronary artery disease (CAD) stratification of low–intermediate-risk patients, such as asymptomatic diabetics, and was recommended according to American Heart Association/American College of Cardiology Foundation guidelines (Grundy 2019).

For this review, PubMed and Google Scholar were searched for potentially relevant articles published from January 1, 1990 to December 31, 2020. The markers will be divided in structural and functional markers of atherosclerosis.

5.1 Structural markers

5.1.1 cIMT-Carotid atherosclerosis

The use of B-mode ultrasound for cIMT assessment is a noninvasive, sensitive and reproducible technique, which can be used to identify and quantify subclinical vascular disease and detect carotid plaques. It is considered a strong predictor for cardiovascular morbidity and mortality in T2DM and is used in numerous studies to detect patients at high risk of developing these complications.

Normally, cIMT increases with age, while male sex, as already menitoned and is associated with higher values (Ebrahim 1999). The Atherosclerosis Risk In Communities (ARIC) study found a 0.07 mm increase of mean cIMT in all age groups of diabetic patients compared to nondiabetics after adjustment for other cardiovascular risk factors (Nambi 2010).

Interestingly, HbA1c is independently associated with cIMT values even in persons without diagnosed diabetes, suggesting the significance of glycemic control in development and progression of atherosclerosis (Huang 2011). It has become clear that, even in the prediabetic state, the risk of CVD is modestly increased *(*Klupp 2015, Levitan 2004*)*. In this direction, Di Pino *et al* (Di Pino 2014) reported that, even in patients with prediabetes or newly onset diabetes, cIMT is impaired and correlates significantly with HbA1c. Moreover, higher HbA1c values are associated with higher cIMT values in subjects with normal glucose tolerance (NGT). Therefore,

HbA1c is better than fasting glycemia or oral glucose tolerance tests as a surrogate marker to identify patients at high CVD risk.

In T2DM patients with near-normal HbA1c levels (5.8%–6.4%), further improvement of glycemic control prevents cIMT progression (Kawasumi 2006). Recently, it has been suggested that poor glycemic control (*i.e*. HbA1c > 7%) and longer diabetes duration (> 1 year) independently exert adverse effects on cIMT in a smaller population (n = 45) of younger patients with diabetes (aged 10–25 years). The presence of hypertension and higher body mass index are predisposing factors as well (Sharma 2017).

Interestingly, Di Flaviani *et al* (di Flaviani 2011) investigated the effect of glucose variability and overall glucose load on CVD risk by monitoring blood glucose and pressure continuously for 24 h in patients with optimal glycemic control. Glucose fluctuations appear to activate the oxidative stress pathway, but cIMT is affected by chronic and postprandial hyperglycemia rather than glucose variability. The prognostic information of postprandial glucose in CVD was previously suggested by the DECODE Study group (DECODE STUDY GROUP 1999).

In theory, metabolic control could potentially attenuate or reduce cIMT. Twelve to fourteen weeks after treatment with the peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, pioglitazone, cIMT was found to be significantly reduced. This effect was independent of improved glycemic control (Langenfeld 2005). Pioglitazone is superior to glimepiride in terms of insulin resistance (IR) improvement and cIMT reduction. PPAR-γ activation has both antiatherogenic and proatherogenic properties.

The effect of sitagliptin on cIMT has been reported from several studies. In the PROLOGUE study, sitagliptin was not superior to conventional treatment in terms of cIMT progression, despite significant improvement of glycemic control (Oyama 2016). Alogliptin, a dipeptidyl peptidase-4 inhibitor (DDP-4i), was also found to attenuate cIMT progression (Katakami 2013). It must be noted, though, that in the PROLOGUE trial, insulin-treated patients were excluded, and the HbA1c changes were lower compared to that in other studies.

Last but not least, cIMT progression is inhibited after metformin use. Metformin has several metabolic effects. It modulates hepatic glucose, improves IR and has recently been found to

decrease the plasma DDP-4 activity with subsequent increase in glucagon-like peptide-1 (GLP-1) concentrations (Green 2006). In T2DM without former CVD, the combination of liraglutide, a GLP-1 analogue, with metformin decreased cIMT after 8 mo. These changes could not be attributed entirely to the HbA1c improvement or lipid changes, suggesting a possible beneficial role in reducing plaque formation and inflammation, as previously reported (Rizzo 2014). Interestingly, the addition of metformin to insulin treatment, aiming at achievement of HbA1c < 7%, did not reduce cIMT in the Copenhagen Insulin and Metformin Therapy (CIMT) trial, a fact attributed partially to the smaller-than-expected final study size (Lundy-Christensen 2016).

Table 5-1 summarizes the interventional and observational studies on cIMT outcomes after glycemic control in T2DM patients.

Table 5-1. Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and carotid intima media thickness outcomes. α-GI: Alpha-glucosidase inhibitors; CHD: Coronary heart disease; cIMT: Carotid intima media thickness; CVD: Cardiovascular disease; DM: Diabetes mellitus; LDL: Low-density lipoprotein; RCT: Randomized controlled trial; SD: Standard deviation; T2DM: Type 2 diabetes mellitus.

Ref.	**Year**	**HbA1c (%), mean ± SD**	**Type of study**	**Intervention**	**Sample size**	**Main findings**
Nambi *et al*	2010	Glucose levels 105 ± 30.7 mg/dL	Population-based cohort	Risk prediction model: Whether cIMT and plaque improves CHD risk prediction when added to traditional risk factors	13145	0.07 mm greater cIMT in the presence of DM
Kawasumi *et al*	2006	5.8-6.4	Cohort	Insulin, sulfonylureas, nateglinide, metformin, pioglitazone, α-GI for 3 yr	100	HbA1c improvement > 0.2% prevents cIMT increase
Di Pino *et al*	2014	5.7-6.4 or > 6.5	Cohort	Subjects without a previous history of diabetes were stratified into three groups	274	Impaired cIMT even in pre-diabetes

				according to HbA1c levels		
Sharma and Pandita	2017	> 7 or < 7	Cohort	T2DM duration > 1 yr or newly diagnosed, age 10-25 yr	45	HbA1c and longer diabetes duration affect cIMT
Di Flaviani *et al*	2011	6.7 ± 1.3	Cohort	Continuous glucose monitoring; Diet and/or metformin	26	No association was observed between cIMT any glucose variability or overall glycemic load
Langenfeld *et al*	2005	7.5 ± 0.9	RCT	Pioglitazone 45 mg/d *vs* glimepiride 2.7 ± 1.6 mg/d for 12-24 wk	173	Pioglitazone reduces cIMT independently of improvement in glycemic control
Oyama *et al*	2016	6.2 < HbA1c < 9.4%	Multicenter PROBE	Sitagliptin 25 to 100 mg/d *vs* conventional treatment over 2 yr	442	Sitagliptin had no additional effect on cIMT progression
Rizzo *et al*	2014	8.4 ± 0.8	Prospective pilot	Liraglutide added on metformin over 8 mo	64	Beneficial role in plaque formation and inflammation

5.1.2 Coronary artery calcification *score (CAC)*

The coronary artery calcification score (CACS) is a well-established marker for the assessment of CVD risk in the general population.

Several researchers have reported CAC progression in T2DM. In a subanalysis of the Multiethnic Study of Atherosclerosis involving 5662 patients with T2DM or metabolic syndrome (MetS) without evident CVD, both categories were found to have greater incidence and

accelerated progression of CAC compared to healthy individuals, which in turn can predict future CVD events (Wong 2012).

The link between CAC and HbA1c or plasma glucose levels is well established. Anand *et al* (Anand 2007) showed that suboptimal HbA1c levels (> 7%) are associated with increased risk for CAC progression. In an asymptomatic Korean population without T2DM, higher HbA1c levels predicted CAC, with the association being more prevalent in women (Chang 2013). The ARIC study showed higher relative risk for coronary heart disease for the highest quantile of HbA1c (Selvin 2005).

Although data for prediabetes are inconsistent, it is suggested that even without glycemic transition from impaired fasting glucose to T2DM, in the presence of IR, higher CAC prevalence is observed (Rhee 2015).

Moreover, symptomatic CAD patients (angina) with T2DM and poor glycemic control appear to have higher plaque volume (Yang 2013). The presence of noncalcified plaques and higher plaque burden are confirmed in asymptomatic T2DM patients as well (Li 2012, Rodriguez 2015). This underlies the importance of extended screening, even at the onset of diabetes.

A recent study from Germany showed that in established T2DM, poor glycemic control is associated with CAC progression. This progression is inevitable and rather unaffected by the burden of risk factors (Erbel 2014). The question of whether tight glycemic control exerts beneficial effects on CAC, either regression or attenuation, remains unanswered because, even now, very few data are available. A small study from Schindler *et al* (Schindler 2009) showed that, after 1 year of treatment, effective glycemic control defined as fasting plasma glucose ≤ 126 mg/dL resulted in lower progression of both cIMT and CACS in treatment-naive, relatively newly-diagnosed T2DM patients (*i.e*. mean DM duration of 25 mo) without known CVD.

In the Veterans Affairs Diabetes (VADT) trial, after a 7.5-year follow-up period, intensive glucose lowering reduced cardiovascular events in patients with less extensive calcified coronary atherosclerosis, implying that aggressive glycemic control may be less effective in more advanced atherosclerosis. This was the case in other major trials such as the ACCORD study, which showed that patients without CVD and HbA1c < 8% are the ones who benefit the most

from intensive treatment (Reaven 2009). However, the extended follow-up to the VADT trial revealed that intensive glycemic control in patients with T2DM of > 5 years duration with previous cardiovascular events resulted in 8.6-fold fewer major cardiovascular events *per* 1000 person-years than those assigned to standard therapy (Hayward 2015). This might suggest that a longer observation period is needed so that the beneficial effect becomes clinically apparent.

Yang *et al* (Yang 2015) showed that long-term HBA1c variability plays an important role because metabolic stabilization for longer periods and at earlier stages of the disease might prevent subclinical coronary atherosclerosis. This is in agreement with the presence of the so-called legacy effect, a hypothesis that supports that early metabolic control has beneficial effects in terms of CVD prevention (Won 2018). Recently, it has been shown that in asymptomatic CAD patients with known T2DM, optimal glycemic control attenuates CAC progression, whereas those patients with more calcified coronary lesions (defined as CAC > 400) appear to benefit the most.

At the tissue level, advanced end-glycation products were found to accelerate calcification in microvascular pericytes (Yamagishi 1999). Other experimental data suggest a positive feedback loop of calcification and inflammation that plays an important role in disease progression, induced by the very same atherosclerotic lesions (Nadra 2005). It is possible, though, that intensive treatment might be able to stop this vicious cycle triggered by baseline calcification.

Funck *et al* (Funck 2017) showed higher burden and a greater number of atherosclerotic plaques in asymptomatic T2DM patients compared to healthy controls, despite optimal control of classical risk factors (hyperglycemia, BP, hyperlipidemia). They hypothesized that intensive risk factor control could not adequately control atherosclerosis progression, probably due to higher burden at baseline, in accordance with data supporting higher CVD risk in T2DM patients with known macrovascular complications.

Malik *et al* (Malik 2017) found that CAC score could improve long-term risk stratification to prevent CVD in T2DM and MetS. Importantly, CACS of 0 is associated with lower CVD risk independent of T2DM duration, glycemic control or insulin treatment. Even in T2DM patients with disease duration of more than 10 years, the absence of CACS was associated with low risk

for future events, as in those with shorter disease duration. Finally, a recent study by Razavi *et al* (Razavi 2021) found that long-term absence of CAC during a follow-up period of 10 years in patients with T2DM and MetS was associated with baseline CAC of 0. Optimal multifactorial control is needed for healthy arterial aging. These data suggest that although T2DM is a CVD risk equivalent, it demonstrates considerable heterogeneity (Ahlqvist 2018). Table 5-2 summarizes the effect of glycemic control on CAC in T2DM patients.

Table 5-2. Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and coronary artery calcification outcomes. CAC: Coronary artery calcification; cIMT: Carotid intima media thickness; MetS: Metabolic syndrome; PWV: Pulse wave velocity; SD: Standard deviation; T2DM: Type 2 diabetes mellitus.

Ref.	**Year**	**HbA1c (%), mean ± SD**	**Type of study**	**Intervention**	**Sample size**	**Main findings**
Razavi *et al*	2021	Fasting glucose > 126 mg/dL	Multiethnic cohort	Two CAC scans with a 10-yr interval	574	More than 40% of adults with MetS or T2DM and baseline CAC = 0 had long-term absence of CAC
Schindler *et al*	2009	9.8 ± 2.7	Prospective	Glyburide 10-20 mg/d ± metformin 500-1000 mg/d; Observation for 14 ± 2 mo	39	Lower progression of cIMT and CAC with glucose-lowering treatment
Won *et al*	2018	7.5 ± 1.2 and 6.4 ± 0.9	Retrospective, single-ethnicity, multicenter observational	Data on the impact of optimal glycemic control on CAC progression	1637	Attenuation of CAC progression, especially if CAC > 400
Funck *et al*	2017	6.5 ± 0.7	Prospective cohort	Observational, 5-yr follow-up	106	CAC progression in DM compared to healthy.

						Independently associated with PWV
Malik *et al*	2017	HbA1c measurements were not available at baseline	Prospective cohort	Observational	6814	Baseline CAC values most important progression determinant

5.2 Functional markers

5.2.1 Flow-mediated dilatation

Originally described in 1992, Flow-mediated dilatation (FMD) is a noninvasive functional marker of subclinical atherosclerosis that utilizes ultrasound to record the reaction of brachial artery to an ischemic stimulus. It describes the vascular response to elevated blood flow, which is mediated by the produced vasoactive nitric oxide. FMD has been found to correlate with the severity and extent of coronary atherosclerosis (Raitakari 2000).

In T2DM, postprandial hyperglycemia occurs early in the course of the disease and is thought to be a better marker of glycemic burden regarding associated complications (Ceriello 2004).

In a study with 30 T2DM patients, the investigators measured FMD and circulating endothelial cells (CECs), a marker of vascular damage. Patients were found to have impaired endothelial function compared to healthy controls, while HbA1c > 7% was associated with higher levels of CECs and lower FMD, suggesting the crucial role of glycemic control in diabetes management (Kotb 2012). Some data suggest that glycemic control may result in improved vasodilatory responses and that certain glucose-lowering agents can improve FMD.

Watanabe *et al* (Watanabe 2000) hypothesized that improvement of IR, a major metabolic cause of atherosclerosis, following treatment with troglitazone for 4 weeks would improve endothelial dysfunction as well. At the end of the study, improvements in fasting glucose, insulin levels and FMD were documented. Similar results were confirmed in recent-onset diabetes without

macrovascular complications, meaning that improvement of fasting insulin concentrations might be the underlying mechanism (Caballero 2003).

It seems that PPAR-γ agonists exert their antiatherogenic effect independently of their glucose-lowering effects, given that pioglitazone improves FMD irrespective of significant changes in insulin, C-reactive protein (CRP), free fatty acids, and adiponectin levels (Martens 2005). In nondiabetic patients with IR and recent history of stroke or transient ischemic attack, pioglitazone appears to reduce the risk for future CVD events (Kernan 2016). The important role of reduction in IR is more apparent after comparison with insulinotropic sulfonylureas that achieve similar HbA1c effects without improvement of FMD (Asnani 2006, Papathanassiou 2009). The PROactive study did not shed light on the mechanism by which pioglitazone exerts its vascular benefit; it did, however, show a reduction in primary cardiovascular outcomes by almost 16% (Dormandy 2005). The reported improvement in fat cell metabolism is another argument toward the positive effects of thiazolidinediones beyond glycemic control (Wajcberg 2007). Interestingly, gliclazide, unlike glimepiride, is reported to improve IR, CECs and FMD in a small group of T2DM patients (Chen2011, Shimabujuro 2004).

FMD, glucose, and insulin levels were recorded prior to and after a dietary tolerance test in 30 newly diagnosed T2DM patients after treatment with acarbose (300 mg/d), nateglinide (270 mg/d), or placebo for 12 weeks. Fasting FMD responses remained similar at follow-up in all groups. Despite comparable improvement in glycemic control, treatment with acarbose, unlike insulinotropic nateglinide, was associated with higher postprandial FMD responses. Major-Pedersen *et al* (Major Pedersen 2008) reported that single administration of nateglinide initially improves postprandial endothelial dysfunction, but the effect disappears after 12 weeks. This might imply that insulin secretion in the long term obviates the beneficial effects of controlled postprandial hyperglycemia on endothelial dysfunction.

Naka *et al* (Naka 2012) compared the effect of two insulin sensitizers, metformin and pioglitazone, on poorly controlled T2DM already treated with sulfonylureas. Despite similar improvements in glycemic control, homeostatic model assessment insulin resistance index (HOMA-IR) and changes in FMD, only in the pioglitazone group did FMD and IR improve significantly. The authors concluded that treatment-induced changes in FMD are not associated

with the effects on glycemic control or IR. Nevertheless, the reduction in IR achieved in this study was smaller compared to others, while the additional role of longer diabetes duration cannot be ignored.

Alpha-glucosidase inhibitors are also superior to nateglinide in terms of FMD improvement, IR index and markers of atherogenic dyslipidemia, despite similar HbA1c reduction (Sawada 2014).

Incretin-based treatments have been available for over a decade, and there is now evidence of important effects on cardiovascular outcomes beyond their glucose-lowering effects, such as antiatherogenic properties, modulation of arterial inflammation and endothelial function (Hirano 2016).

Improvement of both HbA1c and FMD in T2DM with stable CAD is reported after infusion of recombinant GLP-1. Interestingly, IR, as assessed by the hyperinsulinemic isoglycemic clamp technique, was not improved. Therefore, it is rather unlikely that GLP-1 exerts its beneficial effects on endothelium through improvement of insulin sensitivity index (Nyström 2004).

The beneficial effects of GLP-1 analogue treatment are possibly the result of improved insulin secretion and effect on postprandial glycemic control (Hagemann 2007). Based on this observation, Irace *et al* (Irace 2013) tested the differences between intensification of metformin treatment with exenatide *vs* glimepiride and found that this combination ameliorates FMD through improvements of glycemic control and glycemic variability. The role of glucose variability and its possible deleterious effects on vascular endothelium were suggested earlier because glucose swings appeared to be more damaging for the endothelium than constantly high glucose levels (Ceriello 2008). Further large-scale trials are needed to establish the validity of this notion.

A randomized controlled trial comparing the effects of insulin glargine and of the GLP-1 analogue liraglutide failed to show an improvement in FMD after 14 weeks, although liraglutide appeared to protect □-cell function and reduce oxidative stress. Low plasma concentration of liraglutide, the last dose of which was given 1 d prior to FMD measurement, might explain the negative results (Nomoto 2015). A similar conclusion regarding FMD is drawn after comparison of sitagliptin with glimepiride (Nomoto 2016), which resulted in similar HbA1c improvements.

That said, in a study from Egypt, sitagliptin improved endothelial dysfunction, insulin sensitivity, BP and hyperlipidemia in newly diagnosed T2DM patients (Amira 2017). A beneficial effect on FMD after sitagliptin is suggested by other authors without superiority against the α-glucosidase inhibitor voglibose (Kubota 2012).

More recently, Lambadiari *et al* (Lambadiari 2019) showed that in patients with poorly controlled T2DM, treatment intensification with incretin-based treatment improves not only FMD but other markers of subclinical atherosclerosis and cardiac function as well. This improvement is more profound in patients who achieve optimal glycemic control.

Linagliptin (at a dose of 5 mg daily) does not improve large-vessel endothelial function despite decreasing inflammation in patients with longer T2DM duration after 12 wk of treatment. Mitochondrial function and muscle oxygenation were not increased either (Baltzis 2016). This suggests that diabetes duration might play an additional, important role. Surprisingly, Ayaori *et al* (Ayaori 2013) showed a deterioration of FMD after both alogliptin and sitagliptin. This was rather unexpected because positive effects had been reported, as mentioned earlier. A possible explanation for this phenomenon is that DPP-4 enzyme physiologically degrades GLP-1 (7-36) into the non-insulinotropic GLP-1 (9-36), which might be inactive but possibly exerts nitric oxide-mediated vasodilatory effects translated into worse FMD values.

In summary, studies with incretin-based agents are mostly relatively small, nonrandomized trials, and therefore observed differences on vascular function with the various agents are difficult to interpret.

The newest class of glucose-lowering agents, sodium-glucose co-transporter-2 (SGLT2) inhibitors, have been shown to reduce cardiovascular mortality in patients with T2DM. Several scholars have investigated whether glycemic control with these agents has any beneficial effects on the progression of atherosclerosis. In poorly controlled T2DM with CAD, administration of 100 mg canagliflozin for 4 wk improved HbA1c [from 9.2 (mean ± SD) ± 1.4 to 8.6 ± 1.1%, $P < 0.01$] and FMD (Takase 2018). In this study, none of the patients were treated with insulin.

Improvement of HbA1c and FMD was found in two recent studies after treatment with dapagliflozin. The first study included newly diagnosed metformin-treated T2DM patients with

HbA1c of < 8% (Shigiyama 2017), whereas the second included T2DM patients with established ischemic heart disease. In the latter, surrogate markers of endothelial dysfunction and inflammation, such as adhesion molecule 1, endothelial nitric oxide synthase and high-sensitivity CRP, decreased with dapagliflozin therapy, and FMD correlated negatively with HbA1c[75]. Assessment of the Dapagliflozin Effect on Diabetic Endothelial Dysfunction of the Brachial Artery (ADDENDA-BHS2) trial, in which patients with poor glycemic control were randomized to receive either dapagliflozin or glibenclamide, shed more light on the effect of this agent (Zainordin 2020).

A Mediterranean diet, except for reducing acute hyperglycemia and increasing antioxidant defenses and the protective action of GLP-1, improves FMD as well. Similarly, insulin sensitivity and FMD improves in T2DM patients with training, especially interval aerobic exercise (Okada 2010).

Glycemic variability is believed to have unfavorable effects on macro- and microvascular events as well as all-cause mortality in T2DM. Wei *et al* (Wei 2016) showed that glycemic visit-to-visit variability correlates with impairment of renal and endothelial dysfunction, as assessed by FMD.

In an attempt to elucidate the underlying mechanism, Costantino *et al* (Consantino 2017) examined the role of glycemic variability and mitochondrial oxidative stress in endothelial dysfunction. The investigators showed that glucose fluctuations rather than HbA1c cause epigenetic changes. This chromatin remodeling favors a proatherosclerotic phenotype. Owing to overexpression of stress molecules, FMD impairment and oxidative stress persist even after improvement of HbA1c. Table 5-3 summarizes interventional and observational studies on FMD outcomes after glycemic control in T2DM patients.

Table 5-3. Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and flow-mediated dilatation outcomes. α-GI: Alpha-glucosidase inhibitor; CAD: Coronary artery disease; DM: Diabetes mellitus; EC: Endothelial cell; FMD: flow-mediated dilatation; SD: Standard deviation; T2DM: Type 2 diabetes mellitus; IL: Interleukin.

Ref.	Year	HbA1c (%), mean ± SD	Type of study	Intervention	Sample size	Main findings
Watanabe *et al*	2000	Fasting glucose 4.9 ± 0.3 mmol/L	Prospective cohort	Troglitazone 400 mg/d for 4 wk in non-DM	13	Improvement on fasting glucose, insulin and FMD
Caballero *et al*	2003	7.5 ± 1.2 to 7.9 ± 1.5	Prospective randomized double-blinded	Troglitazone 600 mg/d for 12 wk	87	Improvement of FMD in newly diagnosed without CAD
Martens *et al*	2005	7.1 ± 0.3	Prospective, randomized, crossover, placebo-controlled, double-blinded	Pioglitazone 30 mg/d for 4 wk	20	Improvement of FMD and adiponectin levels
Asnani *et al*	2006	10 ± 2.3	Prospective randomized double-blinded	Pioglitazone 30 mg/d for 16 wk	20	Improvement of FMD
Chen *et al*	2011	7.4 ± 1.3	Prospective controlled	Gliclazide 30-90 mg/d for 12 wk	58	Improvement of FMD, ECs and insulin resistance
Naka *et al*	2012	7.8 ± 0.9 and 8.1 ± 1.3	Open-label randomized	Pioglitazone 30 mg/d or metformin 850 mg/d	36	Improvement of FMD and insulin resistance

				added to sulfonylureas for 6 mo		
Sawada *et al*	2014	6.9 ± 0.7 *vs* 7.0 ± 0.4	Randomized prospective	Miglitol 150 mg/d or nateglinide 270 mg/d for 16 wk	104	Improvement of FMD, insulin resistance index and markers of atherogenic dyslipidemia in the α-GI miglitol group
Irace *et al*	2013	8.9 ± 1.2 and 8.2 ± 1.2	Observational	Exenatide 10-20 μg/d plus metformin *vs* glimepiride 2-4 mg/d plus metformin for 16 wk	20	Improvement of FMD; Better control on glycemic variability
Nomoto *et al*	2015	8.6 ± 0.8 and 8.7 ± 0.8	Multicenter, prospective randomized parallel-group comparison	Liraglutide 0.3-0.9 mg/d *vs* glargine added on metformin and/or sulfonylurea for 14 wk	31	Similar FMD changes and □-cell function protection
Amira *et al*	2017	Median (range): 8.7 (8.03 –	Prospective controlled	Sitagliptin 100 mg/d for 24 wk	80	Improvement of FMD, insulin sensitivity blood pressure

		9.15)				and hyperlipidemia
Kubota *et al*	2012	7.3 ± 0.8	Open-labeled prospective observational single-arm	Sitagliptin 50 mg/d for 12 wk	40	Improvement of FMD and plasma adiponectin increase
Lambadiari *et al*	2019	8.9 ± 1.8	Prospective cohort	Incretin-based treatment	100	Improvement of FMD and subclinical atherosclerosis after optimal glycemic control
Baltzis *et al*	2016	7.1 ± 0.8	Randomized, double-blind, placebo-controlled	Linagliptin 5 mg/d *vs* placebo for 12 wk	40	No improvement in large vessel endothelial function
Takase *et al*	2018	9.2 ± 1.4	Retrospective preliminary cross-sectional single-center pilot	Canagliflozin 100 mg/d for 4 wk	11	FMD improvement
Shigiyama *et al*	2017	6.8 ± 0.5 and 6.9 ± 0.5	Prospective, randomized, open-label, blinded end-point, parallel-group, comparative	Dapagliflozin 5 mg/d added on metformin 1500 mg/d for 16 wk	80	Improvement of FMD in newly diagnosed T2DM

Zainordin *et al*	2020	9.7 ± 1.9	Prospective, randomized, crossover, placebo-controlled, double-blind	Dapagliflozin 10 mg/d *vs* placebo added on metformin and insulin over 12 wk	81	No difference in FMD between the two groups observed; Significant reduction in surrogate marker of the endothelial function ICAM-1

5.2.2 Pulse wave velocity

Pulse wave velocity (PWV) is a marker of AS, which in turn expresses the reduced flexibility and elasticity of blood vessels. It is assessed noninvasively and predicts future cardiovascular events and all-cause mortality (Sequi-Dominguez 2020). Reduced elasticity occurs naturally with increasing age or under the rather destructive effect of metabolic disorders, such as T2DM or hypertension. T2DM and hypertension seem to have a synergistic effect on the progression of AS. PWV is a useful marker in the investigation of hypertension (Mancia 2014).

The prognostic value of PWV in T2DM is the subject of several studies. It has been estimated that for every 1 m/s increase in PWV, there is a 14.5% increase in the risk of CVD in patients with T2DM (Oliver 2003). The association of glycemic control with PWV was earlier supported by Yokoyama *et al* (Yokoyama 2004), who showed that along with conventional risk factors, such as hyperlipidemia, hypertension and age, microalbuminuria is a determinant of cIMT and PWV and should, therefore, be taken into account for the detection of subclinical atherosclerosis and treatment stratification.

Control of hyperglycemia, even in the short term, appears to improve AS. De Pascale *et al* (De Pascale 2016) showed that good glycemic control is associated with lower AS and increased number of endothelial progenitor cells, which reflects the endothelium's regenerating capacity after damage.

Another study with 1675 participants in rural Brazil showed that increase of HbA1c by 1% was associated with increase of 54% in the odds of increased AS in the diabetic group. Both HbA1c and fasting blood glucose (FBG) had higher discriminatory power in the risk assessment for increased AS in nondiabetics compared to diabetics. Therefore, HbA1c elevation, even within the normal range, might cause endothelial dysfunction (de Oliveira 2015). Similarly, Lee *et al* (Lee 2016) concluded that even in the nondiabetic population, higher HbA1c levels were associated with increased brachial-ankle PWV. Therefore, early detection and management are essential to avoid atherosclerosis progression. There are additional studies that support the prognostic value of PWV in both diabetes and IGT (Cruickshank 2002).

In an older study, Webb *et al* (Webb 2010) aimed to investigate the impact of glucose metabolism and IR on PWV. For this purpose, they enrolled 570 participants in the large ADDITION-Leicester program, who were divided into the three groups after a standard 75-g oral glucose tolerance test, namely NGT, IGT and T2DM. HbA1c as well as PWV gradually worsened from NGT to IGT and T2DM (all $P < 0.01$). Multivariate models demonstrated a strong relationship among PWV, fasting and 2-h postprandial glucose levels as well as HOMA-IR. Moreover, although all three indices contribute to PWV increase of about 3%–6%, postprandial glucose appears to be the most significant determinant. The effect of postprandial glucose on PWV was later supported by Li *et al* (Li 2012), who found that AS was increased in patients with IGT and newly diagnosed T2DM but not in those with impaired fasting glucose tolerance. Again, the hypothesis was that glycemic control, especially by targeting postprandial hyperglycemia, might reverse this phenomenon or even improve PWV.

Improvement of glycemic control after glimepiride, unlike glibenclamide, is associated with improved brachial ankle PWV and Augmentation Index (AIx). Notably, glimepiride decreases proinflammatory markers such as tumor necrosis factor-α, interleukin and CRP, with

improvement of IR (Koshiba 2006). In that study, insulin-treated T2DM patients were used as the control group.

Beneficial effects on PWV through normalization of IR, as well as reduction of inflammation, are reported after glycemic control with rosiglitazone even in patients with established CAD (Yu 2007).

The effect of hyperinsulinemia on other markers of subclinical atherosclerosis has already been discussed. In patients without evident macrovascular disease, higher insulin levels remain a significant predictor of PWV, even after adjustment for other well-established risk factors, concerning AS (Hansen 2004). Interestingly, in this study, only 2% of the participants had a history of diabetes.

Another argument toward this hypothesis is that the use of insulin-sensitizer metformin in patients with nonalcoholic fatty liver disease (NAFLD)—a condition associated with IR—was associated with a significant reduction of both PWV and AIx in this population, with marginal improvements in fasting glucose, triglycerides, alkaline phosphatase and high-density lipoprotein cholesterol. These favorable effects of metformin were also observed in NAFLD patients without T2DM (Sofer 2011).

In obese patients, treatment with metformin, rosiglitane or a combination of both with lifestyle modification improved not only glycemic control but PWV as well. In this study, however, only femoral PWV, known to assess peripheral stiffness rather than central AS, was associated with HbA1c (Shah 2018). On the contrary, reduction in the body fat mass of obese T2DM patients after exenatide improved lipid profile and aortic PWV, whereas PWV of the extremities did not change. The authors concluded that visceral fat reduction affecting the measurements could explain these findings (Hing 2016). Using a different method, weight loss was found to strongly and independently reduce AS (Petersen 2015). Because several classes of glucose-lowering agents are associated with weight gain, this should be considered prior to treatment stratification. Nevertheless, the beneficial effects of exenatide on PWV do not rely solely on weight reduction because improvements were shown after HbA1c reduction in T2DM patients without evident CAD, treated with metformin (sulfonylurea was prescribed in 5 patients only) (Scalzo 2017).

Regarding the effects of DDP-4i on PWV, data are variable. When added to metformin, in T2DM with suboptimal HbA1c (> 7%), neither sitagliptin nor glibenclamide demonstrated any PWV benefits. Neither drug significantly influenced oxidative stress (Koren 2012). Zografou *et al* (Zografou 2015) showed no increase of PWV in drug-naive patients with T2DM, despite changes in HbA1c after treatment with vildagliptin. These patients had suboptimal HbA1c (7%–9%) at baseline and, moreover, a significant improvement in 24-h BP or waist circumference, all of which are important in terms of endothelial dysfunction.

Conversely, Duvnjak and Blaslov (Duvnjak2016) studied 51 T2DM patients with good glycemic control, assigned to receive either sitagliptin or vildagliptin (100 mg/d). Both drugs were associated with improved PWV and AIx despite insignificant HbA1c changes. The authors concluded that the positive effects on AS are beyond glucose control. Favorable effects of linagliptin treatment for 26 wk with minimal yet significant HbA1c reduction (-0.4%, $P < 0.001$) were recently reported in newly diagnosed T2DM patients. Interestingly, after a 4-wk washout period, PWV returned to pre-intervention levels (de Boer 2017).

Large trials on DDP4-is support a neutral effect on CVD outcomes, although data show an important positive effect on PWV (Savarese 2016). It is, therefore, possible that not all agents of this class share the same beneficial effects. Additionally, these studies differ in terms of baseline HbA1c as well as diabetes duration.

It has been proposed that a HbA1c of > 7% and diabetes duration of > 5 years are important cutoffs, above which stiffening of the arteries accelerates, especially in the presence of hypertension (Chen 2009). A recent observational study from Chang *et al* (Chang 2018) rather confirms this observation. In hypertensive, poorly controlled T2DM (HbA1c³ 9%), reduction of HbA1c was not accompanied by significant differences in PWV or AIx in the short term. In a subanalysis based on cutoff HbA1c level of 7%, those with better HbA1c values had lower PWV, yet not significantly, and shorter T2DM duration. Even in high-risk middle-aged to elderly patients, PWV can be attenuated after improvements of glycemic control, BP, and heart rate. Furthermore, the rate of HbA1c reduction is associated with reduced risk for increased PWV (Ferreira 2015).

Based on the aforementioned facts, it would be reasonable to hypothesize that improvement of glycemic and systemic BP control would attenuate or even improve PWV in T2DM. Amongst the various antidiabetic agents, SGLT2 inhibitors may efficiently lower both HbA1c and BP. Available data suggest that the reduction in CVD mortality is beyond their glycemic and antihypertensive effects, both factors being important for AS. Animal models show improvement in AS after the use of empagliflozin by promoting glycosuria and reducing systemic and renal AS based on improvements of periarterial and tubulointerstitial fibrosis (Gangadharan 2014). Given that numerous studies support the relationship among AS, albuminuria and kidney injury (Woodard 2015), this could be a possible additional mechanism beyond glycemic control. In animal models, dapafliflozin improves hyperglycemia, AS and smooth muscle cell function, meaning that the positive effects on CVD mortality in diabetes are possibly owing to improvements in generalized vascular function (Lee 2016). The newest agent in this category, tofogliflozin, attenuated PWV in T2DM without history of CVD (Katakami 2021). A recent metanalysis on newer agents showed that both GLP-1 analogues as well as DDP-4i effectively reduce PWV (Batzias 2018).

An association between HbA1c and PWV has been previously reported from some cross-sectional studies (Teoh 2013). The implication that hyperglycemia alters material within the arterial wall and contributes to atherosclerosis was reinforced by other similar data. As Ferreira *et al* (Ferreira 2015) reported, early intervention aiming to improve glycemic control might at least partially affect PWV, probably before the structural alterations occur. Table 5-4 summarizes interventional and observational studies on PWV outcomes after glycemic control in T2DM patients.

Table 5-4. Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and pulse wave velocity outcomes. α-GI: Alpha-glucosidase inhibitor; AIx: Augmentation Index; BMI: Body mass index; CAD: Coronary artery disease; cIMT: Carotid intima media thickness; DDP-4i: dipeptidyl peptidase-4 inhibitor; HCT: Hydrochlorothiazide; IFG: Impaired fasting glucose; IR: Insulin resistance; NAFLD: Nonalcoholic fatty liver disease; PWV: Pulse wave velocity; RCT: Randomized controlled trial; SD: Standard deviation; T2DM: Type 2 diabetes mellitus.

Ref.	Year	HbA1c (%), mean ± SD	Type of study	Intervention	Sample size	Main findings
Koshiba *et al*	2006	7.8 ± 2.0 and 7.7 ± 1.9	Prospective, randomized	Glibenclamide followed by glimepiride for 28 wk *vs* continuous administration of glibenclamide *vs* insulin therapy	34	Improvement of PWV, AIx, IR in the glimepiride group
de Oliveira *et al*	2015	5.6 ± 0.7 and 6.3 ± 1.1	Prospective cohort	Observational	1675	Higher HbA1c levels are associated with higher PWV
Yu *et al*	2007	6.5 ± 0.2	Prospective, randomized	Rosiglitazone 4 mg/d for 12 wk in diabetic patients with CAD	123	Decrease in PWV
Sofer *et al*	2011	Fasting glucose: 132 ± 51 mg/dL	Prospective, randomized, placebo-controlled, double-blind	Metformin in patients with NAFLD with or without T2DM/IFG for 4 mo	63	Decrease in PWV and AIx
Shah *et al*	2018	7.7± 2.0	Subanalysis of an RCT	Obese patients with metformin *vs* metformin plus intensive lifestyle intervention *vs* metformin plus rosiglitazone for 7.6 yr post-randomization	453	PWV increased; Attenuation possible
Scalzo *et al*	2017	7.3 ± 1.1	Prospective, randomized, placebo-controlled, double-blind	Exenatide 20 μg/d subcutaneously, 30-60 min prior to meals, for 3 mo	23	Decrease in PWV
Koren *et al*	2012	Fasting glucose: 169 ± 12	Prospective, controlled, open labeled,	Sitagliptin 100 mg/d or glibenclamide 5 mg/d for 3 mo, cross-over switch for an	34	No PWV benefits; Beneficial BMI effects of

		mg/dL	crossover	additional 3 mo		sitagliptin
Zografou *et al*	2015	8.1 ± 0.8	Prospective randomized open-label	Vildagliptin 100 mg/d plus metformin 1700 mg/d *vs* metformin monotherapy 1700 mg/d	64	No effect on arterial stiffness in drug-naive patients with T2DM
Duvnjak and Blaslov	2016	6.9 ± 1.1	Prospective, uncontrolled, open label, parallel-arm, randomized	Sitagliptin 100 mg/d or vildagliptin 100 mg/d for 3 mo	51	Decrease in PWV and Aix; No HbA1c reduction
De Boer *et al*	2017	6.3 ± 0.4	Prospective, randomized, placebo-controlled, double-blind	Linagliptin 5 mg/d *vs* placebo for 26 wk	45	PWV improvement disappears after 4-wk washout period in newly diagnosed T2DM
Chen *et al*	2009	6.9 ± 1.3	Prospective cohort	Observational	1000	PWV correlates with HbA1c and diabetes duration in patients with T2DM and hypertension
Chang *et al*	2018	11.7 ± 1.9	Prospective cohort	Insulin or oral hypoglycemic agents (metformin, sulfonylurea, α-GI, DDP-4i) or combined insulin and oral agents for 12 wk	64	No PWV improvement
Ferreira *et al*	2015	7.6 ± 1.4	Prospective cohort	Metformin, sulfonylureas or insulin for 4.2 yr	417	Attenuation of PWV progression

5.2.3 Large and small artery elasticity

Arterial elasticity is also a noninvasive measure for the assessment of cardiovascular risk. It reflects the extent of vascular injury owing to cardiovascular risk factors and allows risk stratification with considerable prognostic value (Wang 2008).

It is suggested that small arteries compared to large ones—that is, the aorta—contain a more repairable component regarding arterial elasticity, where fixed fibrotic tissue is more prominent and requires a longer repair period (Wilkinson 2004). Interestingly, in a group of nondiabetic patients with CAD, only small (C2) and FMD appeared to be impaired compared to healthy controls, suggesting that C2 is a surrogate marker for the clinical evaluation of endothelial function (Tao 2007).

Shargorodsky *et al* (Shargorodsky 2003) showed that 6 months of treatment with rosiglitazone in 52 patients with moderate CVD risk and poor glycemic control (longer disease duration of 5–28 years, 24% on insulin) resulted in impressive improvement of C2, which was attributed to improvements in IR and hyperinsulinemia. Though there was a tendency toward improvement for large (C1), it did not reach statistical significance. The authors mentioned that a longer follow-up period was probably needed. After an extended follow-up period of 2 years, improvements in both C1 and C2 after rosiglitazone treatment were confirmed, and the beneficial effect deteriorated after treatment discontinuation. Moreover, this was independent of glycemic control, implying the central role of hyperinsulinemia in AS. Last but not least, with data supporting that rosiglitazone inhibits cIMT progression in nondiabetic individuals, the antiatherogenic effect of PPAR-γ activators is independent of glycemic control (Shargorodsky 2007).

Prisant *et al* (Prisant 2006) aimed to investigate the relationship of HbA1c and arterial elasticity by performing measurements of both C1 and C2 in 111 subjects with longer diabetes duration (12 years) and poor glycemic control (HbA1c 8.9%). Increasing age and HbA1c were found to be associated with small, but not large, artery elasticity, whereas women with T2DM had lower C2 compared to men. In this study, 26% of participants were type 1 DM patients.

That said, McVeigh *et al* (McVeigh 1993), in an older study analyzing intra-arterial brachial artery pulse waves in T2DM, suggested reduced C2 in T2DM but no correlations with fasting glucose or HbA1c, whereas C1 was not reduced. They used mostly diet intervention and/or sulfonylurea and metformin to achieve glycemic control.

Several data support that C2 can be raised by means of improvement of HbA1c, such as through fish oil supplements (McVeigh 1994) or telmisartan (Asmar 2002). Mourot *et al* (Mourot 2009) investigated the effect of a cardiovascular rehabilitation program on arterial compliance in patients with T2DM and CAD. These patients had suboptimal or poor glycemic control at the time of admission. After 6 wk, improvement of arterial compliance was observed, probably thanks to regular exercise, optimal glucose-lowering, and hypolipidemic treatment. Because arterial compliance was improved in the subpopulation with no change in antihypertensive treatment as well, the observed increase extended beyond antihypertensive treatment. Moreover, decrease of IR through training and amelioration of glycemic control, which was achieved by insulin, oral agents, or a combination of both, appear to have contributed to this improvement as well.

It is evident that the additional use of these noninvasive markers of atherosclerosis strengthens the predictive risk for developing CAD beyond traditional risk assessment and enables the monitoring of selected treatment in T2DM. The use of proper medication and intervention with proven CVD benefit is of crucial importance in the management of these patients, especially at an early stage of the disease, as later reflected on updated guidelines. By the time of the study, these agents (ie SGLT2- inhibitors) were not available, but still we aimed to assess CVD risk in patients with and without known CVD and and examine the effect of intensive glycemic control, which remains both clinically and scientifically relevant.

Chapter 6 *: Special Part*

6.1 Baseline Study

6.1.1 Background and Hypothesis

Atherosclerotic cardiovascular disease (ASCVD) remains the main cause of death in persons with type 2 diabetes mellitus (T2DM) and results in significant morbidity and increasing health care costs (ADA 2021). Many persons are in fact already predisposed to significant cardiovascular risk even at the prediabetic stage (Haffner 1990). Moreover, they often remain asymptomatic and when they develop clinically evident CVD, their prognosis and outcomes are worse compared to individuals with CVD but without T2DM (Raza 2015, Arnold 2014).

Large epidemiological studies have established the role of hyperglycemia as a risk factor for the development of both microvascular and macrovascular complications (Colaboration E.R.F 2010, Nathan 1993). Hyperglycemia alters the protective mechanisms of the endothelium and leads to inflammatory damage of the endothelial wall, increased permeability, as well as reduction of antiatherogenic vasodilators such as nitric oxide (Williams 1996). Endothelial dysfunction has been previously reported in personswith T2DM as well as prediabetes compared to healthy subjects (Mapanga 2016, Su 2008, de Jager 2006, Liye 2011), while comorbidities such as hypertension and dyslipidemia seem to have a synergistic effect leading to accelerated atherogenesis (Sitia 2010, Munro 1988, Li 2014).

The aim of the present study was to assess vascular dysfunction by using brachial artery flow-mediated dilation (FMD), carotid-femoral pulse wave velocity (cfPWV), central augmentation index(AIx), large (C1) and small (C2) artery compliance, carotid intima-media thickness (cIMT), and ankle-brachial index (ABI) in poorly controlled DM2 persons with and without overt CVD as well as to examine the role of DM2 duration.

6.1.2. Material and Methods

Patients who participated in our study with T2DM were prospectively recruited from the Department of Endocrinology, Ioannina University Hospital and the Diabetes Outpatient Clinic, Chatzikosta General Hospital of Ioannina, between July 2011 and December 2014.

The diagnosis of T2DM was defined according to the American Diabetes Association and the European Association for the Study of Diabetes criteria (ADA 2014). Disease duration was defined as the time interval since diagnosis and study entry, as recorded in patients' clinical records.

Eligible subjects were >50 and <80 years old, with poor glycemic control (HbA1c ≥7.5%, 58 mmol/mol). The presence of established macrovascular complications was evaluated on the basis of the patients' past medical history obtained by experienced cardiologists and endocrinologists.

Hypertension was defined as blood pressure >140/90 mmHg or antihypertensive medication use, and hypercholesterolemia as a level of total cholesterol >200 mg/dl or lipid-lowering medication use. Active smokers or those who had stopped smoking during the previous 12 months were defined as smokers. Physical activity level was recorded in a three-point scale: 1) no exercise or circumstantial exercise less than once per week, 2) brisk walking for at least 30 min for no more than 5 d/wk, and 3) daily exercise schedule, either brisk walking or more intense exercise. Presence of overt macrovascular disease was evaluated by experienced physicians based on review of medical records, history and physical examination.

Exclusion criteria included symptomatic heart failure (Stage C and D), presence of implantable cardioverter-defibrillator or pacemaker, chronic atrial fibrillation, chronic liver disease, chronic kidney disease stage 4 or 5, active malignancies or autoimmune disorders, systematic use of glucocorticoids, drug and alcohol abuse as well as severe mental illness. Informed consent was obtained from all participants. The study was approved by the Ioannina University Hospital Research Ethics Committee and complies with the Helsinki Declaration.

6.1.2.2 .Procedures

The following indices of vascular function were measured in all patients: common carotid intima-media thickness, (cIMT), brachial artery flow-mediated dilation (FMD), carotid-femoral pulse wave velocity (cfPWV), central augmentation index (AIx@75), large (C1) and small artery elasticity indices (C2) and ankle-brachial index (ABI). All measurements were performed by the same experienced operator, early in the morning. Patients were asked to refrain from smoking and remain fasted for at least eight hours before the measurements. To prevent the effect of possible hypoglycemia, glucose levels were measured before the study.

In case of significant hypoglycemia (<70 mg/dl) or hyperglycemia (>300 mg/dl), they were asked to return on a different day. The patients remained in the supine position in a quiet environment with stable temperature (20^oC) for at least 15 minutes before measurement.

Height and weight were measured and body mass index (BMI) was calculated (kg/m^2). Waist-to-hip-ratio was calculated by measuring waist and hip circumference (at the midpoint between last rib and the iliac crest and at the widest point of the hips respectively).

Blood pressure was measured in the sitting position with an automated sphygmomanometer (Omron, Omron Healthcare, Japan).

6.1.2.3. Assessment of arterial structure and function indices

Brachial artery FMD, an established index of vascular endothelial dysfunction was assessed with scans of the brachial artery and measurement of the vasodilatory response. A 5-12 MHz linear transducer (Vivid-I, General Electric) of an Echo-Doppler ultrasound was used to obtain brachial artery scans of the dominant arm at baseline and at 5 minutes after cuff inflation at 250 mm Hg, as well as at four minutes after administering a 400 μg of sublingual glyceryl trinitrate for measurement of the endothelium independent nitrate–mediated vasodilatation (NMD), as previously described and according to published guidelines (Coretti 2002, Antoniou 2021). FMD and NMD were calculated as the percentage increase of the brachial artery diameter after hyperemia and nitrate administration at end diastole (R wave of the electrocardiogram).

The measurement of the common carotid artery IMT was assessed by using B-Mode ultrasound with semi–automated edge detection software (Vivid-I, General Electric, IL, USA). Both the average and maximal cIMT measurement at a length of at least 1cm on the distal wall of the common carotid artery were recorded according to recent consensus of the American Society of Echocardiography [Stein 2008].

Applanation tonometry to measure carotid femoral pulse wave velocity (cfPWV) was used to non-invasively assess arterial stiffness (Sphygmocor system, Cor Medical, Sydney, Australia), as previously described (Wilkinson 2000, Naka 2002). The cfPWV measures the speed of transmission of the pulse wave between carotid and femoral arteries. Transmission time was calculated with a special system software, that uses the R-wave of an electrocardiogram as reference frame and a given distance from stable anatomical positions (suprasternal notch-femoral artery). The Augmentation Index (AIx@75), which is related to the extent and speed of both central and peripheral wave reflection, was calculated with the same method using radial artery pressure waveform, by using the latter systolic pressure peak of the obtained waveform and was normalized for a heart rate of 75 bpm, as previously described.

The HDI Pulse Wave CR2000 was used to assess small and large artery compliance, by obtaining non-invasively radial arterial measurements using an oscillometric technique, as previously described (Vlahos 2011).

ABI was calculated as the ratio of average systolic blood pressure at the ankle of each leg divided by the average systolic blood pressure of the arm using portable doppler device. Values ≤ 0.90 were defined as abnormal and values >1.4 were considered to indicate the presence of stiff arteries.

6.1.2.4. Laboratory and other measurements

Venous blood samples were obtained to measure serum glucose, total cholesterol, high density lipoprotein (HDL), triglycerides, urea and creatinine. LDL–C was calculated using the Friedewald formula (LDL-C= Total Cholesterol- HDL- Triglycerides/5) (Friedewald 1972). HbA1c levels were measured with a point-of-care analyzer (DCA Analyzer, Siemens 2007), that requires a small capillary blood sample (1μl) and provides reliable results within 6 minutes.

6.1.2.5. Statistical Analysis

Normal distribution was tested for all continuous variables using the Kolmogorov Smirnov test. Continuous variables are presented as mean±standard deviation (SD) or median (interquartile range – IQ). The paired t-test and Wilcoxon signed rank test were used to compare continuous variables with normal and not normal distribution respectively at baseline and follow-up. Categorical variables were compared using the McNemar's test. Repeated measures ANOVA (RM-ANOVA) was used to compare changes at baseline vs follow-up between various subgroups of patients (i.e. according to history of CVD, diabetes duration, insulin treatment). A two-sided p-value of <0.05 was considered statistically significant. Based on our previous data in patients with type 2 diabetes (Naka 2002) we anticipated an improvement in FMD of ca. one SD (or ~70% compared with baseline) following treatment. We estimated that a sample size of 50 patients would provide a power of approximately 80% to detect an increase in FMD of at least 0.5 SD (or 35% compared to baseline) following improvement in glycemic control ($\alpha = 0.05$). Data analyses were performed using IBM SPSS version 21.0.

6.1.3. Results

One hundred eight patients were prospectively recruited over the study time period. Thirteen patients did not meet the inclusion criteria and were, therefore, excluded from the study. The total study population consisted of 95 DM2 patients, 53 males (56%) and 42 females (44%). Histogram plots, box plots and Shapiro-Wilk test demonstrated a normal distribution appearance for all continuous variables.

Thirty-eight patients had established CVD (Group 1), whereas 57 had no known history or symptoms suggestive of CVD and comprised Group 2. Mean age was 65 (9) and 62 (10) respectively. The mean duration of diabetes was comparable in both groups [16 (10) vs 13 (9) years, p=0.14), although 14 patients of the second group had a shorter disease duration of <5 years. The impact of diabetes on the vasculature of this subgroup was analyzed separately. Glycemic control was similar between the two groups [mean HbA1c 9.2 (1.5) vs 9.4 (1.8) %, p=0.44). Patients with CVD, had a higher prevalence of hypertension and hypercholesterolemia

(100% vs 74% and 87% vs 54%, p<0.05), but also higher HDL levels [42.7 (8.8) mg/dl vs 20.2 (12.8) mg/dl, p<0.05]. Demographic, clinical, biometric and laboratory parameters of all study participants are summarized in Table 6-1.

Table 6-1. Characteristics of the study population. All continuous variables are normally distributed and therefore expressed in mean-deviation form. Categorical variables are expressed in terms of absolute (n) and relative (%) frequencies. Statistical significance: p-value<0.05. NS= not significant. ***Abbreviations.*** *BMI: Body mass Index; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; LDL, Low-Density Lipoprotein; TRG: triglycerides.*

Category	**Parameter**	**DM2 with CVD (Group 1, n=38)**	**DM2 without CVD (Group 2, n=57)**	**p-value**
Demographics	Age (years)	65 (9)	62 (9.6)	0.163
	Gender (male)	23 (61%)	30 (53%)	0.53
	Diabetes Duration (years)	15.6 (9.6)	12.8 (8.9)	0.138
Comorbidity	Hypertension	38 (100%)	42 (74%)	p<0.05
	Hypercholesterolemia	33 (87%)	31 (54%)	p<0.05
	Smoking	18 (47%)	19 (33%)	0.2
	Exercise	24 (63%)	37 (65%)	1
Family history	Family history of CVD	11 (29%)	10 (18%)	0.214
	Family history of DM2	17 (45%)	32 (56%)	0.301
Biometrics	BMI (kg/m^2)	29.5 (5.7)	30.7 (7)	0.37
	Hip (cm)	106.2 (10.8)	106.1 (14.0)	0.974
	Waist (cm)	105.1 (13.3)	103.4 (14.0)	0.56
	Waist-to-hip ratio	0.99 (0.07)	0.96 (0.08)	0.068

Laboratory measurements	HbA1c (%)	9.2 (1.5)	9.4 (1.8)	0.444
	Chol (mg/dl)	187.9 (51.3)	197.3 (38.8)	0.314
	HDL (mg/dl)	42.7 (8.8)	20.2 (12.8)	$p<0.05$
	LDL (mg/dl)	110.6 (45.1)	114.1 (32.4)	0.657
	TRG (mg/dl)	177.6 (125)	164.9 (128.8)	0.636
	Creatinine (mg/dl)	1.1 (0.29)	1.0 (0.29)	0.101

Patients with CVD were treated more often with antihypertensive medications, insulin, b-blockers, antiplatelet drugs and statins ($p<0.05$) (Table 2). Use of glimepiride and metformin was similar but other sulfonylureas were more common among patients without CVD (40% vs 24%) (Table 6-2).

Table 6-2. Drug intake in our study population, expressed in terms of absolute (n) and relative (%) frequencies. Statistical significance: p-value<0.05. NS= not significant. **Abbreviations.** ACE Angiotensin-converting enzyme; ARBs, Angiotensin II receptor blockers; DPP4, dipeptidyl peptidase 4.

Drugs	**DM2 with CVD (Group 1, n=38)**	**DM2 without CVD (Group 2, n=57)**	**p-value**
Insulin, n (%)	22 (58%)	17 (30%)	p<0.05
Sulfonylureas, n (%)	9 (24%)	23 (40%)	0.122
Glimepiride, n (%)	6 (16%)	10 (18%)	1
Metformin, n (%)	27 (71%)	40 (70%)	1
DPP4-inhibitors, n (%)	8 (21%)	13 (23%)	1
Pioglitazone, n (%)	1 (3%)	4 (7%)	0.645
Meglitidines, n (%)	1 (3%)	2 (4%)	1
Alpha-glucosidase inhibitors	0	1 (2%)	1
Calcium channel blockers, n (%)	10 (26%)	13 (23%)	0.808
ACE inhibitors, n (%)	10 (26%)	11 (19%)	0.457
Beta-blockers, n (%)	27 (71%)	12 (21%)	p<0.05
ARBs, Angiotensin II receptor blockers, n (%)	18 (47%)	26 (46%)	1
Diuretics, n (%)	17 (45%)	16 (28%)	0.124
Antiplatelets, n (%)	27 (71%)	10 (18%)	p<0.05
Statins, n (%)	32 (84%)	24 (42%)	p<0.05
Fibrates, n (%)	7 (18%)	4 (7%)	0.109

Hemodynamic parameters and markers of subclinical atherosclerosis are presented in Table 6-3. Systolic and diastolic blood pressure and heart rate were comparable in both groups. Patients with CVD had higher cIMT ([0.82 (0.2) vs 0.69 (0.2) mm, p<0.05] and lower FMD [3.13 (2.6) vs 4.7 (3.4) %, p<0.05), whereas PWV was similar in both groups [12.1 (3.4) vs 11.3 (3), p=0.1). HbA1c did not correlate with any markers of subclinical atherosclerosis.

Table 6-3. Basic hemodynamic parameters and markers of subclinical atherosclerosis in mean-(deviation) form. Statistical significance: p-value<0.05. **Abbreviations.** ABI, ankle-brachial index; AIx@75, augmentation index; AcfPWV, carotid-femoral pulse wave velocity; cIMT, carotid intima-media thickness, C1, large artery elasticity index; C2, small artery elasticity index; DBP, diastolic blood pressure; HR, heart rate; FMD, flow-mediated dilation.

Parameter	**DM2 with CVD (Group 1, n=38)**	**DM2 without CVD (Group 2, n=57)**	**p-value**
cfPWV, m/s	12.1 (3.4)	11.3 (3)	0.099
HR, bpm	67 (11.3)	69 (11.5)	0.34
SBP, mmHg	136.2 (20.1)	130.4 (17.5)	0.16
DBP, mmHg	75.8 (10.3)	74.5 (12.8)	0.59
C1, ml/mmHg x 100	14.3 (5.7)	13.9 (5.6)	0.74
C2, ml/mmHg x 10	4 (1.59)	4.1 (2.1)	0.86
ABI	0.91 (0.13)	0.95 (0.1)	0.15
FMD, %	3.13 (2.6)	4.7 (3.4)	p<0.05
cIMTaver, mm	0.82 (0.24)	0.69 (0.19)	p<0.05
cIMTmax, mm	1.09 (0.29)	0.96 (0.24)	p<0.05

The second group was divided into two subgroups, based on diabetes duration. In 43 patients the disease had been diagnosed > 5 years prior to enrollment (Group 2a), whereas 14 patients had a

shorter disease duration (diagnosed < 5 years prior to enrollment) (Group 2b). One way ANOVA analysis between the three groups (i.e. group 1, 2a and 2b) demonstrated statistically significant differences in age, HDL levels, diabetes duration and all measured markers of endothelial dysfunction (Table 6-4).

Table 6-4. Continuous parameters with statistically significant differences between patients with DM2 and CVD (group 1) and patients with long and short diabetes duration without CVD (group 2 and 2b respectively), utilizing one- way ANOVA analysis (between groups).

Source of variation	**df**	**Parameter**	**Sums of Squares**	**Mean Square**	**F**	**p-value**
Between Groups	2	Age	655.6	327.8	4.06	$p<0.05$
		Diabetes Duration	2213.1	1106.6	17.6	$p<0.05$
		HDL, mg/dl	1636.9	818.5	6.4	$p<0.05$
		PWV, m/s	63.6	31.8	3.3	$p<0.05$
		FMD, %	84.6	42.3	4.41	$p<0.05$
		IMTaver,mm	0.65	0.32	7.78	$p<0.05$
		IMTmax, mm	0.73	0.37	5.59	$p<0.05$

Post hoc analysis with Tukey's pair-wise comparison procedure revealed that groups 1 and 2a differed only in the HDL level at an alpha level of 0.05. In other words, vascular function indices did not differ between patients suffering from DM2 with CVD and patients with DM2 of long duration without CVD. These indices however, differed significantly between groups 1 and 2b. In addition, patients with DM2 of short duration and no CVD had significantly lower PWV and

cIMT and higher FMD compared to patients with CVD ($p<0.05$ for all, Table 6-5). No other significant correlations were observed.

Framingham risk score was positively associated with aPWV, Aix@75 (r=0.21 and 0.22, both p=0.04) and negatively with C1 and C2 (r= -0.21, p=0.04 and -0.26, p=0.01) No significant associations were observed with FMD, IMT or ABI. (Table 6-6).

Table 6-5. Subgroup analysis between diabetics with long vs short diabetes duration in subjects without CVD, by means of post hoc analysis (Tukey's pair-wise comparison).

Parameter	**Group 2a**	**Group 2b**	**p-value**
Age, years	64 (8)	57 (12.5)	p<0.05
Diabetes Duration, years	16.2 (7.5)	2.3 (1.7)	p<0.05
HDL, mg/dl	48.9 (13.5)	20.2 (12.8)	p=0.25
Creatitine	1.0 (0.31)	0.9 (0.13)	p=0.21
PWV, m/s	11.8 (3.1)	9.9 (1.98)	p=0.14
FMD, &	4.3 (3.2)	5.9 (3.8)	p=0.21
IMTaver, mm	0.73 (0.19)	0.57 (0.13)	p<0.05
IMTmax, mm	1.00 (0.25)	0.83 (0.17)	p=0.07

Table 6-6. Associations of Framingham risk score (FRS) and markers of subclinical atherosclerosis.

	r	p
PWV, m/s	0.21	0.04
FMD, %	-0.15	0.16
IMTaver, mm	-0.01	0.92
IMTmax, mm	0.05	0.65
C1-Large, ml/mmHg x 100	-0.21	0.04
C2-small, ml/mmHg x 10	-0.26	0.01
ABI	0.06	0.55
AIx@75	0.22	0.04

Moreover, in Group 1 FRS was significantly higher compared to group 2 (13.4 ±8) % vs 9.7 ±7%), p= 0.02). When group 2 was divided in further subgroups according diabetes duration,

FRS gradually increased from group 2b to 2a and finally group 1 [7.3 (6.5) vs 10.5 (7)] vs 13.4 (8) %, p=0.02]. Last but not least, FRS did not differ significantly between those with CVD and longer duration of Diabetes [(13.4 (8) vs. 10.5 (7) %, p= 0.08)] (Table 6-7).

Table 6-7. Framingham risk score (FRS) expressed as (%) in mean-(deviation) form in Group 1, 2b and 2c. Statistical significance: * p-value <0.05

	DM2 with CVD (Group 1,n= 37)	**DM2 without CVD and longer diabetes duration** (Group 2a, n=43)	**DM2 without CVD and shorter diabetes duration** (Group 2a, n=14)
FRS, %	13.4 (8) *	10.5 (7) *	7.3 (6.5)*

6.1.4. Discussion

Diabetes is a known risk factor for CVD and relates to subclinical atherosclerosis. Through the years it has become more evident that not all diabetics are at the same risk. Therefore, a proper risk stratification might help to identify those who are more likely to benefit from risk factor modifications.

Previous studies comparing healthy controls with DM2 patients have shown that DM2 is an independent risk factor for endothelial dysfunction (Liye 201). The endothelium is recognized as an active metabolic organ that maintains the vascular homeostasis through its numerous metabolic and synthetic functions (Khazaei 2008). The development of accelerated diabetic atherosclerosis and, subsequent macrovascular complications, are not only a result of chronic hyperglycemia, but also of the presence of the other components of the metabolic syndrome, such as hypertension and hyperlipidemia. These seem to have a synergistic damaging effect on the endothelium, which is forced to functional and structural changes, resulting in reduced production of important antiatherogenic vasodilators, such as nitric oxide (NO) (Munro 1988, Li 2014).

FMD expresses the percentage of increase of the brachial artery diameter after induced hyperemia that increases NO release. FMD offers a noninvasive endothelial function assessment in conduit arteries, which are affected by the development of atherosclerotic disease. Since its

introduction in the early 1990s by Patel et al (Patel 2006) the method has been used as a surrogate end point in many clinical trials. A recent meta-analysis concluded that FMD varies from 0.20 to 19.2 % in healthy populations, while in subjects with CHD and DM2 FMD ranged from -1.3 to 14% and from 0.75 to 12 % respectively (Bots 2005).

Significant associations have been previously found between FMD and diabetes duration by our group (Naka 2012). Although FMD-detectable endothelial dysfunction has been reported even in newly diagnosed patients (Tian 2011), the synergistic effect of CHD and DM2 on FMD has been previously questioned. Simova et al showed that the presence of DM2 was associated with differences in FMD in patients with different degrees of coronary artery stenosis (Simova 2009). Ito et al showed that when compared to healthy subjects, the presence of DM2, CHD or both resulted in lower FMD levels. Although there was no clear difference in the FMD between subjects with DM2 and CHD versus those with DM2 and no CHD, the authors concluded that FMD could be useful for the detection of subclinical atherosclerosis in these patients (Ito 2015).

Zhang XG et al studied patients with DM2 and documented CHD and demonstrated that blood glucose fluctuations affect inflammatory response and possibly induce CHD in these patients, as reflected by impairment of FMD (Zhang 2014). In our study, individuals with DM2 and documented CVD had significantly lower FMD compared to DM2 individuals without CVD. Post hoc analysis suggested that this difference may have been attributed to diabetes duration, as FMD was significantly impaired in DM2 individuals with longer diabetes duration.

Carotid Intima Media thickness measurement (cIMT) is another non-invasive method used to identify subclinical vascular carotid disease. cIMT reflects structural rather than functional changes of the arterial wall (Stein 2008, Van de Veire 2010). Normally, values increase with age; However, evidence of accerelation of cIMT progression has been shown in the presence of metabolic disorders such as diabetes; in a meta-analysis including 24,111 patients from 24 studies, Brohall et al showed an additional mean cIMT increase of 0.13 mm in DM2 patients, suggesting a higher relative risk for cardiovascular events (Brohall 2006). Tasic et al reported that the presence of hypertension in diabetic subjects is associated with a higher incidence of asymptomatic carotid artery disease and left ventricular hypertrophy as measured by cIMT and left ventricular mass in echocardiography (Tasic 2015). Furthermore, the Insulin Resistance

Atherosclerosis Study showed, that subjects with DM2 and coronary artery disease had a greater cIMT, compared to those without DM2 (Haffner 2000). In accordance with these findings, we showed that in patients with CVD and DM2, the mean cIMT was significantly higher compared to those with DM2 but no CVD (0.82 vs 0.7 respectively, $p<0.05$). An even larger difference was observed when DM2 patients with known CVD and long duration of the disease were compared to those with no history of CVD and shorter (<5 years since diagnosis) disease duration (0.57mm, $p<0.05$).

Pulse Wave Velocity (PWV) is another useful tool commonly used to assess arterial stiffness. Arterial stiffness reflects functional wall properties and expresses the propagation speed of the pulsation along the arterial tree (Mitchell 2009, Benetos 2002, Oliver 2003). Normally, it increases with age; but this process is accelerated by the presence of risk factors for CVD such as hypertension or DM2 (Djaberi 2008, Benetos 1993, Safar 2003). Previous studies have shown that PWV is higher among patients with DM2 and can be observed even prior to the onset of diabetes, in patients with impaired glucose tolerance (Simons 1999, Henry 2003, Hatsuda 2006). Tomiyama et al showed, in an observational study with 2080 subjects, that high blood pressure and plasma glucose levels, even below those defining hypertension and diabetes, may synergistically lead to progression of arterial stiffening (Tomiyama 2006). In the same direction, Bruno al compared hypertensive patients with and without DM2 and showed that the coexistence of hypertension and DM2 worsens arterial stiffness, as indicated by higher PWV and lower FMD (Bruno 2012). In our study PWV was not significantly higher in subjects with DM2 and CVD, compared to those without CVD, probably reflecting the early onset if arterial stiffness in DM2 even in the absence of overt CVD.

FRS has been a useful yet simple tool for CVD prediction by using gender, age, blood pressure, cholesterol levels and smoking status. Nevertheless, even in asymptomatic patients classified as low risk according to FRS subclinical atherosclerosis has been reported. Moreover, its role in diabetes has been questioned, since it appears to underestimate the CVD risk in this population (Coleman 2007). Older studies comparing it with other risk factor engines, such as United Kingdom Prospective Diabetes Study (UKPDS) cardiovascular risk equations, showed that in newly diagnosed diabetics both FRS and UKDS risk engines are only moderately effective at identifying those at high risk and therefore not suitable for predicting CVD risk (Guzder 2005,

Davis 2009). Other authors tried to combine FRS with subclinical markers of atheroclerosis. It appears that addition of IMT but not PWV, FRS improves CVD prediction in asymptomatic DM2 patients (Yoshida 2012). A more recent metanalysis showed that incorporating PWV in FRS leads to an improvement in the efficacy of CVD prediction in patients free of CVD. In our study, FRS was associated with PWV and indices of arterial elasticity, but not FMD or cIMT. A possible explanation might be that both FRS and PWV are strongly affected by arterial pressure, which could serve as a common component. Despite its limitations, though, even in patients with DM2, FRS was, as expected, better among those with shorter duration of disease and better macrovascular profile, whilst the presence of known CVD was associated with higher FRS score and more pronounced atherosclerosis. The use of a combined assessment tool that utilizes both FRS and markers of subclinical atheroclerosis might be promising in detecting those who might profit the most by treatment intensification.

The role of strict glycemic control in the prevention of macrovascular complications has been previously investigated in large randomised controlled clinical trials. (Skyler 2009). These failed to show an improvement in macrovascular outcomes in subjects assigned to receive intensive treatment, despite improvement of microvascular complications. However, all these studies enrolled patients with long-standing diabetes (Brown 2010). Zoungas et al investigated the association of glycemic control with the presence of vascular complications in patients with DM2. They demonstrated that a "threshold" may exist, above which there is an almost linear association of HbA1c increase and risk of both macrovascular and microvascular complications. Interestingly, these associations were more evident among patients with longer duration of diabetes (Zoungas 2012). Encouraging data from the multicenter Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes trial (EMPA-REG OUTCOME) demonstrated significantly lower rates of death from any cause and hospitalization for heart failure for patients with DM2 at high risk of cardiovascular events, although no differences were observed in the rates of myocardial infarction or stroke. However, patients in this study were relatively younger, their glycemic control was slightly better and the reduction in cardiovascular deaths was partially driven by reduction in deaths from heart failure, while rates of myocardial infarction remained similar (Zinman 2015).

Similar results were published in the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial, that showed a trend towards a reduction, in the incidence of myocardial infarction, stroke, and hospitalization for heart failure for patients with DM2 at high cardiovascular risk (Marso 2013). In our most recent study we showed an improvement of vascular indices and cIMT deterioration after short term aggressive control in patients with long standing diabetes (Antoniou 2019). Taking into consideration the positive results in terms of subclinical atherosclerosis after use of newer antidiabetic agents, the early detection and careful risk stratification might play a crucial role to avoid macrovascular complications.

For more than a decade, DM2 has been considered to be a CHD equivalent, due to the increased rates of cardiovascular events in comparison to healthy subjects. However, several meta-analyses have shown that, although patients with diabetes of both early or late onset, are at increased risk of future CHD events, the duration of the disease may play a decisive role. These two groups of patients seem to differ in terms of cardiovascular risk only in the first five years since diagnosis of DM2 (Hadaegh 2010). Therefore, only DM2 of longer duration appears to be a CHD-equivalent, reaching the levels of risk of subjects with established CVD and prior cardiovascular events (Bulugahapitiya 2009, Rana 2016, Wannamethee 2011).

These findings are largely in agreement with our results, as in our sample of poorly controlled DM2 patients, the presence of CVD was associated with worse indices of vascular function. Further subgroup analysis based on DM2 duration showed that subjects with shorter disease duration had significantly better vascular profile regarding PWV, FMD and cIMT. Markers of subclinical atherosclerosis tend to be worse among DM2 patients with overt macrovascular complications and better in those at an earlier stage of the disease process. Further research is needed to investigate whether interventions to prevent progression of vascular lesions in DM2 patients may be more effective at an early stage of the disease. This was indicated by the extended follow up of UKPDS that, unlike ACCORD and ADVANCE, included newly diagnosed patients and demonstrated a reduction in diabetes-related and all-cause mortality in the group of patients allocated to receive intensive treatment. The clinical usefulness of vascular markers as potential tools for assessing cardiovascular risk in large patient populations, remains to be proven.

6.1.5 Limitations of the study

Our study has several limitations that should be considered. First, the number of participants studied was relatively small. Secondly, subjects with a disease duration of less than five years, were considered as newly diagnosed, although for many patients, the exact time of initial diagnosis remains unclear. Moreover, patients with short diabetes duration were also younger, which could potentially bias our results.

Another yet important limitation has to do with the utility of Framingham risk score in Diabetes, which is already mentioned to be ineffective in detecting a serious amount of patients with asymptomatic CVD.

6.1.6. Conclusions

We conclude that in poorly controlled DM2 patients, the presence of macrovascular disease is associated with more pronounced vascular dysfunction, as assessed with non-invasive markers of subclinical atherosclerosis. Furthermore, patients with relatively short duration of diabetes (<5 years since diagnosis) had a significantly better vascular profile.

6.2. FOLLOW-UP STUDY

Background and Hypothesis

Epidemiologic studies suggest a strong association between both microvascular and macrovascular complications of type 2 diabetes mellitus (T2DM) and the degree and duration of hyperglycemia, like already mentioned (Davies 2018). Despite the evidence linking hyperglycemia (even in the pre-diabetic range) with CVD, several large randomized controlled trials have failed to show a cardiovascular benefit from strict glycemic control in patients with long-standing T2DM at high CVD risk (DECODE 1999, UKPS 1998, Skyler 2009).

Endothelial dysfunction is an early event in the process of atherosclerosis and has been shown to predict cardiovascular outcomes (Calles-Escandon 2001) Although some information on the effect of specific antidiabetic agents on indices of arterial function exists, little is known about the possible effects of glycemic improvement per se, on these indices. Whether the effects of glycemic control on markers of arterial function may be influenced by specific patient characteristics such as the duration of diabetes or the presence of established macrovascular disease is also not known.

In some older studies, pioglitazone and metformin appeared to improve large artery endothelial function, mostly in patients with recently diagnosed T2DM and no clinical evidence of macrovascular disease (Papathanassiou 2009, Mather 2001). Some studies have shown that insulin treatment may have beneficial effects on endothelial function in subjects with T2DM and no macrovascular or clinically significant microvascular disease (Vehkavaara 2004), whereas others failed to demonstrate any effect on FMD despite a reduction of HbA1c from 10.8% to 8.2% (95 to 66 mmol/mol) in patients with T2DM with vascular complications, on multiple medications (Bagg 2001). To this end, we aimed to investigate whether intensive glucose lowering treatment in T2DM patients with poor glycemic control may improve indices of arterial structure and function. The effect of diabetes duration and presence of established macrovascular disease as well as insulin treatment were also studied.

6.2.1 Material-Methods

Population Study

Patients with T2DM were prospectively recruited from the Department of Endocrinology, Ioannina University Hospital and the Diabetes Outpatient Clinic, Chatzikosta General Hospital of Ioannina, between July 2011 and December 2014. Eligible subjects were >50 and <80 years old, with poor glycemic control (HbA1c ≥7.5%, 58 mmol/mol). Disease duration was defined as the time interval since diagnosis. Patients were studied at baseline and following the achievement of optimal glycemic control, during a 12-month follow-up period. The general HbA1c target was <7% (53 mmol/mol), according to guidelines. Patients who did not achieve an absolute HbA1c reduction of at least 0.5% (5.5mmol/mol), were excluded from the final analysis. They were regularly seen as required, during the follow-up period. Apart from antidiabetic treatment intensification, clinical management, including lifestyle modification advice and prescription of antihypertensive or lipid-lowering medications, was based on standard medical care guided by clinical judgement.

We performed the same measurements for the assessment of vascular indices as already described, at baseline and follow up.

6.2.2 Statistical analysis

Normal distribution was tested for all continuous variables using the Kolmogorov Smirnov test. Continuous variables are presented as mean ± standard deviation (SD) or median (interquartile range – IQ). The paired t-test and Wilcoxon signed rank test were used to compare continuous variables with normal and not normal distribution respectively at baseline and follow-up. Categorical variables were compared using the McNemar's test. Repeated measures ANOVA (RM-ANOVA) was used to compare changes at baseline vs follow-up between various subgroups of patients (i.e. according to history of CVD, diabetes duration, insulin treatment). A two-sided p-value of <0.05 was considered statistically significant. Based on our previous data in patients with type 2 diabetes [Naka 2002] we anticipated an improvement in FMD of ca. one SD

(or ~70% compared with baseline) following treatment. We estimated that a sample size of 50 patients would provide a power of approximately 80% to detect an increase in FMD of at least 0.5 SD (or 35% compared to baseline) following improvement in glycemic control ($\alpha = 0.05$). Data analyses were performed using IBM SPSS version 21.0.

6.2.3. Results

Ninety-seven consecutive patients were screened but only 80 subjects agreed to a follow-up visit; the final study population consisted of 62 patients who achieved an HbA1c reduction of at least 0.5% and underwent a thorough assessment of vascular function both at baseline and follow-up. The desired glycemic control was achieved in a period of 6-12 months at follow-up (median time 9 months). Eighteen patients were excluded due to insufficient HbA1c reduction (i.e. <0.5% or 5.5 mmol/mol). The demographic characteristics of patients that were included in the study are presented in Table 6-8.

Table 6-8. Baseline demographic characteristics of the study population (n=62). Continuous variables are presented as mean±standard deviation. Abbreviations: CVD, cardiovascular disease; DM, diabetes mellitus.

Age, years	64±9
Female/Male gender, n (%)	27/35 (44/56)
History of CVD, n (%)	25 (40)
Dyslipidemia, n (%)	44 (71)
Hypertension, n (%)	54 (87)
Current Smoking, n (%)	12 (19)
Duration of diabetes (years)	14±9.6
Exercise, n (%)	49 (79)
Family history of CVD, n (%)	14 (23)
Family history of DM, n (%)	38 (61)

Medications, laboratory and vascular parameters at baseline and follow-up are presented in Table 6-9.

Table 6-9. Characteristics of the study population at baseline and follow-up.Continuous variables are presented as mean±standard deviation or median (interquartile range). **Abbreviations:** AIx@75, Aortic augmentation index; cfPWV, carotid-femoral pulse wave velocity; C1, large artery elasticity index; C2, small artery elasticity index; cIMT, carotid intima-media thickness; DPP4, Dipeptidyl peptidase-4; GLP1,Glucagon-like Peptide 1; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; hs-CRP, high sensitivity C-Reactive Protein; RAAS, Renin angiotensin aldosterone system.

	Baseline (n=62)	**Follow-up (n=62)**	**P value**
Body Mass Index, kg/m²	30.2±6.1	30.5±5.8	0.496
Waist circumference, cm	103±14	102±14	0.119
Waist-to-Hip-Ratio	0.97±0.10	0.96±0.10	0.307
Serum creatinine, mg/dl	1.04±0.32	1.03±0.31	0.700
Total Cholesterol, mg/dl	190±47	186±41	0.475
HDL cholesterol, mg/dl	47±13	46±10	0.592
Triglycerides, mg/dl	128 (91, 205)	119 (89, 164)	**0.034**
Fasting glucose, mg/dl	192±69	146±56	**<0.001**
HbA1c, %	8.8 (8.1, 10.1)	7.4 (6.9, 7.8)	**<0.001**
hsCRP, mg/l	2.65 (1.15, 3.52)	1.35 (0.71, 2.91)	**<0.001**
Antidiabetic Medications, n (%)			
Insulin	24 (39)	32 (52)	**0.008**
Metformin	41 (66)	55 (89)	**0.001**
Sulfonylureas	21 (34)	20 (32)	1.000
Glitazones	2 (3)	8 (13)	0.070

Incretin based	15 (24)	21 (34)	0.146
DPP4-inhibitors	15 (24)	17 (27)	0.730
GLP1-receptor agonists	5 (8)	5 (8)	1.000
Other Medications, n (%)			
RAAS inhibitors	34 (55)	29 (47)	0.383
Calcium channel blockers	15 (24)	19 (31)	0.388
Beta-blockers	22 (35)	25 (40)	0.508
Diuretics	19 (31)	27 (44)	**0.039**
Statins	36 (58)	44 (71)	**0.039**
Systolic blood pressure, mmHg	132±18	132±20	0.962
Diastolic blood pressure, mmHg	75±11	74±10	0.551
Vascular indices			
$cIMT_{average}$, mm	0.71±0.19	0.74±0.21	0.091
$cIMT_{max}$, mm	0.97±0.25	1.03±0.27	**0.014**
Flow-mediated dilation, %	3.06 (1.96, 6.33)	3.08 (1.53, 5.73)	0.194
C1, ml/mmHg x 100	13.7±4.7	12.6±4.1	0.123
C2, ml/mmHg x 10	3.3 (2.7, 4.2)	4.2 (3.2, 5.4)	**0.026**
cfPWV, m/s	11.7±3.4	11.4±4.0	0.373
AIx@75, %	23.2±8.5	22.0±10.3	0.362
Ankle brachial index	0.98 (0.91, 1.00)	0.98 (0.90, 1.00)	0.350

There was a significant reduction in HbA1c by ~20% ($p<0.001$). Triglycerides and hsCRP levels were also significantly decreased (by ca. 7% and 49% respectively, $p<0.001$ for both). With regards to the antidiabetic medications, an increase in the use of metformin (66% vs 89%, baseline vs follow-up) and insulin (39% vs 52%, baseline vs follow-up) was observed ($p<0.01$ for both) at follow-up, whilst the total daily insulin dose increased by ca. 100% (15.6±3.2 vs 31.8±7.1 IU, baseline vs follow-up, $p=0.002$). The use of diuretics (31% vs 44%, baseline vs follow-up) and statins (58% vs 71%, baseline vs follow-up) also increased at follow-up ($p<0.05$ for both) (Table 2). Regarding vascular indices, maximal cIMT (0.97±0.25 to 1.03±0.27 mm, $p=0.014$ baseline vs follow-up) and C2 [3.3 (IQ 2.7, 4.2) to 4.2 (IQ 3.2, 5.4) ml/mmHg x 10, $p=0.028$ at baseline vs follow-up] significantly increased (Table 2) at follow-up.

Subgroup analysis

In patients with longer (>5 years) disease duration, maximal cIMT increased at follow-up (0.99±0.26 to 1.07±0.26 mm, $p=0.016$), while it did not significantly differ in those with T2DM of ≤5 years duration (0.91±0.20 to 0.93±0.28, $p=0.581$) (Table 3). C2 did not change significantly in patients with longer (>5 years) disease duration [3.4 (IQ 2.8, 5.0) to 3.8 (IQ 3.1, 5.7) ml/mmHg x 10, $p=0.282$], while it significantly increased in those with T2DM of ≤5 years duration [3.1 (IQ 2.5, 3.9) to 4.6 (IQ 4.0, 5.0) ml/mmHg x 10, $p=0.004$]. However, when RM-ANOVA was performed, the above changes in cIMT and C2 were not significantly different between the two subgroups (Table 6-10).

Table 6-10. Changes in vascular indices at follow-up according to (A) diabetes duration, (B) history of cardiovascular diseases or (C) insulin treatment. Continuous variables are presented as mean±standard deviation or median (interquartile range). Abbreviations: AIx@75, Aortic augmentation index; cfPWV, carotid-femoral pulse wave velocity; C1, large artery elasticity index; C2, small artery elasticity index; CVD, Cardiovascular diseases; cIMT, carotid intima-media thickness; RM-ANOVA, Repeated measures ANOVA.

	Baseline	**Follow-up**	**P value**	**Baseline**	**Follow-up**	**P value**	**RM ANOVA**
A.	**Diabetes duration**						
	Duration ≤5 years, n=15			***Duration >5 years, n=47***			
$cIMT_{average,}$ mm	0.64±0.16	0.66±0.19	0.791	0.74±0.20	0.77±0.21	0.083	0.643
$cIMT_{max}$, mm	0.91±0.20	0.93±0.28	0.581	0.99±0.27	1.06±0.26	**0.016**	0.360
Flow-mediated dilation, %	5.50 (2.69, 6.95)	4.64 (1.97, 6.56)	0.733	2.86 (1.84, 6.25)	3.07 (1.51, 5.06)	0.195	0.588
C1, ml/mmHg x 100	13.6±3.2	14.1±3.4	0.641	13.7±5.1	12.1±4.3	0.065	0.199
C2, ml/mmHg x 10	3.1 (2.5, 3.9)	4.6 (4.0, 5.0)	**0.004**	3.4 (2.8, 5.0)	3.8 (3.1, 5.7)	0.282	0.161
cfPWV, m/s	9.4±1.6	8.9±3.0	0.378	12.4±3.6*	12.2±4.0†	0.583	0.679
AIx@75, %	21.8±13.1	15.9±11.8	0.128	23.7±6.5	23.9±9.0†	0.861	0.055
Ankle brachial index	0.97 (0.90, 1.00)	0.98 (0.90, 1.00)	0.689	0.98 (0.91, 1.00)	0.98 (0.90, 1.00)	0.211	0.581

** p<0.05 vs diabetes duration ≤5 years at baseline, † p<0.05 vs diabetes duration <5 years at follow-up*							
B.	**History of CVD**						
	CVD (-), n=37			***CVD (+), n=25***			
$cIMT_{average,}$ mm	0.67±0.14	0.69±0.16	0.240	0.78±0.23*	0.82±0.24†	0.237	0.738
$cIMT_{max}$, mm	0.94±0.21	0.98±0.25	0.152	1.02±0.30	1.10±0.29†	**0.044**	0.363
Flow-mediated dilation, %	4.00 (2.18, 6.59)	3.11 (2.09, 6.63)	0.551	3.03 (1.86, 5.64)	2.25 (0.61, 4.88)†	0.212	0.397
C1, ml/mmHg x 100	14.2±5.0	12.5±4.0	0.106	13.0±4.2	12.8±4.4	0.801	0.309
C2, ml/mmHg x 10	3.2 (2.6, 3.9)	3.1 (2.1, 6.6)	0.164	3.6 (2.8, 5.0)	4.4 (3.3, 5.0)	0.065	0.520
cfPWV, m/s	10.9±3.3	11.0±3.8	0.767	13.0±3.4*	12.1±4.3	0.193	0.149
AIx@75, %	22.1±9.5	20.8±11.9	0.526	24.9±6.6	23.7±7.2	0.474	0.973
Ankle brachial index	0.97 (0.92, 1.00)	0.98 (0.92, 1.00)	0.724	0.98 (0.85, 1.00)	0.92 (0.82, 1.00)†	0.052	0.112
** p<0.05 vs patients without history of CVD at baseline, †p<0.05 vs patients without history of CVD at follow-up*							

C.	**Insulin treatment**						
	Non-insulin treatment, n=30			**Insulin treatment, n=32**			
$cIMT_{average,}$ mm	0.64±0.13	0.67±0.15	0.315	0.78±0.22*	0.81±0.23†	0.179	0.788
$cIMT_{max}$, mm	0.90±0.19	0.95±0.22	0.063	1.04±0.29*	1.11±0.29†	0.094	0.765
Flow-mediated dilation, %	5.28 (2.37, 6.97)	3.60 (2.13, 6.59)	0.206	2.81 (1.75, 5.91)	2.94 (0.85, 5.02)	0.517	0.677
C1, ml/mmHg x 100	14.0±4.1	12.5±3.9	0.140	13.4±5.3	12.7±4.4	0.474	0.600
C2, ml/mmHg x 10	3.4 (2.8, 4.2)	4.5 (3.3, 5.3)	0.133	3.1 (2.5, 4.8)	3.9 (3.1, 6.0)	0.104	0.629
cfPWV, m/s	10.7±2.7	10.1±3.4	0.151	12.7±3.8*	12.6±4.2†	0.969	0.392
AIx@75, %	22.5±10.4	22.2±11.7	0.879	23.9±6.3	21.8±8.9	0.161	0.533
Ankle brachial index	1.00 (0.94, 1.00)	0.99 (0.95, 1.00)	0.503	0.96 (0.85, 1.00)	0.94 (0.81, 1.00)	0.084	0.114
** $p<0.05$ vs patients not on insulin treatment at baseline, † $p<0.05$ vs patients not on insulin treatment at follow-up*							

Both groups had similar characteristics at baseline, including the HbA1c, although metformin and insulin were less frequently used in those with shorter disease duration (Table 6-11); they also had lower cIMT and cfPWV values at baseline compared to patients with longer T2DM duration. (Table 6-10).

Table 6-11. Changes in various studies parameters at follow-up according to diabetes duration. Continuous variables are presented as mean±standard deviation or median (interquartile range). * $p<0.05$ vs diabetes duration ≤5 years at baseline; † $p<0.05$ vs diabetes duration <5 years at follow-up. Abbreviations: BMI, Body mass index; BP, Blood pressure; CVD, Cardiovascular diseases; HbA1c, Glycated hemoglobin; HDL, High density lipoprotein; HsCRP, High sensitivity C-reactive protein; RAAS, Renin angiotensin aldosterone system; RM-ANOVA, Repeated measures ANOVA.

	Duration ≤5 years, n=15			***Duration >5 years, n=47***			
	Baseline	**Follow-up**	**P value**	**Baseline**	**Follow-up**	**P value**	**RM ANOVA**
Age, years	59±11	-		65±7*	-		-
Female gender, n (%)	6 (40)	-		21 (45)	-		-
History of CVD, n (%)	5 (33)	-		20 (43)	-		-
Dyslipidemia, n (%)	12 (80)	-		43 (92)	-		-
Hypertension, n (%)	11 (73)	-		43 (92)	-		-
Current Smoking, n (%)	3 (20)	-		9 (21)	-		-
BMI, kg/m²	28.8±5.9	29.4±6.3	0.624	30.7±6.1	30.9±5.6	0.640	0.741
Waist circumference, cm	100±12	97±11	**0.017**	104±14	103±14	0.578	0.107
Waist-to-Hip-Ratio	0.97±0.08	0.94±0.07	**0.022**	0.97±0.11	0.96±0.10	0.749	0.272
Serum creatinine, mg/dl	0.94±0.17	0.95±0.24	0.969	1.07±0.35	1.06±0.33	0.655	0.797
Total Cholesterol, mg/dl	194±53	199±45	0.689	188±45	181±39	0.298	0.387
HDL cholesterol, mg/dl	50±12	48±10	0.543	46±14	45±10	0.771	0.743
Triglycerides, mg/dl	127 (78,186)	100 (78, 170)	0.263	129 (91, 210)	119 (95, 161)	0.076	0.762
Fasting glucose, mg/dl	213±76	137±43	**0.001**	186±67	148±59	**0.001**	0.075
HbA1c, %	8.8 (8.1, 10.0)	6.9 (5.6, 7.3)	**0.001**	8.8 (9.1, 10.6)	7.5 (7.1, 7.9)†	**<0.001**	0.169
hsCRP, mg/l	2.27 (0.86,	1.50 (0.72,	**0.031**	2.69 (1.20,	1.30 (0.69,	**0.002**	0.229

	5.60)	2.50)		3.45)	3.19)		
Systolic BP, mmHg	126±16	121±16	0.052	134±18	136±21†	0.603	0.274
Diastolic BP, mmHg	76±11	71±9	**0.043**	74±11	75±10	0.744	0.080
Antidiabetic Medications, n (%)	1 (7)	3 (20)	0.500	23 (49)*	29 (62) †	**0.031**	-
Insulin	4 (27)	13 (87)	**0.004**	37 (79)*	42 (89)	0.125	
Metformin	3 (20)	6 (40)	0.375	18 (38)	14 (30)	0.289	
Sulfonylureas	2 (13)	2 (13)	1.000	0 (0)*	6 (13)	0.031	
Glitazones	3 (20)	4 (27)	1.000	12 (26)	17 (36)	0.180	
Incretin based							
Other Medications, n (%)							-
RAAS inhibitors	6 (20)	7 (47)	1.000	28 (60)	22 (47)	0.238	
Calcium channel blockers	2 (13)	3 (20)	1.000	13 (28)	16 (34)	0.508	
Beta-blockers	3 (20)	5 (33)	0.500	19 (40)	20 (43)	1.000	
Diuretics	5 (33)	6 (40)	1.000	14 (30)	21 (45)	0.065	
Statins	6 (40)	10 (67)	0.125	30 (64)	34 (72)	0.289	

A significant increase in maximal cIMT (1.02±0.30 to 1.10±0.29, p=0.044) was observed at follow-up in patients with pre-existing CVD whilst no difference was found in those with no pre-existing CVD (0.94±0.21 to 0.98±0.25, p=0.152) (Table 10). However, in RM-ANOVA, this change was not significantly different between the two subgroups. Changes in HbA1c were also similar in the two subgroups (Table 6-12). Interestingly, patients with known CVD presented with worse cIMT and cfPWV at baseline compared to those without CVD (Table 3). Patients with known CVD were more frequently treated with statins, beta-blockers and insulin.

Table 6-12. Changes in various studies parameters at follow-up according to the history of known cardiovascular diseases. Continuous variables are presented as mean±standard deviation or median (interquartile range).* *p<0.05 vs patients without CVD at baseline;* † *p<0.05 vs patients without CVD at follow-up.* Abbreviations: BMI, Body mass index; BP, Blood pressure; CVD, Cardiovascular diseases; HbA1c, Glycated hemoglobin; HDL, High density lipoprotein; HsCRP, High sensitivity C-reactive protein; RAAS, Renin angiotensin aldosterone system; RM-ANOVA, Repeated measures ANOVA.

	CVD (-), n=37			***CVD (+), n=25***			
	Baseline	**Follow-up**	**P value**	**Baseline**	**Follow-up**	**P value**	**RM ANOVA**
Age, years	63±9	-		66±7	-		-
Female gender, n (%)	17 (46)	-		10 (40)	-		-
Diabetes duration, years	13±10	-		15±10	-		-
Dyslipidemia, n (%)	31 (84)	-		24 (96)	-		-
Hypertension, n (%)	29 (78)	-		25 (100)*	-		-
Current Smoking, n (%)	8 (22)	-		4 (16)	-		-
BMI, kg/m²	30.8±7.1	31.2±6.7	0.557	29.4±4.2	29.5±4.0	0.731	0.777
Waist circumference, cm	102±14	101±13	0.345	104±14	103±14	0.119	0.873
Waist-to-Hip-Ratio	0.97±0.11	0.96±0.11	0.667	0.96±0.09	0.95±0.08	0.154	0.655
Serum creatinine, mg/dl	0.99±0.32	0.95±0.29	0.253	1.12±0.32	1.14±0.30†	0.624	0.283
Total Cholesterol, mg/dl	197±39	185±35	0.055	178±56	186±49	0.524	0.108
HDL cholesterol, mg/dl	51±14	49±9	0.3553	41±9*	42±10†	0.471	0.286
Triglycerides, mg/dl	118 (87, 184)	100 (78, 135)	**0.011**	152 (104, 236)	156 (102, 191) †	0.648	0.569
Fasting glucose, mg/dl	189±65	149±55	**0.003**	197±75	141±58	**<0.001**	0.451
HbA1c, %	8.9 (8.2, 10.4)	7.3 (6.9, 7.8)	**<0.001**	8.4 (7.9, 9.8)	7.5 (6.9, 7.8)	**<0.001**	0.338
hsCRP, mg/l	2.30 (0.85, 3.38)	1.30 (0.77, 3.25)	**0.039**	2.80 (1.93, 3.99)	1.40 (0.62, 2.30)	**0.001**	**0.043**
Systolic BP, mmHg	129±17	129±19	0.827	138±19	137±22	0.922	0.840
Diastolic BP, mmHg	74±11	73±9	0.510	76±10	76±10	0.862	0.816

Antidiabetic Medications, n (%)							-
Insulin	10 (27)	15 (41)	0.063	14 (56)*	17 (68) †	0.250	
Metformin	26 (70)	35 (95)	**0.004**	15 (60)	20 (80)	0.125	
Sulfonylureas	14 (38)	16 (43)	0.754	7 (28)	4 (16) †	0.250	
Glitazones	0 (0)	5 (14)	0.063	2 (8)	3 (12)	1.000	
Incretin based	9 (24)	13 (35)	0.289	6 (24)	8 (32)	0.625	
Other Medications, n (%)							-
RAAS inhibitors	19 (51)	13 (35)	0.180	15 (60)	16 (64) †	1.000	
Calcium channel blockers	11 (30)	12 (32)	1.000	4 (16)	7 (28)	0.250	
Beta-blockers	8 (22)	8 (22)	1.000	14 (56)*	17 (68) †	0.453	
Diuretics	11 (30)	15 (41)	0.125	8 (32)	12 (48)	0.289	
Statins	17 (46)	21 (57)	0.219	19 (76)*	23 (92) †	0.219	

No differences were found in vascular markers' changes at follow-up between insulin-treated patients and those who did not receive insulin (Table 6-13), whilst HbA1c improved similarly in both subgroups. Of note, insulin-treated patients had higher HbA1c and systolic BP at baseline, as well as higher cIMT, cIMTmax and PWV compared to those treated with antidiabetic agents other than insulin.

Regarding the small group of patients who were excluded from the study, due to heterogenity of this group no safe conclusions can be made.

Table 6-13. Changes in various studies parameters at follow-up according to the use of insulin treatment. Continuous variables are presented as mean±standard deviation or median (interquartile range). ** p<0.05 vs patients not on insulin treatment at baseline, † p<0.05 vs patients not on insulin treatment at follow-up.* Abbreviations: BMI, Body mass index; BP, Blood pressure; CVD, Cardiovascular diseases; HbA1c, Glycated hemoglobin; HDL, High density lipoprotein; HsCRP, High sensitivity C-reactive protein; RAAS, Renin angiotensin aldosterone system; RM-ANOVA, Repeated measures ANOVA.

	Insulin (-), n=30			***Insulin (+), n=32***			
	Baseline	Follow-up	P value	Baseline	Follow-up	P value	RM ANOVA
Age, years	62±9	-		65±8	-		-
Female gender, n (%)	15 (50)	-		12 (38)	-		-
Diabetes duration, years	10±8	-		17±10*	-		-
History of CVD, n (%)	8 (27)			17 (53)*			
Dyslipidemia, n (%)	25 (83)	-		30 (94)	-		-
Hypertension, n (%)	25 (83)	-		29 (91)	-		-
Current Smoking, n (%)	5 (17)	-		7 (22)	-		-
BMI, kg/m²	30.5±5.6	30.7±4.7	0.779	30.0±6.5	30.3±6.7	0.327	0.885
Waist circumference, cm	103±12	101±10	0.033	103±16	103±16	0.704	0.322
Waist-to-Hip-Ratio	0.95±0.08	0.94±0.08	0.177	0.98±0.12	0.98±0.11	0.650	0.817
Serum creatinine, mg/dl	0.94±0.21	0.92±0.18	0.677	1.14±0.38*	1.13±0.37†	0.911	0.809
Total Cholesterol, mg/dl	188±47	185±37	0.708	191±48	186±44	0.547	0.815

HDL cholesterol, mg/dl	49±14	48±9	0.486	45±13	45±10	0.984	0.601
Triglycerides, mg/dl	124 (89, 187)	108 (82, 144)	0.116	151 (98, 225)	129 (95, 182)	0.155	0.986
Fasting glucose, mg/dl	180±48	130±38	<0.001	204±83	161±66†	0.007	0.698
HbA1c, %	8.4 (8.0, 9.5)	6.9 (5.7, 7.5)	<0.001	9.3 (8.3, 11.3)*	7.6 (7.2, 8.4) †	<0.001	0.985
hsCRP, mg/l	2.50 (0.98, 3.25)	1.45 (0.67, 2.91)	0.032	2.78 (1.36, 3.99)	1.11 (0.71, 3.00)	0.001	0.345
Systolic BP, mmHg	127±15	126±15	0.772	138±19*	139±23†	0.804	0.714
Diastolic BP, mmHg	75±12	72±9	0.081	75±10	76±10	0.450	0.079
Antidiabetic Medications,n (%)							-
Metformin	21 (70)	30 (100)	0.004	20 (63)	25 (78) †	0.125	
Sulfonylureas	14 (47)	18 (60)	0.289	7 (22)*	2 (6) †	0.063	
Glitazones	2 (7)	6 (20)	0.219	0 (0)	2 (6)	0.500	
Incretin based	9 (30)	13 (43)	0.289	6 (19)	8 (25)	0.625	
Other Medications, n (%)							-
RAAS inhibitors	15 (50)	13 (43)	0.727	19 (59)	16 (50)	0.582	
Calcium channel blockers	5 (17)	7 (23)	0.688	10 (31)	12 (38)	0.688	
Beta-blockers	9 (30)	10 (33)	1.000	13 (41)	15 (47)	0.688	
Diuretics	9 (30)	11 (37)	0.625	10 (31)	16 (50)	0.070	
Statins	16 (53)	21 (70)	0.125	20 (63)	23 (72)	0.375	

6.2.4 Discussion

In patients with long-standing T2DM, aggressive glycemic control, as reflected by improvement in HbA1c at a median follow up period of 9 months, was not associated with an improvement in endothelial function (FMD) or central aortic/large artery stiffness (cfPWV, C1) and in fact a mild deterioration of carotid subclinical atherosclerosis (cIMT) was found. However, an improvement in small artery elasticity (C2) was observed at follow-up. Moreover, the duration of established diabetes or the presence of CVD did not seem to alter the effects of glycaemic control on these markers in this cohort. To our knowledge, this is the first study to report on the effects of intensive glycemic control on several markers of subclinical atherosclerosis in T2DM patients of variable disease duration, including patients with established macrovascular disease.

Subclinical markers of atherosclerosis are impaired in patients with T2DM, as already mentioned. Their measurement potentially represents a cost-effective alternative compared to classic complex cardiac testing, allowing the assessment of vascular alterations that precede atherosclerotic changes that are related to overt clinical disease in high risk patients, and have been long used for this purpose in observational and intervention trials. Importantly, each of these vascular indices may be differentially affected by metabolic alterations or pharmacological interventions.

Our results are in accordance with findings of randomized controlled trials in T2DM, that failed to show a reduction of cardiovascular events when hyperglycaemia was intensively treated aiming at near-normal glucose values (HbA1c 6.4 to 6.9 %, 46 to 52 mmol/mol) [16]. It has been proposed that this lack of benefit might be due to relatively short follow-up, presence of advanced atherosclerotic disease, higher rates of hypoglycemia in the intervention arms of the trials or adverse cardiovascular effects of insulin or sulphonylureas (Skyler 2009). The fact that none of the vascular indices related to macrovascular arterial function improved, despite the marked HbA1c reduction in our study population, might suggest that longer treatment may be required before the potential benefits of the metabolic control translate into measurable

improvements in the surrogate vascular markers of atherosclerosis or, even more so, into improved CVD outcomes. In support of this hypothesis, in the UKPDS post-trial follow-up study, a significant reduction in overall mortality, diabetes-related mortality, and myocardial infarction during the ~17 year follow-up period was shown in subjects initially assigned to intensive therapy (Almourani 2019).

On the other hand, the importance of glycaemic control in the prevention and treatment of microvascular disease has been established in several studies in the past (Holman 2008, Terry 2012). Hyperglycemia has been considered the main factor related to impaired intracellular signaling dysfunction, increased toxic metabolites (e.g. advanced glycated products) and altered redox state that ultimately lead to microvascular dysfunction and disease (i.e. retinopathy, neuropathy, nephropathy) (Barrett 2017). Currently, we showed that intensive glycaemic control in poorly controlled T2DM patients improved small artery function as suggested by the increase in small artery elasticity index (C2), a finding that is consistent with previous large clinical studies that showed a benefit in the incidence of microvascular disease following an intensive glycemic control regimen (Reichard 1993, UKPDS Lancet 1998, Patel 2008). A previous study in children with type 2 diabetes showed that plasma glucose was negatively associated with small artery elasticity (C2) (Tryggestad 2013), while in adults with type 2 diabetes increasing HbA1c levels were associated with decreasing C2 values (Prisant 2006). Whether the improvement in small artery function observed in our study would result in improved clinical outcomes of microvascular disease in these patients is not known and requires further study.

Furthermore, in our study, carotid atherosclerosis as assessed by maximum IMT in common carotids was shown to deteriorate in the short term following aggressive glycaemic control. Poor glycemic control leads to worse cIMT by increasing age and duration of diabetes (Tabatabaei-Malazy 2015), whereas some evidence suggests that strict glycemic control may ameliorate the progression of c IMT (Kawasumi 2006). Various antidiabetic agents such as metformin, gliclazide, nateglinide, pioglitazone and more recently alogliptin and exenatide appear to delay or even reverse cIMT progression in T2DM and this effect is commonly independent of glycemic control (Katakami2004, Mita 2016, Mita 2007, Yasunari 2011, Patti 2019). In a study from Denmark, treatment with insulin alone in patients with long-standing T2DM reduced cIMT over an 18-month period, but the combination of insulin and metformin had no effect, despite the

larger reduction in HbA1c, less weight gain, and smaller insulin dose (Lundby-Christensen 2016). Notably, the progression of atherosclerosis in diabetes is probably related not only to hyperglycemia but also to the presence and control of other cardiovascular risk factors. The observed deterioration in cIMT in our study may therefore be partly explained by the fact that despite the use of statins and anti-hypertensive medications in the large majority of our patients, lipid (non-HDL cholesterol ca. 140 mg/dl) and blood pressure levels were suboptimal. In addition, most patients had long-standing diabetes and a significant portion were overweight.

There is increasing evidence that the duration of diabetes is a strong marker for risk stratification among patients with type 2 diabetes (Cosentino 2020). Longer duration of type 2 diabetes has been associated with a deterioration of vascular function (Naka 2002), but also a greater risk for CVD (Wannamethee 2011, Venuraju 2019). In addition, disease duration is related to aging and a longer exposure to hyperglycemia and other metabolic derangements in these patients and could potentially lead to lower and/or delayed benefit of various interventions. In support of this notion, it is suggested that earlier application of metabolic therapies in the course of diabetes may be more effective in cardiovascular prevention, an effect described as metabolic memory (Skyler 2009, Almourani 2008). In the present study, although there was a trend for a greater improvement of C2 marker and a deceleration of IMT progression in response to intensive glycemic control in patients with shorter duration of T2DM (<5 years) the overall effects of aggressive glycaemic control on vascular indices in T2DM patients with shorter or longer than 5 years of established disease did not differ significantly. This may be due to the relatively small sample size and the fact that even in patients with shorter duration of established diabetes, the disease may had pre-existed for several years prior to diagnosis.

Similarly, results of vascular indices did not differ for those with and without known CVD. It has been previously reported that various antidiabetic treatments may improve arterial function indices mostly in patients with T2DM and no clinical evidence of macrovascular disease (Papathanassiou 2009, Mather 2001, Vehkavaara 2004, Chugh 2010). On the contrary, no benefit was observed in patients with T2DM and established CVD. In the present study, although patients with diabetes and CVD had different cardiometabolic and vascular (worse cIMT and cfPWV) profiles at baseline compared to patients with diabetes without CVD, both groups behaved similarly following intensification of glycemic control. Importantly, since this study

was conducted prior to the introduction of newer antidiabetic agents with proven cardiovascular benefit, treatment intensification was mostly based on insulin therapy, since the use of GLP1Ra was limited and SGLT2 inhibitors were not available at the time.

Ongoing controversy exists on the cardiovascular effects of insulin therapy in T2DM; several observational studies have shown dose-dependent associations for insulin with increased cardiovascular risk and mortality (Herman 2017). On the other hand, large trials such as the UKPDS and ORIGIN study did not show any cardiovascular harm (Dongerkery 2017). Insulin effects on the vasculature are complex, since insulin signaling in endothelial cells can be mediated via the PI3K (vasodilation, anti-thrombotic effects) and/or the MAPK pathway (vasoconstriction, prothrombotic effects). It has been proposed that when insulin is administered in poorly controlled T2DM patients, selective insulin resistance in the PI3K pathway occurs, resulting in unopposed signalling through the MAPK pathway, thus increasing the risk of vascular events (Nolan 2015). Our results show that treatment intensification mostly based on insulin initiation or intensification had a neutral effect on vascular indices during the study period, although the concomitant use of metformin and other medications may have blunted any negative effect of insulin on the vasculature. Importantly, none of our patients experienced episodes of severe hypoglycemia requiring medical intervention or admission to hospital.

In recent years, several well designed, large clinical trials have demonstrated CVD benefits in T2DM patients at high cardiovascular risk or established CVD treated with newer antidiabetic agents such as GLP1-RAs (liraglutide, semaglutide, albiglutide, dulaglutide), and SGLT2 inhibitors (empagliflozin, canagliflozin, dapagliflozin) (Sfairopoulos 2018, Home 2019). Since these studies were designed as cardiovascular safety trials, any differences in glycemic control between the active treatment and control groups were small. Although the exact mechanisms are not yet clear, accumulating evidence suggests that SGLT2 inhibitors exert their beneficial effects by reducing heart failure and cardiovascular mortality through haemodynamic and/or metabolic effects and GLP1 RAs reduce major vascular events via antiatherosclerotic mechanisms. The effect of SGLT2 inhibitors and GLP-1 RA on endothelial function and arterial stiffness remains controversial. A recent meta-analysis on the effects of these newer antidiabetic drugs on endothelial function and arterial stiffness concluded that in contrast to DPP-4 inhibitors or GLP-1 RA, SGLT-2 inhibitors significantly improved FMD, while both GLP-1 RA and, to a lesser

extent, DPP-4 inhibitors significantly decreased PWV (Batzias 2018). Whether these observations are due to direct glucose-lowering effects or to indirect effects driven by the modulation of other cardiovascular risk factors is however unclear.

Limitations of the study.

The number of enrolled patients was relatively small and precluded analysis with adjustments for multiple confounders as this would confer biases with overfitting models. Most of the patients had long-standing T2DM and were not treatment naïve at baseline while they received many different antidiabetic medications that may have confounded the results. However, the aim of the present study was to investigate the effect of glycemic control on vascular markers and not that of any individual antidiabetic agent. In subgroup analysis, differences in the baseline metabolic profile of the subgroups may have affected our findings. Finally, the lack of a control group without any change of HbA1c or a deterioration in glycaemic control may hide potential beneficial effects of our intervention in terms of amelioration of a deterioration of vascular indices in the control group.

We conclude that in T2DM patients, short term aggressive glycemic control without a concomitant improvement in other cardiovascular risk factors was associated with an improvement in the elasticity of small arteries (i.e. microvascular function) without any effect on endothelial function, elasticity or arterial stiffness of large arteries and central aorta (i.e. macrovascular function). Carotid atherosclerosis deteriorated in the short term despite the intensive glycaemic control. A multifactorial approach including improvement of lipid profile, arterial blood pressure and lifestyle interventions may be more appropriate in order to achieve a delay in atherosclerosis progression and induce further improvements in other vascular indices. The duration of diabetes, the history of established cardiovascular atherosclerotic disease as well insulin treatment did not significantly affect the changes produced by our intervention. Further studies are needed to investigate whether multifactorial interventions, improvement of glycemic control with newer antidiabetic agents (i.e. GLP-1 agonists and SGLT2 inhibitors) and a longer duration of intervention may be more effective in improving vascular indices in patients with T2DM.

Chapter 7 : General conclusions

The baseline study demonstrated the following key points:

- in poorly controlled T2DM patients, the presence of macrovascular disease was associated with more pronounced vascular dysfunction, as assessed with non-invasive markers of subclinical atherosclerosis
- markers of subclinical atherosclerosis tend to be worse among T2DM patients with overt macrovascular complications and better in those at an earlier stage of the disease process
- clinical usefulness of vascular markers as potential tools for assessing cardiovascular risk in large patient populations, remains to be proven.

- Framingham risk score was lower in the presence of CVD, as expected. The latter was comparable with those with longer duration of diabetes and significantly better for those with shorter duration
- FRS appeared to be positively associated with arterial stiffness and indices of elasticity. Therefore, the use of subclinical markers could add important information in the initial risk stratification

The follow up study resulted also in important key conclusions:

- Markers of endothelial dysfunction might improve after glycaemic control in patients with shorter diabetes duration
- Endothelial function worsened in presence of CVD despite HbA1c-decrease
- short term aggressive glycemic control without a concomitant improvement in other cardiovascular risk factors was associated with an improvement in the elasticity of small

arteries (i.e. microvascular function) without any effect on endothelial function or macrovascular function)

- The duration of diabetes, the history of established cardiovascular atherosclerotic disease as well insulin treatment did not significantly affect the changes produced by our intervention.
- Multifactorial interventions targeting all cardiovascular risk factors are needed to maximize cardiovascular benefits
- further studies and for longer duration of intervention are needed to investigate whether multifactorial interventions, improvement of glycemic control with newer antidiabetic agents (i.e. GLP-1 agonists and SGLT2 inhibitors) may be more effective in improving vascular indices in patients with T2DM.

This is evidentafter the review of the existing literature on these markers, like already mentioned in chapter 5.

The additional use of these noninvasive markers of atherosclerosis strengthens the predictive risk for developing CAD beyond traditional risk assessment and enables the monitoring of selected treatment in T2DM. Progression or even regression of atherosclerosis is possible in T2DM, especially in patients with newly diagnosed diabetes, with relatively good glycemic control and use of newer agents, such as DDP4-is and SGLT2 inhibitors. This is best reflected in the updated guidelines, which support their use after metformin treatment, which also has beneficial effects. A multifactorial intervention with improvement of classical risk factors, such as hypertension and BP, should always be considered. Both structural and functional markers are easily accessible and could be an additional tool for clinicians to screen high-risk patients, with CAC, cIMT and PWV showing less intra-observer variability compared to FMD and small artery elasticity index. Despite considerable evidence for predictive value especially for cIMT, CAC

and PWV, larger studies and studies over longer periods are needed to correlate clinical outcomes with improvement of subclinical atherosclerosis.

Abstract in english

The purpose of this study was to assess vascular function in patients with Type 2 Diabetes mellitus (DM2) with and without established cardiovascular disease (CVD) by using non invasive markers of subclinical atherosclerosis, as well as to examine the association of endothelial dysfunction with diabetes duration and glycemic control.

The prevalence of diabetes mellitus has been increased significantly and has been considered as a growing epidemic. The need for effective intervention is therefore urgent. Atherosclerotic cardiovascular disease (ASCVD) remains the main cause of death in persons with type 2 diabetes mellitus (T2DM), resulting in significant morbidity and increasing health care costs. Even in the pre-diabetic range, individuals suffering from T2DM are predisposed to significant cardiovascular risk. Moreover, they often remain asymptomatic and when they develop clinically evident CVD, their prognosis and outcomes are worse compared to individuals with CVD but without DM2.

Large epidemiological studies have established the role of hyperglycemia as a risk factor for the development of both microvascular and macrovascular complications. Hyperglycemia alters the protective mechanisms of the endothelium and leads to inflammatory damage of the endothelial wall, increased permeability, as well as reduction of antiatherogenic vasodilators, such as nitric oxide. Endothelial dysfunction has been previously reported in persons with T2DM as well as prediabetes compared to healthy subjects, while comorbidities such as hypertension and dyslipidemia seem to have a synergistic effect leading to accelerated atherogenesis.

Despite the evidence linking hyperglycemia (even in the pre-diabetic range) with CVD, several large randomized controlled trials have failed to show a cardiovascular benefit from strict glycemic control in patients with long-standing T2DM at high CVD risk (DECODE 1999, UKPS 1998, Skyler 2009). Although DM2 was initially characterized as CVD risk equivalent, through the years it has become more evident that not all diabetics are at the same risk. Therefore, a proper risk stratification might help to identify those who are more likely to benefit from risk factor modifications.

So, the aim of the initial baseline study was to assess vascular dysfunction by using brachial artery flow-mediated dilation (FMD), carotid-femoral pulse wave velocity (cfPWV), central augmentation index(AIx), large (C1) and small (C2) artery compliance, carotid intima-media thickness (cIMT), and ankle-brachial index (ABI) in poorly controlled DM2 persons with and without overt CVD as well as to examine the role of DM2 duration.

Patients who participated in our study with T2DM were prospectively recruited from the Department of Endocrinology, Ioannina University Hospital and the Diabetes Outpatient Clinic, Chatzikosta General Hospital of Ioannina. A careful evaluation was made regarding diabetes duration and macrovascular complications. The following indices of vascular function were measured in all patients: common carotid intima-media thickness, (cIMT), brachial artery flow-mediated dilation (FMD), carotid-femoral pulse wave velocity (cfPWV), central augmentation index (AIx@75), large (C1) and small artery elasticity indices (C2) and ankle-brachial index (ABI).

The baseline study included 95 persons with T2DM and poor glycemic control were included and divided into two groups, with and without known CVD. The latter were further subdivided into two subgroups according to diabetes duration (longer duration > 5 years after T2DM diagnosis). The results showed that the presence of macrovascular disease was associated with more pronounced vascular dysfunction, as assessed with those non-invasive markers of subclinical atherosclerosis. Furthermore, patients with relatively short duration of diabetes (<5 years since diagnosis) had a significantly better vascular profile.

The follow -up study aimed to investigate the effect of intensive antidiabetic treatment on vascular indices in patients with DM2. The same markers of subclinical atherosclerosis were assessed before and after improvement of glycemic control. Patients who did not achieve an absolute HbA1c reduction of at least 0.5% were excluded from the final analysis. They were regularly seen as required, provided with all necessary prescriptions regarding lipid-lowering treatment etc, and were encouraged to follow lifestyle modification as well. The study showed that in patients with long-standing T2DM, short-term aggressive glycemic control at a median follow up of 9 months was associated with an improvement of microvascular function (C2) and deterioration of carotid atherosclerosis (IMT) without any effect on the elastic properties of large

arteries. Moreover, the duration of established diabetes or the presence of CVD did not seem to alter the effects of glycaemic control on these markers in this cohort. To our knowledge, this was also the first study to report on the effects of intensive glycemic control on several markers of subclinical atherosclerosis in T2DM patients of variable disease duration, including patients with established macrovascular disease.

A multifactorial approach including improvement of lipid profile, arterial blood pressure and lifestyle interventions may be more appropriate in order to achieve a delay in atherosclerosis progression and induce further improvements in other vascular indices. The duration of diabetes, the history of established cardiovascular atherosclerotic disease as well insulin treatment did not significantly affect the changes produced by our intervention. Further studies are needed to investigate whether multifactorial interventions, improvement of glycemic control with newer antidiabetic agents (i.e. GLP-1 agonists and SGLT2 inhibitors) and a longer duration of intervention may be more effective in improving vascular indices in patients with T2DM.

Περίληψη στην ελληνική

Ο σκοπός της μελέτης ήταν να μελετηθεί η αγγειακή λειτουργία με τη βοήθεια μη επεμβατικών δεικτών υποκλινικής αθηροσκλήρυνσης σε ασθενείς με διαβήτη τύπου (ΣΔ2), με ή χωρίς εγκατεστημένη μακροαγγειοπάθεια. Επιπλέον να μελετηθεί η σχέση της ενδοθηλιακής δυσλειτουργίας με την διάρκεια σακχαρώδους διαβήτη και την επίδραση του γλυκαιμικού ελέγχου σε αυτούς τους δείκτες.

Η καρδιαγγειακή νόσος εξακολουθεί να αποτελεί τη βασική αιτία θανάτου αυτών των ασθενών, οι οποίοι μάλιστα εμφανίζουν έως και τετραπλάσια θνητότητα σε σχέση με τον γενικό πληθυσμό. Καθώς η επίπτωση του ΣΔ2 αυξάνεται με δραματικούς ρυθμούς ώστε να χαρακτηρίζεται πλέον ως πανδημία, η αναγκη για αποτελεσματικές θεραπευτικές παρεμβάσεις ώστε να αποφευχθούν αυτές οι επιπλοκές και η ανεύρεση των ασθενών που έχουν υψηλό κίνδυνο και που ενδεχομένως επωφεληθούν περισσότερο απο αυτές τις παρεμβάσεις είναι επιτακτική. Αυτό είναι ιδιαιτέρως σημαντικό, ειδικά εάν λάβει κανείς υπόψην ότι οι ασθενείς αυτοί παραμένουν ασυμπτωματικοί μέχρι να αναπτύξουν έκδηλη καρδιαγγειακή νόσο, η δε πρόγνωση είναι χειρότερη σε σχέση με αυτούς που δεν έχουν ΣΔ2.

Μεγάλες επιδημιολογικές μελέτες έχουν καταδείξει τον ρόλο της υπεργλυκαιμίας στην ανάπτυξη μικρο- και μακροαγγειακών επιπλοκών. Η υπεργλυκαιμία αλλοιώνει τους προστευτικούς μηχανισμούς του αγγειακού ενδοθηλίου, οδηγώντας σε ενδοθηλιακή δυσλειτουργία, η οποία αποτελεί και την αρχική βλάβη της αθηροκλήρυνσης. Η ύπαρξη ενδοθηλιακής δυσλειτουργίας έχει επιβεβαιωθεί ακόμη και σε ασθενείς με διαταραχή ανοχής γλυκόζης, δηλαδή πολύ πριν την εμφάνιση και του ΣΔ2. Η παρουσία υπέρτασης, δυσλιπιδαιμίας φαίνεται να ασκούν επιπλέον συνεργικό ρόλο στην καταστροφή του ενδοθηλίου και άρα επιτάχυνση της προόδου της αθηροσκλήρυνσης.

Δυστυχώς παρά τη συσχέτιση του βαθμού υπεργλυκαιμίας με την καρδιαγγειακή νόσο, μεγάλες μελέτες παρέμβασης απέτυχαν να δείξουν καρδιαγγειακό όφελος μετά από εντατική υπογλυκαιμική θεραπεία. Το κοινό αυτών των μελετών ήταν ενδεχομένως το ότι συμπεριέλαβαν ασθενείς με μακρά διάρκεια νόσου και υψηλό κίνδυνο για εμφάνιση καρδιαγγειακών επιπλοκών.

Λαμβάνοντας υπόψιν αυτά τα βιβλιογραφικά δεδομένα, αποφασίστηκε η διενέργεια μελέτης των ακόλουθων μη επεμβατικών δεικτών αθηροσκλήρυνσης σε ασθενείς με πτωχό γλυκαιμικό έλεγχο, με η χωρίς εγκατεστημένη μακροαγγειοπάθεια και η εξέταση του ρόλου της διάρκειας διαβήτη.

Οι δείκτες αυτοί είναι συγκεκριμένα ροοεξαρτώμενη αγγειοδιαστολή βραχιονίου αρτηρίας (Brachial artery flow-mediated dilation,FMD), η μέτρηση αρτηριακής σκληρίας (carotid-femoral pulse wave velocity, PWV), η καταγραφή του χρόνου μετάδοσης σφυγμικού κύματος (central augmentation index), του πάχους έσω-μεσου χιτώνα κοινής καρωτίδας (carotid intima-

media thickness), δείκτες αρτηριακής ευενδοτότητας (large and small (C2) artery compliance) καθώς και του σφυροβραχιονίου δείκτη.

Στην μελέτη αυτή συμμετείχαν εθελοντικά ασθενείς των εξωτερικών ιατρείων της Κλινικής της Ενδοκρινολογίας, Διαβήτη και Μεταβολισμού καθώς και ασθενείς από το Διαβητολογικό Ιατρείο του Γενικού Νοσοκομείου Ιωαννίνων Χατζηκώστα. Κατόπιν λεπτομερούς ανάλυσης του ιστορικού για την καταγραφή τόσο της διάρκειας ΣΔ2 όσο και της εγκατεστημένης μακροαγγειοπάθειας, συμπεριλήφθηκαν στην αρχική μελέτη 95 ασθενείς.

Η βασική μελέτη έδειξε ότι η παρουσία μακροαγγειοπάθειας σχετίστηκε με πιο εκτεταμένη ενδοθηλιακή δυσλειτουργία. Επιπλέον, οι ασθενείς με μικρότερη διάρκεια ΣΔ 2 (< 5 έτη) χωρίς μακροαγγειακές επιπλοκές φάνηκε να έχουν καλύτερο αγγειακό προφίλ.

Η μελέτη παρακολούθησης συμπεριέλαβε 63 άτομα, τα οποία κατόπιν εντατικού ελέγχου για ένα διάστημα περίπου 9 μηνών παρουσίασαν σημαντική βελτίωση της γλυκαιμικής ρύθμισης, όπως αυτή ορίστηκε από μείωση του επιπέδου γλυκοζυλιωμένης αιμοσφαιρίνης τουλάχιστον κατά 0.5%. Πέραν της βέλτιστης γλυκαιμικής αγωγής, οι ασθενείς ενθαρρύνθηκαν να ακολουθήσουν πιο υγειινό τρόπο ζωής, ενώ δόθηκαν οδηγίες για βελτίωση και άλλων παραγόντων κινδύνου, όπως της αρτηριακής πίεσης, της δυσλιπιδαιμίας κλπ. Οι αγγειακοί δείκτες μελετήθηκαν πριν και μετά τη βελτίωση γλυκαιμικού ελέγχου.

Το τελικό συμπέρασμα ήταν ότι σε αυτό το διάστημα βελτιώθηκαν δείκτες ευενδοτότητας μικρών αγγείων, καθυστέρησε η πρόοδος πάχυνσης του τοιχώματος της κοινής καρωτίδας αρτηρίας στους ασθενείς με μακρά διάρκεια νόσου, ενώ δεν φάνηκαν άλλες σημαντικές αλλαγές σχετικά με τους υπόλοιπους δείκτες. Επιπλέον, η διάρκεια ΣΔ2 και η ύπαρξη εγκατεστημένης μακροαγγειοπάθειας δεν φάνηκε να παίζουν κάποιον περαιτέρω ρόλο σε ο,τι αφορά αυτούς τους δείκτες. Αυτή ήταν και η πρώτη μελέτη η οποία αναφέρει το ρόλο του γλυκαιμικού ελέγχου με τη βοήθεια όλων αυτών των δεικτών σε ασθενείς με ποικίλη διάρκεια ΣΔ2, συμπεριλαμβανομένων αυτών με γνωστή καρδιαγγειακή νόσο.

Τα νεότερα δεδομένα που προκύπτουν από τελευταίες μελέτες που χρησιμοποίησαν νεότερα αντιδιαβητικά σκευάσματα, όπως οι ινκρετίνες ή συν-μεταφορέας 2 γλυκόζης νατρίου (SGLT2) και σε πιο αρχικά στάδια της νόσου φαίνεται να ασκούν θετική επίδραση σε ό,τι αφορά τον καρδιαγγειακό κίνδυνο. Μεγαλύτερες μελέτες και για πιο εκτεταμένο διάστημα παρακολούθησης τόσο σε νεοδιαγνωσθέντες όσο και σε ασθενείς με μακρά διάρκεια νόσου στο μέλλον είναι απαραίτητες προκειμένου να δοθούν περισσότερες πληροφορίες σχετικά με το καρδιακό όφελος.

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ΙΩΑΝΝΙΝΑ, 9/1/2023
Αριθμ. Πρωτ.: 18

ΒΕΒΑΙΩΣΗ ΠΕΡΑΤΩΣΗΣ ΔΙΔΑΚΤΟΡΙΚΗΣ ΔΙΑΤΡΙΒΗΣ

Βεβαιώνεται η ακρίβεια των εξής στοιχείων:

Στοιχεία Ταυτότητας

Όνομα:	**ΣΟΦΙΑ**	Επώνυμο:	**ΑΝΤΩΝΙΟΥ**
Πατρώνυμο:	ΚΩΝΣΤΑΝΤΙ ΝΟΣ	Μητρώνυμο:	ΓΕΡΑΣΙΜΟΥΛΑ
Τόπος Γέννησης:	ΚΕΡΚΥΡΑ	Έτος Γέννησης:	1985

Στοιχεία Εγγραφής

Ημερομηνία:	8/2/2011	Ακαδ. Έτος:	2010-2011
Κατηγορία:	ΕΠΙΛΟΓΗ ΔΙΔΑΚΤΟΡΩΝ	Αριθμ. Μητρώου:	**26**
Αίτηση Εγγραφής:	21/01/2011	Αποφ. Εγγραφής:	Γ.Σ. 705α/8-2-2011

Η φοιτήτρια ολοκλήρωσε την εκπόνηση της Διδακτορικής Διατριβής με τίτλο «Η επίδραση του αυστηρού γλυκαιμικού ελέγχου σε ασθενείς με Σακχαρώδη Διαβήτη τύπου 2 στους πρώιμους, μη επεμβατικούς δείκτες αθηροσκλήρυνσης. Ο ρόλος της διάρκειας νόσου και της ύπαρξης εγκατεστημένης μακροαγγειοπάθειας», και στις 31/10/2022 την παρουσίασε και υποστήριξε δημόσια, ενώπιον των μελών της επταμελούς εξεταστικής επιτροπής.

Η Επταμελής Εξεταστική Επιτροπή, η οποία ορίστηκε σύμφωνα με τις κείμενες διατάξεις, ενέκρινε την εκπονηθείσα διδακτορική διατριβή και πρότεινε την απονομή του τίτλου της Διδάκτορος με βαθμό «**ΑΡΙΣΤΑ**».

Επιβλέπων της Διδακτορικής Διατριβής ήταν ο κ. ΣΤΥΛΙΑΝΟΣ ΤΙΓΚΑΣ , Αναπληρωτής Καθηγητής του ΤΜΗΜΑΤΟΣ ΙΑΤΡΙΚΗΣ του Πανεπιστημίου Ιωαννίνων.

Εκκρεμεί η διαδικασία της αναγόρευσης.

Η βεβαίωση αυτή χορηγείται για κάθε νόμιμη χρήση.

ΙΑΤΡΙΚΗ · ΣΧΟΛΗ · ΠΑΝΕΠΙΣΤΗΜΙΟΥ ΙΩΑΝΝΙΝΩΝ 1977

Η Γραμματέας του Τμήματος

ΜΑΡΙΑ ΚΑΠΙΤΟΠΟΥΛΟΥ