Dissertation

The impact of multifactorial risk factor intervention, vitamin D and probiotic supplementation on novel and established cardiovascular surrogate measures in subjects with disturbed glucose metabolism

submitted by
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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis.

Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of „Good Scientific Practice“.

Graz, August 2012

____________________________
**Abbreviations**

ACAPS  *asymptomatic carotid artery plaque study, Asymptomatic carotid artery plaque study*

ACCF  *American College of*

ACE  *angiotensin converting enzyme*

AGES  *Austrian Agency for Health and Food Safety*

AHA  *American Heart Association*

ANOVA  *analysis of variance*

ARB  *angiotensin receptor blocker*

AUC  *Area under the curve*

BMI  *body mass index, Body Mass Index*

CHD  *coronary heart disease*

CI  *confidence intervals*

cIMT  *carotid intima-media thickness*

CKD  *Chronic kidney disease*

cRP  *c-reactive protein*

CVD  *cardiovascular disease, cardiovascular disease*

EAS  *European Atherosclerosis Society*

ELISA  *Enzyme-linked immunosorbent assays*

ESC  *European Society of Cardiology*

Eudra-CT  *European Union Drug Regulating Authorities Clinical Trials*

FAO  *Food and Agriculture Organization of the United Nations*

FPG  *Fasting plasma glucose*

FDA  *Food and Drug Association*

FHS  *Framingham Study*
FMD  *Flow mediated dilatation*

GOT  *glutamat-oxalacetat-transaminase*

GPT  *Glutamat-Pyrovat-Transaminase*

Hb\textsubscript{A1c}  *Glycosylated hemoglobin*

HDL  *High density lipoproteins*

HOMA-IR  *Homeostasis model assessment for insulin resistance*

HR  *Hazard ratios*

hsCRP  *high sensitive c-reactive protein*

ICH-GCP  *International Conference on Harmonization of Good Clinical Practice*

IDF  *International Diabetes Federation*

IFG  *Impaired Fasting Glucose*

IgG  *Immunoglobulin G*

IGT  *Impaired Glucose Tolerance*

IL  *Interleukin*

IMT  *Intima media thickness*

ISI  *Insulin sensitivity index*

LBP  *Lipopolysaccharide-binding protein*

LcS  *Lactobacillis casei Shirota*

LDL  *Low density lipoproteins*

LPS  *Lipid bodies*

LURIC  *Ludwigshafen Risk and Cardiovascular Health*

MDA-LDL  *Malondialdehyde-modified low-density lipoprotein*

NCEP-ATP-III  *National Cholesterol Education Program and the Adult Treatment Panel-III*

NGT  *normal glucose tolerance*

NO  *nitric oxide*
NYHA  new york heart association
oGTT  oral glucose tolerance test
OGTT  Oral glucose tolerance test
ox-LDL  oxidized LDL
PAI  plasminogen activator inhibitor
PAT  Peripheral arterial tone
PCH  post-challenge hyperglycemia
PLAC  Pravastatin, Lipids, and Atherosclerosis in the Carotids
PTH  parathyroid hormone
QUICK  Quantitative Insulin-sensitivity Check Index
RAAS  Renin angiotensin aldosterone system
RCT  Randomized control trial
RHI  reactive hyperemia index
SD  Standard deviation
sICAM  Soluble intercellular adhesion molecule
sVCAM  Soluble vascular cell adhesion molecule
TNF  Tumor necrosis factor
ULN  upper limit of normal
VDR  vitamin d receptor
vWF  von Willebrand factor
WHO  World Health Organisation, World Health Organisation
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Ergebnisse: CARDIONOR-Studie: Die intensivierte Behandlung von Risikofaktoren verbesserte signifikant die globale Arginin-Bioverfügbarkeit (GABR) [0.33±0.12 zu Studienbeginn vs. 0.38±0.14 nach 3 Monaten; p=0.018] und das
Verhältnis von Arginin zu Ornithin (AOR) [0.39±0.15 vs. 0.46±0.19 nach 3 Monaten; p=0.039]. Eine signifikante Verbesserung wurde jedoch nur bei Patienten mit kurzer Diabetesdauer (<5 Jahre) festgestellt, während bei Patienten mit längerer Erkrankungsdauer die Verbesserung keine statistische Signifikanz aufwies. Ergänzend korrelierte die Veränderung von GABR mit der Intima Media Dicke reziprok (r=-0.381; p=0.014). Von Baseline bis zu 2 Jahren gab es eine signifikante Reduktion der durchschnittlichen IMT der Karotiden (cIMT) (0.88±0.12 mm vs. 0.86±0.13 mm; p=0.021). Die durchschnittliche cIMT korrelierte zu Studienbeginn mit dem Alter (r=0.365; p<0.001), der Diabetesdauer (r=0.273; p=0.007) wie auch dem systolischen Blutdruck (r=0.320; p=0.001). Im Gegensatz dazu konnten wir keine signifikanten Veränderungen in der Zahl von CD34+CD133+VEGFR2 und dem reaktiven hyperämischen Index von Studienbeginn bis zu 3 Monaten feststellen.

*Interventionsstudie (Lactobacillus casei Shirotatra):* Der Insulin Sensitivitätsindex (ISI) wurde durch eine 12-wöchige Probiotika-Verabreichung signifikant verbessert (0.058±0.021 vs. 0.038±0.025, p<0.01), allerdings ohne signifikanten Unterschied zur Kontrollgruppe. Es konnten jedoch keine Verbesserungen bei weiteren Indices der Insulinsensitivität (QUICKI, ISOGT und HOMA-IR) und der Beta-Zellfunktion (1° und 2° Phase Insulin Sekretion und HOMA-β) festgestellt werden.

*Interventionsstudie (Vitamin D):* Die Studie musste aufgrund von Rekrutierungsproblemen und von aktuell publizierten Forschungsergebnissen, welche eine Fortführung der Studie nicht rechtfertigen würden, vorzeitig abgebrochen werden. Zu Studienende konnten keine signifikanten Veränderungen der flussvermittelten Vasodilatation (FMD) und der nitratvermittelten Vasodilatation (NMD) im Vergleich der Vitamin D-Gruppe mit der Plazebo-Gruppe, beobachtet werden.

*Kohortenanalyse:* Patienten mit Diabetes Mellitus (DM) hatten die niedrigsten durchschnittlichen 25(OH)-Vitamin D Werte (14.5±8.1 ng/ml), gefolgt von Patienten mit postprandialer Hyperglykämie (pcHG) (17.4±9.4 ng/ml) und der Gruppe mit normaler Glukosetoleranz (NGT) (18.7±9.6; p< 0.001) (DM vs. pcHG p< 0.001, DM vs. NGT p< 0.001, pcHG vs. NGT p=0.002). Nach einem medianen Follow-up von 7.7 Jahren starben total 582 Personen (22.7%), 242 (41.7%) in der
Diabetes-Gruppe, 217 (20.9%) in der postprandialen Gruppe und 123 (12.8%) in der Gruppe mit normaler Glukosetoleranz.

4. Abstract

Introduction: Individuals with so-called pre-diabetes (impaired glucose tolerance or impaired fasting glucose) are at high risk to experience an adverse cardiovascular event (stroke, myocardial infarction, cardiovascular death) in their life. Therefore, treatments and interventions aiming to reduce these events are of great interest. In the first part of my thesis programme, we focused on novel cardiovascular surrogate parameters and how they are affected by multifactorial risk factor intervention. For the second part we investigated the impact of vitamin D and probiotic supplementation, respectively on surrogate measures.

Methods: We carried out a prospective, open 2 years clinical trial (CARDIONOR-study) including 97 patients with type 2 diabetes (T2DM). Whilst we have investigated the impact of the multifactorial intervention on intima media thickness (IMT) in the whole population, we have assessed 3 novel cardiovascular surrogate measurements (arginine bioavailability ratios, the reactive hyperaemia index (RHI) and endothelial progenitor cells) in subsets of our study population. In addition, we performed a randomized-controlled pilot study including 30 subjects with metabolic syndrome to investigate the impact of Lactobacillus casei Shirota (LcS) supplementation on glucose tolerance and indices of insulin sensitivity and beta-cell function. Moreover, we performed a randomized-controlled study in coronary artery disease patients with post-challenge hyperglycemia and vitamin D deficiency to investigate the effect of vitamin D supplementation on endothelial function. Additionally, an analysis of 2565 patients of the LURIC study was performed to elucidate whether the association of 25(OH) vitamin D levels with mortality is the same in various stages of glucometabolic disturbances.

Results: CARDIONOR-study: Intensified risk factor management significantly improved global arginine bioavailability ratio (GABR) [0.33±0.12 at baseline vs. 0.38±0.14 after 3 months; p=0.018] and arginine to ornithine ratio (AOR) [0.39±0.15 vs. 0.46±0.19 after 3 months; p=0.039]. A significant improvement was only seen in patients with short diabetes duration (<5 years) whereas in patients with longer diabetes duration improvement did not reach statistical significance. In
addition the change of GABR was inversely correlated with mean intima media thickness (r=-0.381; p=0.014). The mean carotid IMT significantly reduced from baseline to 2 year (0.88±0.12 mm vs. 0.86±0.13 mm; p=0.021). Mean cIMT correlates at baseline significantly with age (r=0.365; p<0.001), and duration of diabetes (r=0.273; p=0.007) as well as systolic blood pressure (r=0.320; p=0.001). In contrast, no significant changes in the number of CD34+CD133+VEGFR2 and the reactive hyperemia index could be observed from Baseline to 3 months while risk factor management was intensified.

**Intervention study (LcS):** Insulin sensitivity index (ISI) was significantly improved after 3 months of probiotic supplementation (0.058±0.021 vs. 0.038±0.025, p<0.01) but not significantly different to the control group. No improvements were seen in further indices of insulin sensitivity (QUICKI, ISoGTT and HOMA-IR) and beta-cell function (1st and 2nd phase insulin secretion and HOMA-β).

**Intervention study (vitamin D):** The study was terminated early due to inability to recruit and newly published data, which did not justify the further conduction of the trial. At the end of the trial, no significant changes in the vitamin d group in flow mediated dilatation (FMD) and nitrate mediated dilatation (NMD) compared to the placebo group were observed.

**Cohort analysis:** Patients with conventional diabetes (DM) had the lowest mean 25(OH)-vitamin D levels (14.5±8.1 ng/ml) followed by the post-challenge hyperglycemia (pcHG) group (17.4±9.4 ng/ml) and normal glucose tolerance group (NGT) (18.7±9.6; p< 0.001) (DM vs. pcHG p< 0.001, DM vs. NGT p< 0.001, pcHG vs. NGT p=0.002). After a median follow-up of 7.7 years, a total of 582 participants (22.7%) died, 242 (41.7%) in the DM group, 217 (20.9%) in the pcHG group and 123 (12.8%) in the NGT group, respectively.

**Conclusion:** We demonstrate that multifactorial risk factor intervention significantly improved IMT as well as the GABR and AOR in patients with type 2 diabetes. The intake of LcS for 12 weeks in subjects with metabolic syndrome did not clearly affect insulin sensitivity or beta-cell function in our pilot trial. The trial investigating the supplementation of vitamin d for 12 weeks in subjects with CAD, post-challenge hyperglycemia or early type 2 diabetes (T2DM) and vitamin d deficiency had to be terminated early due to the inability to recruit. However, at this
time no trend in favour of vitamin D with regard to FMD has been observed. In our cohort analysis we display that 25(OH)D levels are associated with increased mortality in normal glucose tolerance, post-challenge hyperglycemia as well as diabetic patients. However, further studies with a larger sample size in different patient populations need to be performed to clarify the effects of various treatments on cardiovascular risk factors and surrogate markers of CVDs.
5. Introduction

Type 2 diabetes mellitus (T2DM) is one of the leading causes of morbidity and mortality worldwide (Roper et al. 2001), and is a well-established risk factor for cardiovascular disease (CVD). Already in 1979, the Framingham Study was the first to describe a 2-3 fold increase in the risk of CVD in diabetic compared to non-diabetic subjects (Kannel, McGee 1979). Subsequent, patients with type 2 diabetes have been shown to have a two to six-fold higher incidence of coronary artery disease and stroke and are considered as a risk equivalent of a non-diabetic patient with pre-existing heart disease (Haffner et al. 1998).

Fasting glucose level below the diabetes threshold has also been shown to be a risk factor for CVD (Coutinho et al. 1999). Additionally, there is evidence that impaired glucose tolerance may be associated with increased risk for total mortality as well (DECODE Study Group, the European Diabetes Epidemiology Group. 2001, Barrett-Connor, Ferrara 1998). Prevention and treatment of glucometabolic disturbances is thus important, and has been recommended as a means of reducing the future risk of CVD complications (Ryden et al. 2007). However, since definitive outcome trials take a long to complete and are costly, most of the interventional data from the past relies on the impact of various glucose lowering drugs on cardiovascular surrogate parameters.

5.1 Diabetes mellitus type 2 and intermediate hyperglycemia

Type 2 diabetes mellitus accounts for 85% of cases of diabetes in Caucasian population. The rapid global increase in T2DM will explain almost all of the predicted doubling of diabetes prevalence (to 300 million cases worldwide) by 2025 (King, Aubert & Herman 1998). Today type 2 diabetes is regarded as a disease caused by the two major intertwined core defects insulin resistance and beta-cell dysfunction (Defronzo 2009). Chronic hyperglycemia is associated with long-term dysfunction and failure of various organs, especially eyes, kidney, nerves, heart and blood vessels. Since 1965 the World Health Organisation (WHO) and other large international and national diabetes associations have published regular guidelines for the diagnosis and classification of diabetes.
**Diagnostic criteria**

Every few years, the diabetes community re-evaluates the current recommendations for the classification, diagnosis, and screening of diabetes, reflecting new information from research and clinical practice. In 2005 a joint WHO and International Diabetes Federation (IDF) advisory group updated the current WHO guidelines. The following table (table 1) summarises the 2006 WHO recommendations for the diagnostic criteria for diabetes and intermedia hyperglycemia (WHO/IDF 2006).

**Table 1 Diagnostic criteria for diabetes and intermedia hyperglycemia**

<table>
<thead>
<tr>
<th></th>
<th>Fasting plasma glucose</th>
<th>2-h plasma glucose *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;110 mg/dl</td>
<td>&lt;140 mg/dl</td>
</tr>
<tr>
<td></td>
<td>&lt;6.1 mmol/l</td>
<td>&lt;7.8 mmol/l</td>
</tr>
<tr>
<td>Impaired Fasting Glucose (IFG)</td>
<td>≥ 110 to ≤ 126 mg/dl</td>
<td>&lt;140 mg/dl</td>
</tr>
<tr>
<td></td>
<td>≥ 6.1 to ≤7.0 mmol/l</td>
<td>&lt;7.8 mmol/l</td>
</tr>
<tr>
<td>Impaired Glucose Tolerance (IGT)</td>
<td>&lt;126 mg/dl</td>
<td>≥140 to ≤200 mg/dl</td>
</tr>
<tr>
<td></td>
<td>&lt;7.0 mmol/l</td>
<td>≥7.8 to ≤11.1 mmol/l</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>≥126 mg/dl</td>
<td>≥200 mg/dl</td>
</tr>
<tr>
<td></td>
<td>≥7.0 mmol/l</td>
<td>≥11.1 mmol/l</td>
</tr>
</tbody>
</table>

* Venous plasma glucose 2-h after ingestion of 75g oral glucose load

Glycosylated haemoglobin (HbA$_{1c}$) is a widely used marker of chronic glycaemia, reflecting average blood glucose levels over a 2- to 3-month period of time. Elevated HbA$_{1c}$ levels at or beyond the threshold of 6.5% are considered diagnostic for diabetes, while levels from 5.7% to 6.4% point to high risk for developing both diabetes and cardiovascular disease and are a marker of increased risk for diabetes, according to the American Diabetes Association (ADA) (American Diabetes Association 2010). The oral glucose tolerance test (OGTT) and 2-h plasma glucose has been recommended for screening for more than 30 years, but in practice has not been much used, because it is more difficult to execute, impractical for large numbers, time consuming and more expensive than fasting blood glucose tests. Nevertheless the OGTT should be retained as a diagnostic test for the following reasons (WHO/IDF 2006):
- fasting plasma glucose alone fails to diagnose approximately 30% of cases of post-challenge diabetes,
- an OGTT is the only means of identifying people with IGT,
- an OGTT is frequently needed to confirm or exclude an abnormality of glucose tolerance in asymptomatic people.

This test should be performed in individuals with fasting plasma glucose 110 - 125mg/dL to detect glucose tolerance status (WHO/IDF 2006).

5.2 Glucose metabolism and cardiovascular risk

Recent estimates suggest that 195 million people throughout the world have diabetes. This number is projected to increase to 330 million, maybe even to 500 million by 2030 (King, Aubert & Herman 1998, Wild et al. 2004). About 90% of the diabetic population has type 2 diabetes mellitus. Of concern is that, up to 50% of all patients with type 2 diabetes are undiagnosed (Harris et al. 1998, Rathmann et al. 2003, Anonymous1998d). Type 2 diabetes and cardiovascular disease often appears as two sides of a coin: Many patients with established coronary heart disease suffer from diabetes or its pre-states, and conversely, diabetes has been considered as an equivalent of coronary heart disease (Ryden et al. 2007).

Type 2 diabetes is a well-known risk factor for atherosclerosis. More recently, it has been demonstrated that even the state preceding overt diabetes, called impaired glucose tolerance (IGT), may also predispose subjects to increased cardiovascular risk. For accurate detection of abnormal glucose metabolism in the early stages the oral glucose tolerance test (OGTT) is mandatory. The Euro Heart Survey on Diabetes and the Heart reported that two thirds of patients with positive OGTTs would have remained undiagnosed if only fasting plasma glucose levels had been considered (Bartnik et al. 2007). Several studies (DECODE Study Group, the European Diabetes Epidemiology Group. 2001, DECODA Study Group, International Diabetes Epidemiology Group 2002) showed that a 2-hour post load level measured by OGTT is a better predictor of the dysglycemic state than a fasting glucose level alone and is also a better risk predictor for subsequent cardiovascular complications (DECODE Study Group, the European Diabetes Epidemiology Group. 2001, Qiao et al. 2002).
Three large prospective studies have reported that abnormal glucose metabolism, including pre-diabetes and type 2 diabetes, was more common than normoglycemia in patients with cardiovascular disease (Bartnik et al. 2004, Norhammar et al. 2002, Hu et al. 2006). 2007 the guidelines on diabetes, pre-diabetes and cardiovascular disease stated that all patients with these conditions should be tested if their glucometabolic condition is not already known (Ryden et al. 2007). The use of an OGTT in routine in the cardiology setting is a simple, cost-effective approach that has the potential to improve the detection and management of glucometabolic abnormalities significantly in patients with cardiovascular disease. Actually, a high percentage of these patients have impaired glucose tolerance, although they may be unaware of it (Bartnik et al. 2004, Norhammar et al. 2002).

All stages of glucometabolic abnormalities are associated with an increased risk of cardiovascular morbidity and mortality (DECODA Study Group, International Diabetes Epidemiology Group 2002, Lowe et al. 1997). Shaw et al. reported that people with isolated post-challenge hyperglycemia a doubled risk for CVD mortality compared to on-diabetic people (Shaw et al. 1999). Hence, it is so important to identify them as early as possible to intensify cardiovascular risk factor management accordingly. In people without diabetes mellitus, fasting blood glucose concentration is modestly and non-linearly associated with risk of vascular disease (Emerging Risk Factors Collaboration et al. 2010). Wannamethee and colleagues suggested that both early and late onset of diabetes are associated with increased risk of major CHD events and mortality, but only early onset of diabetes (associated with > 10 years’ duration) appears to be a CHD equivalent (Wannamethee et al. 2011). Several studies have provided evidence that diabetic microangiopathy can be reduced by tight glycemic control (Anonymous1993, Anonymous1996, Anonymous1998b, Anonymous1998a, Gaede et al. 2003). This reduction will also have an convenient influence on CVD {{373 Laakso,M. 1996; 371 Anonym 1998; 374 Selvin,E. 2004}}.

The relation between macrovascular disease and hyperglycemia is less clear than the relation to microangiopathy (Anonymous1993, Laakso, Kuusisto 1996, Stratton et al. 2000, Gaede et al. 2008, Canadian Diabetes Association et al. 2006). In the UKPDS trial, each per cent decline of HbA1c was associated with a 14% lower
rate of myocardial infarction and fewer deaths from diabetes or any cause (Anonymous1998b, Stratton et al. 2000). Numerous prospective observational trials assessing the risk of macrovascular disease in diabetes have shown that this risk is increased already at glycemic levels marginal above the normal range or even within the high normal range (Hu et al. 2006, Stratton et al. 2000, Nathan, Meigs & Singer 1997). Recently, three large scale intervention trials investigated the impact of intensive glucose lowering treatment, aiming for an Hb$_{A1c}$ target of 6.5% or lower compared to conventional glucose lowering treatment with a higher glucose target. All three trials per se did not show a significant reduction in major cardiovascular events by the intensive treatment (Action to Control Cardiovascular Risk in Diabetes Study Group et al. 2008, ADVANCE Collaborative Group et al. 2008, Duckworth et al. 2009). However, a meta-analysis summarizing all available trial data demonstrated a significant 9% and 15% relative risk reduction for major cardiovascular events (95%CI 0.84-0.99) and for myocardial infarction (0.76-0.94), respectively (Control Group et al. 2009).

### 5.3 Cardiovascular risk factors

Risk factors are markers that are statistically related to the risk of morbid events, presumably because they identify or contribute to one or more of the vascular processes that lead to these events, but they do not necessarily identify the disease itself (Cohn 2004). They are crucial to the identification of high-risk individuals who could benefit from targeted preventive measures. Until now, a number of new risk factors have been associated with cardiovascular diseases. Only few of these were consecutively evaluated well enough to determine their definite role in risk prediction compared with classical risk factors. In spite of the large number of known risk factors, the identification of individuals at increased risk for CVD is not a simple undertaking. Although the major clinical risk factors explain a large proportion of the risk, a notable rate of individuals have few traditional risk factors and score low in current prediction models (Herder, Karakas & Koenig 2011) despite having CV events.
Table 2 List of risk factors of cardiovascular diseases used in current prediction models (modified according to (Herder, Karakas & Koenig 2011))

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Ethnicity</th>
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</thead>
<tbody>
<tr>
<td><strong>Anthropometric factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Obesity (weight, BMI, waist circumference, waist-to-hip ratio)</td>
<td></td>
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<tr>
<td>- Blood lipids (triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, use of lipid-lowering drugs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Blood pressure (systolic blood pressure, diastolic blood pressure, use of antihypertensive drugs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Metabolic factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Parameters of glucose metabolism (fasting glucose, 2-h glucose, fasting insulin, estimates of insulin resistance, estimates of β-cell function, HbA1c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Blood pressure</td>
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<td>- Liver enzymes</td>
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<td>- Uric acid</td>
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<td><strong>Lifestyle factors</strong></td>
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<td>- Physical inactivity</td>
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<td>- Dietary components (red meat, fruits, vegetables, fiber, coffee)</td>
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<td>- Smoking</td>
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<td>- Alcohol</td>
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<td><strong>Socioeconomic factors</strong></td>
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<td><strong>Markers of subclinical inflammation</strong></td>
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<td>- Leukocyte count</td>
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<td>- CRP</td>
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<td>- Adiponectin</td>
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<td><strong>Markers of hemodynamic stress (natriuretic peptides)</strong></td>
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<td><strong>Markers of myocardial cell necrosis (troponins)</strong></td>
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<td><strong>Sensitive markers of renal dysfunction (cystatin C)</strong></td>
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<td><strong>Lipid-related markers (phospholipases, lipoprotein subclasses, apolipoproteins)</strong></td>
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<td><strong>Markers of coagulation (fibrinogen, PAI-1, D-dimer)</strong></td>
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<td><strong>Markers of angiogenesis</strong></td>
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<td><strong>Family history of myocardial infarction</strong></td>
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5.3.1 Traditional risk factors

Traditional vascular risk factors may be grouped according to whether they may be modified or not. The major modifiable risk factors are hypertension, lipids, hyperglycemia, smoking behavior and obesity. All of them can be treated by lifestyle modification and pharmaceutically. Contrary, non-modifiable risk factors are age, gender and genetics (family history).

Figure 1 Modifiable risk factors

High blood pressure

In the Framingham Heart Study, even high-normal blood pressure (systolic blood pressure of 130-139 mmHg, diastolic blood pressure of 85-89 mmHg, or both) increased the risk of cardiovascular disease 2-fold, as compared with healthy individuals (Vasan et al. 2001). There is convincing evidence that lowering blood pressure reduces cardiovascular complications in patients with T2DM (Hansson et al. 1998, Anonymous2000, ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group, The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial 2002, Anonymous1998c). However, how low blood pressure targets in patients with T2DM should be set, still needs to be elucidated.

High blood lipids

In the Framingham study was shown that the higher the cholesterol level, the greater the risk of coronary artery disease (Castelli 1988). Raised total cholesterol is a major risk factor for ischemic heart disease and stroke (Ezzati et al. 2002).
Increased triglycerides are an independent risk factor for coronary heart disease after controlling for LDL and HDL cholesterol (Cullen 2000).

Plenty of epidemiologic studies have shown a significant and independent inverse relationship between HDL cholesterol and the risk for cardiovascular disease (Castelli 1988, Gordon et al. 1977, Wilson et al. 1980). Already in 1989, Gordon and colleagues noted that an increase by 1 mg/dl in HDL cholesterol results in a 2-3% risk reduction for CAD (Gordon et al. 1989). Several trials have shown that cholesterol-lowering therapy reduces the incidence of major cardiac events in patients with coronary heart disease (Nissen 2005, Cannon et al. 2004, Law, Wald & Rudnicka 2003). A recent meta-analysis reported that reduction of LDL cholesterol with statin therapy significantly reduces the risk of major vascular events in patients with type 2 diabetes (Cholesterol Treatment Trialists' Ctt 2012). The VA-HIT study (Veterans Affairs Cooperative Studies Program High-Density Lipoprotein Cholesterol Intervention Trial) showed that therapy with Gemfibrozil significantly reduces the risk of major cardiovascular events in patients with coronary disease (Rubins et al. 1999). However, trials specifically investigating the potential benefit of fibrates in T2DM failed to show a significant effect (ACCORD Study Group et al. 2010, Keech et al. 2005)).

Interestingly, a recent trial investigating the cardiovascular benefits of niacin, the most effective agent currently available to raise HDL cholesterol, was stopped prematurely due to futility and a potentially raised stroke rate (AIM-HIGH Investigators et al. 2011).

**Hyperglycemia**

In the UK Prospective Diabetes Study (UKPDS) was reported that there is a significant association between glycemia and the development of micro- and macrovascular complications of T2DM, including mortality, across the wide range of exposure to glycemia that appears in patients with T2DM (Stratton et al. 2000). The UKPDS 33 study showed that intensive glucose therapy (either with insulin or sulfonylureas) in patients with newly diagnosed T2DM reduces the risk of microvascular complications (Anonymous 1998b). Beneficial effects of an early intensive glucose lowering regimen with regard to macrovascular events were shown to become apparent later in the course of the disease, suggesting a glucometabolic legacy effect (Holman et al. 2008).
Smoking
For over six decades studies reported a strong association between cigarette smoke exposure and heart disease. Persons who consume more than 20 cigarettes daily have a 2- to 3-fold increase in total heart disease. Continued smoking is a major risk factor for recurrent heart attacks (Rea et al. 2002). Even though smoking is a risk factor in men and women, Huxley and colleagues suggest in a systematic review and meta-analysis that women had a significant 25% increased risk for coronary heart disease conferred by cigarette smoking compared with men (Huxley, Woodward 2011).

After smokers give up smoking, their risk of mortality and future cardiac events falls off (Aberg et al. 1983, Rigotti, Pasternak 1996). Bakhru and colleagues reported that the smoking-associated inflammatory response abates within 5 years after smoking cessation. This indicates that the vascular effects are reversible and that cardiovascular risk lowers gradually with reduced exposure (Bakhru, Erlinger 2005).

Obesity
Although obesity has deleterious effects on health itself it also significantly increases the risk for co-morbidities such as type 2 diabetes (T2DM) and cardiovascular disease (Lavie, Milani 2003). Obesity has a multifactorial character, which comprises an independent risk factor for cardiovascular disease. Numerous large studies have shown a positive relationship between CVD mortality and body mass index (BMI), a widely used measure of human obesity (Yusuf et al. 2005, Wilson et al. 2002, Stevens et al. 1998, McGee, Diverse Populations Collaboration 2005)). A very recent study showed that bariatric surgery, a weight reductive surgery, was associated with reduced number of cardiovascular deaths and reduced incidence of cardiovascular events (Sjostrom et al. 2012).

Physical activity
Already in 1992, the body of evidence led the American Heart Association to recognize physical activity as a risk factor for CHD and CVD (Fletcher et al. 1992). A great variety of studies reported that active people have lower risk for CHD and cardiovascular disease than inactive ones (Anonymous2009, Sofi et al. 2008, Nocon et al. 2008). A recent systematic review and meta-analysis showed that
there is a large body of evidence clearly supporting reduced risks of CHD and CVD with physical activity, but details of the relationship are less clear (Shiroma, Lee 2010). There is evidence that physical activity and cardiorespiratory fitness training reduce the risk of cardiovascular disease in men and women (Manson et al. 2002, Blair et al. 1996).

5.3.2 Non-traditional risk factors

**Lipoprotein (a)**

Lipoprotein (a) is a major independent genetic risk factor for cardiovascular disease (Emerging Risk Factors Collaboration 2009). Elevated Lp(a) levels associate robustly with increased CVD risk and are causally related to premature development of atherosclerosis and CVD (Nordestgaard et al. 2010). Plasma Lp(a) is not recommended for risk screening in the general population; however, Lp(a) measurement should be considered in all subjects at intermediate or high risk of CVD/CHD (Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS) et al. 2011) who present with:

I. Premature CVD
II. Family hypercholesterolemia
III. A family history of premature CVD and/or elevated Lp(a)
IV. Recurrent CVD despite statin treatment
V. $\geq 3\%$ 10 year risk of fatal CVD according to the European guidelines

According to the EAS guidelines 2010 Lp(a) levels should be $<50\text{mg/dl} (<80^{\text{th}}\text{ percentile})$. Up to now, there is no Lp(a) therapy available, for which a cardiovascular benefit would have been shown.

**c-reactive protein (CRP)**

CRP is a sensitive, non-specific systemic marker of inflammation and tissue damage that has long been used to monitor inflammation in fever and other conditions (Pepys, Hirschfield 2003). According to some studies, elevated levels of CRP are associated with traditional cardiovascular risk factors and with obesity (Miller, Zhan & Havas 2005, Lemieux et al. 2001). The Women’s Health Study
(WHS), Physicians’ Health Study (PHS), Monitoring of Trends and Determinants of Cardiovascular disease (MONICA) study and the Atherosclerosis Risk in Communities study (ARIC) demonstrated that a CRP >3mg/L carried a nearly 2-fold risk of cardiovascular events compared with a CRP level < 1 mg/L, which was independent of other risk factors (Koenig et al. 1999, Ridker, Glynn & Hennekens 1998, Ridker et al. 2000). The 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults states that measurement of CRP can be useful in selecting patients for statin therapy and may be reasonable for cardiovascular risk assessment, depending on the patient’s age and risk level (Greenland et al. 2007).

5.4 Cardiovascular surrogate measurements

5.4.1 Definition

As defined by the FDA (Food and Drug Association), a surrogate end point or marker is a laboratory measurement or physical sign that is used in therapeutic trials as a substitute for a clinically meaningful end point that is a direct measure of how a patient feels, functions, or survives and is expected to predict the effect of the therapy (Katz 2004). In T2DM, Hb_{A1c} is used as a surrogate measurement to predict diabetic microvascular complications (nephropathy, neuropathy and retinopathy) and macrovascular complications (mainly myocardial infarction). Additionally, surrogate measurements such as blood pressure, blood lipids and hsCRP have been used to predict outcomes of cardiovascular disease (Yudkin, Lipska & Montori 2011).

Figure 2 Selected Cardiovascular surrogate marker
5.4.2 Carotid intima-media thickness

Determination of carotid intima-media thickness (cIMT) is a generally accepted research method for detection and quantification of subclinical cardiovascular disease. The intima-media thickness describes the distance from the lumen-intima interface to the media-adventitia interface of the artery wall, as measured on non-invasively acquired ultrasonographic images of the carotid arteries. It is generally measured in the common carotid artery 1cm proximal to the carotid bulb and is accepted as a marker of generalized arterial disease (Bots, Hofman & Grobbee 1994, Geroulakos et al. 1994a, O’Leary et al. 1999).

Measurement of cIMT has several advantages as a risk prediction tool. The measurement is non-invasive, inexpensive, readily applicable and carries no risk for subjects. Hence, cIMT has now become an established surrogate marker of CVD in clinical trials to evaluate the efficacy of interventions with statins, antihypertensives, and antidiabetic medications (Bots et al. 2009, Crouse et al. 2007, Howard et al. 2008, Katakami et al. 2004). Since it has been used as a research tool for nearly three decades, population distributions of cIMT are known for both, men and women, for individuals between 25 and 85 years, as well as most races and ethnicities (Stein et al. 2008). Generally, cIMT measurements >1.20mm are accepted as abnormal. Progression of atherosclerosis is estimated to occur between 0.02 and 0.05 mm per year in population-based studies (Feinstein, Voci & Pizzuto 2002). Several guidelines and consensus statements have recommended measurement of cIMT together with plaque detection (Greenland et al. 2007, Stein et al. 2008, Graham et al. 2007). The presence of carotid plaque is associated with an approximately 3-fold increased risk of future CVD events (Wyman et al. 2006). Carotid plaque burden has been also shown to improve prediction of all-cause mortality (Stork et al. 2004).

5.4.3 Endothelial dysfunction

Endothelial dysfunction can be defined as, “the partial or complete loss of balance between vasoconstrictors and vasodilators, growth promoting and growth inhibiting factors, pro-atherogenic and anti-atherogenic factors” (Quyyumi 1998). Lerman et al. defined endothelial dysfunction as the “ultimate risk of the risk factors” (Lerman, Zeiher 2005). It can be seen as a crucial, initial event in
atherogenesis (Ross 1999) and has been shown to precede development of clinically detectable atherosclerotic plaques in the coronary arteries (Mano et al. 1996).

Actually, there is no standard test to assess endothelial functions in vivo. Invasive assessment of coronary endothelial function by quantitative coronary angiography and coronary Doppler flow measurements, along with graded intracoronary infusions of endothelium dependent vasodilatators such as acetylcholine were considered the “gold standard” for testing endothelial dysfunction (Hasdai, Lerman 1999). Another alternative is to measure levels of biochemical markers of endothelial function, such as Endothelin-1, soluble intracellular adhesion molecules (sICAM), soluble vascular cell adhesion molecule (sVCAM) and other markers of fibrinolysis and coagulation, such as von Willebrand factors (vWF) and markers of low grade inflammation, such as IL-1, IL-6, TNF-alpha and CRP (Winkler et al. 1999). Non-invasive techniques for assessment of endothelial function are to measure flow mediated endothelium dependent dilatation (FMD) of the brachial artery during reactive hyperemia and to measure endothelial dysfunction by a EndoPAT™ device, that quantifies the endothelium-mediated changes in vascular tone, elicited by a 5-minute occlusion of the brachial artery (using a standard blood pressure cuff)(Moerland et al. 2012).

Endothelial dysfunction is a reversible disorder. Non-pharmacological and pharmacological approaches have been shown to improve or reverse endothelial dysfunction, though they target one or more cardiovascular risk factors, such as smoking cessation, antihypertensive therapy, cholesterol lowering therapy, estrogen replacement therapy in postmenopausal women (Beckman, Creager 2006, Beckman et al. 2004, Matsuda et al. 2000, Papanathanassiou et al. 2009, Sourij, Zweiker & Wascher 2006).

5.4.4 Microalbuminuria

Long known to be associated with kidney disease, the importance of protein in the urine is now becoming recognized as cardiovascular risk factor. Microalbuminuria is defined as persistently increased urinary excretion of albumin. Due to different sampling techniques, different albumin detection techniques, and difference in albumin quantity ranges (table 3 shows diagnostic tresholds) there is no single definition of microalbuminuria (de Jong, Curhan 2006). Microalbuminuria is a
complication of diabetes and a strong predictor of subsequent development of overt diabetic nephropathy (Deckert et al. 1992). In addition microalbuminuria does not only predict future risk of renal injury but studies as the HOPE trial (Yusuf et al. 2000), have shown a clear relationship between microalbuminuria and cardiovascular events.

Table 3 Diagnostic thresholds of microalbuminuria

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<th>Microalbuminuria</th>
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<tr>
<td>24h urine</td>
<td>Albumin (mg/24h)</td>
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<td>30 to &lt;300</td>
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<tr>
<td>Overnight urine</td>
<td>Albumin (µg/24h)</td>
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<td>20 to &lt;200</td>
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<tr>
<td>Spot urine</td>
<td>Albumin (mg/L)</td>
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<td></td>
<td>20 to &lt;200</td>
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<td></td>
<td>Albumin/creatinine ratio (male) mg/mmol</td>
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<td></td>
<td>2.5 to &lt;25</td>
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<td>Albumin/creatinine ratio (male) mg/g</td>
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<td></td>
<td>Albumin/creatinine ratio (female) mg/mmol</td>
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<td>3.5 to &lt;35</td>
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<td></td>
<td>Albumin/creatinine ratio (female) mg/g</td>
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<td>30 to &lt;300</td>
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A large number of studies report an association between microalbuminuria and other cardiovascular risk factors, like hypertension (Pedrinelli et al. 2002, Campese, Bianchi & Bigazzi 1999), hyperlipidemia (Campese, Bianchi & Bigazzi 1999), age and smoking (Gerstein et al. 2000, Yuyun et al. 2004). Microalbuminuria is also associated with markers of inflammation, such as c-reactive protein (Barzilay et al. 2004) and has further been linked to abnormalities of the vasculature, such as endothelial dysfunction and reduced vascular dilatation (Malik et al. 2007). Dinneen and Gerstein demonstrated in a meta-analysis a twofold increased risk of total and cardiovascular mortality and morbidity in type 2 diabetes mellitus (Dinneen, Gerstein 1997).

5.4.5 Ankle brachial index

Measurement of the ankle brachial index (ABI) includes the systolic blood pressure in the brachial artery at each elbow and systolic blood pressure in the posterior tibial and the dorsalis pedis arteries at each ankle. The result is reported as the ratio of systolic blood pressure measured at the ankle to that at the arm.
The ABI is calculated for each leg separately and the lower of the two values is taken as a result for the patient (Khan, Farooqui & Niazi 2008). This ratio is quick and easy to measure and has been used in practice to confirm the diagnosis and assess the severity of peripheral artery disease (PAD) in the legs. Subjects with peripheral artery disease in the lower extremities are among the highest-risk vascular patients. PAD is an indicator of widespread atherosclerosis in other vascular territories, such as the carotid, coronary and cerebrovascular arteries (Mautner, Mautner & Roberts 1992, Newman et al. 1993, Salonen, Salonen 1991). Papamichael and colleagues, reported that an ABI <0.90 is an independent predictor of cardiovascular events (cardiac death, nonfatal myocardial infarction, unstable angina) after adjustment for age, LDL-cholesterol and carotid IMT (Papamichael et al. 2000). The ABI is considered a useful tool for prediction of cardiovascular risk, because the procedure is simple, taking less than 10 minutes (Mohler et al. 2004) and can be performed by a suitable trained nurse in the office or clinic setting. The intra-observer variability of the test in trained observers is low (7%) (Matzke et al. 2003). Non-invasiveness of the test and minimal discomfort result in a high patient acceptability. Numerous studies have reported that the ABI, compared to angiography, has a sensitivity of more than 90% and a specificity of more than 95% in diagnosing an at least 50% stenosis of the lower extremity arteries (McDermott et al. 2005, Hirsch et al. 2006, Diehm et al. 2004). Recently, Hanssen and colleagues reported that the association between ABI and cardiovascular and all-cause mortality were similar in individuals with and without T2DM (Hanssen et al. 2012).

5.5 Vitamin D

5.5.1 Source and metabolism of vitamin D

In the early 20th century Vitamin D was classified as a vitamin, but vitamin D is technically not a vitamin, it is not an essential dietary factor; rather, it is a pro-hormone produces photochemically in the skin from 7-dehydrocholesterol (DeLuca 2004). The major source of vitamin D for most humans is exposure to sunlight (DeLuca 2008, Holick 2008).

25-hydroxyvitamin D levels vary by latitude, altitude, geography and seasonality as a result of solar exposure. Caucasians and Hispanics tend to have higher 25-
22

hydroxyvitamin D levels in comparison to African-Americans (Zadshir et al. 2005) due to their greater cutaneous melanin content, which reduces the initial conversion of 7-dihydrocholesterol to vitamin D₃ in the skin by ultraviolet B rays (Clemens et al. 1982).

20 minutes of a daily whole body exposure to UVB radiation (290-315nm) three times a week is able to maintain adequate vitamin D status in people with light skin (Krause et al. 1998). Seasonal variation is found in the major circulating form of vitamin D, 25-hydroxyvitamin D with an abrupt increase in concentration in June and highest values from June to October (Brot et al. 2001).

Austria has only a moderate climate and its geographic location (46°50´N to 49°00´N) is relatively northern. Generally, solar UV-B radiation is assumed to be insignificant small at geographic latitude of 40°N from November until February and from October to April at a latitude of 50°N, respectively (Holick 1994). During that period, maintenance of vitamin D level is dependent on oral vitamin D intake and on the stores of vitamin D built up during the previous summer months. In Austria there is no food fortification with vitamin D, whereas in the United States, milk, some juice products, some breads, yoghurts, and cheeses are fortified with vitamin D (Holick, Chen 2008).

Dietary vitamin D is a second, less important source. There is little vitamin D that occurs naturally in the food supply. Oily, fatty fish such as salmon, mackerel, herring and sardines are good sources of vitamin D₃, as well as beef liver and egg yolk and UV-irradiated and sun-dried mushrooms (Chen et al. 2007).

There are two forms of vitamin D, vitamin D₃ (cholecalciferol) originates from 7-dehydrocholesterol and is produced in the epidermis and dermis in humans from exposure to sunlight and vitamin D₂ (ergocalciferol) which is produced in yeast and plants. The chemical difference between vitamin D₂ and D₃ is in the side chain: vitamin D₂ has a double bond between carbon 22 and 23 and a methyl group on carbon 24, in contrast to vitamin D₃. Vitamin D from the skin and diet is metabolized in the liver to 25-hydroxyvitamin D [25(OH)D], which used to determine a patient’s vitamin D status (DeLuca 2004, Holick 2007). 25(OH)D is then hydroxylated in the kidneys by the 25-hydroxyvitamin D-1α-hydroxylase (1-OHase) to its biologically active form 1,25(OH)₂D₃. Renal synthesis of 1,25(OH)₂D₃ is regulated by plasma parathyroid hormone (PTH), together with serum calcium and negative feedback.
It has also been recognized that many other tissues in the body, including macrophages, brain, colon, prostate, mammary gland, osteoblasts, keratinocytes and others, have the enzymatic machinery to locally produce 1,25(OH)$_2$D$_3$ (Cross et al. 2001, Mawer et al. 1994, Schwartz et al. 1998, Townsend et al. 2005, Tangpricha et al. 2001). Interestingly extra-renal 1α–hydroxylase acts largely independent from confirmed regulatory mechanisms such as serum calcium, PTH or negative feedback (Peterlik, Cross 2005).

The discussion about optimal levels of 25-hydroxyvitamin D as measured in serum is still ongoing. Most experts define a blood level of 25(OH)D <20ng/ml to be vitamin D deficiency, whereas a level from 21 to 29 ng/ml is considered insufficient, and in order to maximize vitamin D´s effect for health, 25(OH)D should be >30 ng/ml (Thomas et al. 1998, Malabanan, Veronikis & Holick 1998, Holick 2006, Bischoff-Ferrari et al. 2006).

### 5.5.2 Effects of vitamin D

Vitamin D is mainly known for its effects on calcium and bone metabolism (Holick 1994, Bouillon et al. 2008). Severe vitamin D deficiency causes a lack of bone mineralization, which manifests as rickets in children and osteomalacia in adults (DeLuca 2004, Holick 1994, Holick 2003). Since vitamin D was discovered in the beginning of the 20$^{th}$ century it has been related to a wide variety of health outcomes, including depression (Hoogendijk et al. 2008, Milaneschi et al. 2010), cancer (Yin et al. 2011, Yin et al. 2010, Yin et al. 2009), asthma (Sutherland et al. 2010) and cardiovascular diseases (Wang et al. 2008, Lavie, Lee & Milani 2011). Additionally, there is evidence that some autoimmune disease, such as multiple sclerosis (Munger et al. 2004, van der Mei et al. 2003), type 1 diabetes (Mathieu, Badenhoop 2005), rheumatoid arthritis (Rossini et al. 2010) and Crohn’s disease (Sentongo et al. 2002) are often caused by a lack of vitamin D.
5.5.3 Vitamin D and cardiovascular risk factors

In the following the author will briefly summarize evidence on the effect of vitamin D status on cardiovascular risk factors.

*Parathyroid hormone* (PTH) levels are inversely correlated with 25(OH)D concentrations. Epidemiological studies showed that elevated and high-normal PTH levels are associated with an increased risk of cardiovascular events and mortality (Pilz et al. 2010b). PTH exerts various effects on the heart including myocardial hypertrophy and pro-arrhythmic actions and increases blood pressure (Fitzpatrick, Bilezikian & Silverberg 2008). Therefore PTH suppression by vitamin D supplementation might reduce cardiovascular risk.

*Arterial hypertension* has been established as one of the most important cardiovascular risk factors for stroke, myocardial infarction, peripheral vascular disease, heart failure, kidney failure or mortality (Messerli, Williams & Ritz 2007). Observational studies have consistently reported cross-sectional associations
between low 25(OH)D levels and elevated blood pressure (Almirall et al. 2010, Schmitz et al. 2009, Scragg, Camargo & Simpson 2010). Witham et al. performed a meta-analysis of eight randomized trials evaluating vitamin D and blood pressure, reporting a statistically significant reduction in diastolic blood pressure, but a non-significant reduction in systolic blood pressure (Witham, Nadir & Struthers 2009). A recent meta-analysis by Pittas et al. did not show an effect of vitamin D supplementation on systolic or diastolic blood pressure compared with placebo (Pittas et al. 2010).

Low 25(OH)D levels are commonly found in patients with both type 1 and type 2 diabetes mellitus (Scragg et al. 1995, Sugden et al. 2008, Joergensen et al. 2010, Cigolini et al. 2006). Several cross-sectional and observational studies reported an association between vitamin D status and the prevalence of type 2 diabetes (Ford et al. 2005, Pittas et al. 2006, Scragg et al. 2004). Further, such associations are not only found in observational studies but low 25(OH)D levels are also associated with a higher probability of future diagnosis of diabetes mellitus in prospective studies (Grimnes et al. 2010, Knekt et al. 2008). There is evidence that vitamin D supplementation might increase pancreatic insulin release and improves insulin resistance (Borissova et al. 2003) and impaired glucose tolerance (Boucher 1998) in patients with T2DM.

**Chronic kidney disease** (CKD) is one of the most powerful predictors of premature cardiovascular disease. Data suggest that the progression of CKD is linked to hypovitaminosis D (Al-Badr, Martin 2008, Jones 2007). Patients with CKD have a high rate of severe vitamin D deficiency that is further aggravated by the reduced ability to convert 25(OH)D vitamin D into the active form 1,25 dihydroxy-vitamin D (Al-Badr, Martin 2008, Jones 2007).

**Dyslipidemia** is an important predictor of cardiovascular disease. A recent systematic review by Zittermann et al. did not show an effect of vitamin D on serum cholesterol levels. However there is evidence for a triglyceride-lowering effect of vitamin D which primarily comes from studies with chronic kidney disease patients, a group with elevated triglyceride levels (Zittermann, Gummert & Borgermann 2011). Currently data from interventional trials were not able to
clearly demonstrate consistent effects of vitamin D on plasma lipids (Zittermann et al. 2009, Jorde, Grimnes 2011, Rajpathak et al. 2010). A number of observational trials have shown a robust association between deficient vitamin D levels and cardiovascular risk, but further interventional studies are urgently needed to determine whether vitamin D supplementation does lower cardiovascular risk (Wang et al. 2008, Dobnig et al. 2008, Kendrick et al. 2009, Pilz et al. 2008).

*Endothelial dysfunction* is an early step in the development of atherosclerosis and is a well-established common pathway of cardiovascular risk factors (Schachinger, Britten & Zeiher 2000, Suwaidi et al. 2000). It is associated with the risk for future cardiovascular events (Akcakoyun et al. 2008, Davignon, Ganz 2004, Yeboah et al. 2007). Observational and interventional studies have shown associations between low circulating levels of vitamin D and endothelial dysfunction (Al Mheid et al. 2011, Jablonski et al. 2011, Tarcin et al. 2009). Sugden et al. showed in a placebo-controlled, randomized trial that a single large dose of vitamin D improves endothelial function in patients with type 2 diabetes mellitus and vitamin D insufficiency (Sugden et al. 2008).

### 5.5.4 Vitamin D and cardiovascular disease (CVD)

In 1981, Robert Scragg and colleagues raised the hypothesis that sunlight and vitamin D may protect against cardiovascular disease (Scragg 1981). Over the last three decades, a vast number of epidemiological and observational studies and a rather small number of interventional studies described vitamin D’s effects on the cardiovascular system, but we still cannot distinctly confirm or reject the hypothesis formulated three decades ago.

Cardiovascular disease is globally the number one cause of death. CVD includes various illnesses such as coronary heart disease (CHD), cerebrovascular disease such as stroke, peripheral arterial disease, and congestive heart failure.

In summary, data from observational studies suggest that CVD is associated with vitamin D deficiency. Numerous epidemiological trials have reported reduced 25(OH)D levels in patients with previous and prevalent cardiovascular diseases (Parker et al. 2010, Pilz et al. 2010a, Pilz et al. 2011b, Brewer, Michos & Reis 2011). Furthermore recent studies have reported that baseline blood levels of 25(OH)D predict subsequent risk of cardiovascular disease (Wang et al. 2008,
Dobnig et al. 2008, Giovannucci et al. 2008). In contrast to the above mentioned findings Hsia et al. could not detect a statistically significant association between vitamin D/calcium and cardiac and cerebrovascular risk, respectively (Hsia et al. 2007). Elamin and colleagues summarized the results from six RCT assessing the effect of vitamin D supplementation on risk of myocardial infarction (Elamin et al. 2011). The pooled relative risk was 1.02 (95% CI 0.93 to 1.13), indicating no effect. However, all these data mainly gained in cross-sectional investigations or prospective association studies have to be proven in prospective, randomised controlled trials. Interventional data from the past are extremely rare, and these studies were done with inadequate low levels of vitamin D supplementation.

5.5.5 Vitamin D and mortality


In patients with T2DM, severe vitamin D deficiency predicts increased risk of all-cause mortality and cardiovascular mortality, independent of urinary albumin excretion rate and conventional cardiovascular risk factors (Joergensen et al. 2010).

Autier and colleagues published a meta-analysis of randomized controlled trials of vitamin D supplementation found that vitamin D reduced all-cause mortality by 7% [relative risk, 0.93; 95% CI, 0.87 – 0.99] (Autier, Gandini 2007). Contrary, four subsequent meta-analyses failed to show a statistically significant impact of vitamin D alone on mortality (Bjelakovic et al. 2011, Chung et al. 2009, Avenell et al. 2009, Rejnmark et al. 2012), while three of these four studies did show a reduced mortality if vitamin D was combined with calcium (Bjelakovic et al. 2011, Avenell et al. 2009, Rejnmark et al. 2012).

Recently, Elamin et al. reported in a meta-analysis that they are unable to show a statistically significant reduction in mortality and cardiovascular risk associated with vitamin D. The quality of the available evidence is low to moderate at best (Elamin et al. 2011).
6. Observational study (CARDIONOR)

6.1 Background and aims

Patients with type 2 diabetes face a substantially elevated risk for cardiovascular and cerebrovascular disease (Haffner, Cassells 2003, Janghorbani et al. 2007). This risk is based on traditional and non-traditional cardiovascular risk factors (Stratton et al. 2000, Schachinger, Britten & Zeiher 2000, Kannel 1996, Collins et al. 2003, Chironi et al. 2006, Hill et al. 2003). It is, thus, partially mediated by modifiable conditions for instance blood pressure, cholesterol, or hyperglycemia. Indeed, controlled prospective intervention studies highlighted that individual lowering of those risk factors resulted in a reduction of vascular events such as acute coronary syndromes or stroke (Stratton et al. 2000, Collins et al. 2003, Neal et al. 2000). The STENO2-study, a long term trial among subjects with type 2 diabetes mellitus, evaluating the impact of an intensified multi-targeted risk intervention compared with conventional treatment, demonstrated the superiority of the intensified intervention arm with regard to cardiovascular event prevention and mortality (Gaede et al. 2003).

Risk normalization for type 2 diabetic patients was not reached in the STENO-2 trial. In addition, it is, up to now, not possible to predict at early treatment stages who was to suffer progressive atherosclerosis and a future event despite intensive treatment of risk factors. Such early identification of treatment non-responders could enable to further intensify treatment or to develop new treatment strategies. The aim of our analysis is to evaluate the effect of an intensified, targeted, multifactorial intervention comprising behavior modification and pharmacologic therapy on potential novel as well as established cardiovascular surrogate parameters.

Whilst we have investigated the impact of the multifactorial intervention on intima media thickness (IMT) in the whole population, we have assessed 3 novel cardiovascular surrogate measurements (arginine bioavailability ratios, the reactive hyperaemia index (RHI) and endothelial progenitor cells) in subsets of the population, mainly due to limited funding resources. Reduced nitric oxide bioavailability is the underlying cause of endothelial dysfunction, which has been shown to be associated with increased cardiovascular risk (Halcox et al. 2002). L-
arginine is the only substrate for nitric oxide (NO) synthesis in vascular endothelial cells and is converted in a multistep reaction by nitric oxide synthetase to NO and L-citrulline. The second enzyme metabolizing L-arginine is arginase which converts the amino acid to ornithine (Morris et al. 2005). Arginase has been shown to be up-regulated in patients with diabetes (Morris et al. 2005, Romero et al. 2008). Recently the global arginine bioavailability ratio (GABR), L-arginine divided by L-ornithine and L-citrulline, has been introduced as a potential new cardiovascular surrogate parameter that is in particular reduced in subjects with diabetes (Sourij et al. 2011, Tang et al. 2009). This new ratio was suggested in order to overcome the poor prognostic value of L-arginine levels alone.

The mobilization of endothelial progenitor cells (EPCs) from the bone marrow into the circulation has been reported after tissue ischemia or endothelial damage (Asahara et al. 1997, Kawamoto et al. 2001, Rafii, Lyden 2003). EPCs can be defined by expression of surface markers, such as CD34, CD133 and VEGFR-2 (Peichev et al. 2000, Gehling et al. 2000) and they are involved in the rejuvenation of the endothelium (Asahara et al. 1997, Shi et al. 1998). Hill and colleagues reported that there is an association between dysfunction and decreased number of EPCs and cardiovascular risk factors (Hill et al. 2003). In addition, there is evidence that there is an inverse association between the number of EPCs and hypertension (Imanishi et al. 2005), family history of coronary artery disease (George et al. 2003), diabetes (Jung et al. 2010) and hypercholesterinaemia (Chen et al. 2004).

Our third surrogate measurement is RHI (reactive hyperaemia index) used to non-invasively measure endothelial function (Bonetti et al. 2004, Kuvin et al. 2003, Philippova et al. 2011, Kuvin et al. 2001). Previous studies have shown that RHI correlates with biomarkers of atherosclerosis (Lieb et al. 2009), presence of cardiovascular risk factors (Mitchell et al. 2005, Hamburg et al. 2008), presence of coronary artery disease (Toggweiler et al. 2010, Neunteufl et al. 1997) and occurrence of cardiovascular events (Rubinshtein et al. 2010). RHI is an independent predictor if coronary atherosclerosis (Reinhard et al. 2011). Several studies have demonstrated an improvement in RHI as a result of lifestyle modification (Schroeter et al. 2006, Barringer, Hatcher & Sasser 2011, Fisher et al. 2003) or pharmacological intervention (Aversa et al. 2008). Especially, ACE-inhibitors have beneficial impact on RHI (Iwatsubo et al. 1997, Okuro et al. 2006).
The advantage of the RHI over other measures of endothelial function is that this technology (Endo-PAT 2000) is less operator dependent, can be more rapidly performed and requires less expensive technology (Axtell, Gomari & Cooke 2010).

Finally we have investigated the impact of multifactorial risk intervention on an established CV surrogate, the intima media thickness. We investigate whether multifactorial risk factor intervention for a period of 2 years improves IMT. The intima media thickness of carotid arteries (de Jager et al. 2006) is closely related to atherosclerotic burden in the coronary arteries and the risk for acute vascular events (Bots et al. 1997, Lorenz et al. 2006).

6.2 Methods

6.2.1 Subjects

Patients with type 2 diabetes mellitus without previous vascular events were identified from the outpatient clinic at the Division of Endocrinology and Metabolism at the Medical University of Graz. The present CARDIONOR study (Treatment of Cardiovascular Risk in Patients with Diabetes Mellitus Type-2: Identification of Treatment Non-Responders) was conducted according to the guidelines laid out in the Declaration of Helsinki as revised in 1996 and all procedures were approved by the Ethical Committee of the Medical University of Graz (18-143 ex 06/07). Written informed consent was obtained from all subjects. 45 to 75 year old patients with type 2 diabetes mellitus with two of the following criteria will included: LDL-cholesterol > 120mg/dl, blood pressure > 140/90 mmHg (either or), HbA1c > 7.5% (all listed parameters could be treated or untreated). Diabetes mellitus was defined as fasting blood glucose of ≥ 126 mg/dl or a history of established diabetes according to WHO criteria (WHO/IDF 2006). Patients with a history of any cardiovascular events, heart failure (>NYHA II), serum creatinine > 265.2 µmol/L, aspartat-aminotransferase/alanine-aminotransferase > 3-fold ULN of the reference range and major psychiatric disorders were excluded.
6.2.2 Study design

We performed a single-center, prospective, open 2 years clinical trial. At baseline, after 3 months and after 2 years patients were seen in the outpatients clinic of the department of endocrinology and metabolism for a detailed examination. Investigations performed on the respective study visits are shown in table 4. Baseline determination of risk factors and measurements of carotid artery intima media thickness was performed in the morning after an overnight fast. Thereafter all patients were treated by a physician specialised in diabetes and a diabetes nurse. After 3 months all modifiable or potentially variable risk factors were evaluated again. 24 months after inclusion into the CARDIONOR-study carotid artery IMT as the primary outcome was measured again.

Standard anthropometric data (height, weight, waist circumference) were obtained from each subject. Blood pressure was measured after subjects with metabolic syndrome have been seated for at least 5 minutes with an automated sphygmomanometer Boso Medicus Uno (Bosch & Sohn GmbH, Juningen, Germany). The body mass index (BMI) was calculated as the weight (kilograms) divided by the square of height (meters). Waist circumference was measured in a standing position midway between the lower costal margin and the iliac crest.

Table 4 Time schedule of study visits

<table>
<thead>
<tr>
<th>Months</th>
<th>0</th>
<th>3</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit no.</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Inclusion/exclusion criteria</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demographics /relevant medical history/current medical conditions</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Prior concomitant meds/therapies</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Physical examination/blood pressure, heart rate</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Body weight/ height</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>SF-36 Health questionnaire</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>EQ-5D questionnaire</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Blood count, liver function tests, renal function tests, lipids,</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Patients received a target oriented treatment of risk factors according to current national treatment guidelines (available at http://www.oedg.org). All patients received comprehensive lifestyle counselling, personalized nutrition and exercise advice.

### 6.2.3 Interventions

#### 6.2.3.1 Blood glucose

$\text{Hb}_{\text{A1c}}$ of $<6.5\%$ was the treatment goal. Patients were treated according to current guidelines of the Austrian Diabetes Association (http://www.oedg.org) and local reimbursement practice, maximizing the “non-insulinotropic” treatment modalities. Thus the first line therapy was metformin followed by a glitazone followed by addition of a third oral antidiabetic drug. If not sufficient insulin therapy was added. If $\text{Hb}_{\text{A1c}}$ was $>8.0\%$ after lifestyle counselling and 0 to 1 oral drugs were used, intensification was performed by use of two additional treatments.

#### 6.2.3.2 Lipids

A LDL cholesterol $<100$ mg/dl was the treatment goal. Patients received statins according to current national diabetes guidelines and reimbursement practice. Statin therapy was intensified or expanded if indicated by failure to reach LDL goal.
6.2.3.3 Blood pressure
A blood pressure of 130/80 mmHg (office reading) was the treatment goal. Patients received treatment according to current national diabetes guidelines and reimbursement practice. First line therapy was inhibition of the renin-angiotensin-system. Diuretics and calcium-channel-blockers were used for additive treatment. Beta-blockers and centrally acting drugs were used as third line drugs.

6.2.4 Assessments

6.2.4.1 Carotid IMT (cIMT) and plaque
Intima media thickness and plaque were measured by using high resolution portable ultrasound Acuson Cypress (Siemens Medical Solutions USA Inc., Moutainview, California). The common carotid artery, carotid bulb, and internal as well as external arteries were examined. CIMT was measured in the common carotid artery in a segment of 2cm below the bulb and considered not to contain any plaque. Subjects fasted the previous night for at least 12 hours and were kept in the supine position in a quiet and shadowed room throughout the study. Fifteen minutes after maintaining the prone position carotid IMT was measured. Far wall measurements of both sides were combined to calculate mean cIMT (Schmoelzer et al. 2003). Carotid atherosclerotic lesions were determined from scans of both, right and left carotid artery segments. Plaque burden was quantified using B-score according to ACAPS (asymptomatic carotid artery plaque study) protocol (Anonymous1992).

6.2.4.2 Reactive hyperemia index
Endothelial function was tested functionally and biochemically. Functionally the Endo-PAT 2000 (Itamar Medical Ltd., Caesarea, Israel) was used to measure endothelium-dependent vaso-reactivity (Bonetti et al. 2004) and is emerging as a useful method for assessing vascular function (Kuvin et al. 2003). Before measurements, the subjects were in supine position for a minimum of 10 minutes, in a quiet, temperature controlled room with dimmed lights. During the measurement the subject lie in a bed with their hands at the level of their heart and fingers hanging freely. Probes were placed on both index fingers and pulse wave amplitudes were detected and recorded during the study. According to previous
studies (Bonetti et al. 2004, Bonetti, Lerman & Lerman 2003), after a five-minute baseline measurement, arterial flow was occluded using a cuff on the non-dominant arm. The cuff was inflated to 60 mmHg above systolic pressure. After five minutes of occlusion, the cuff was rapidly deflated to allow reactive hyperemia. Pulse wave amplitudes were recorded again for at least five minutes. The ratio of the PAT signal after cuff release compared with baseline was calculated through a computer algorithm automatically normalizing for baseline signal and indexed to the contra lateral arm. The calculated ratio reflects the reactive hyperemia index (RHI) (Bonetti et al. 2004). Nohira et al. showed that using Endo-PAT the RHI score reflects NO-bioavailability (Nohria et al. 2006). Augmentation index is also automatically calculated as an average from multiple pulses using the formula \( \frac{P_2 - P_1}{P_1} \) (%) where \( P_1 \) is the peak pressure of the recorded pulse wave and \( P_2 \) is the pressure of the inflection point corresponding to the arrival of the reflected waves. Biochemically, an endothelial function z-score was calculated according to the methods used in the Hoorn study (van Hecke et al. 2005).

**6.2.4.3 Glucose tolerance**

A frequently sampled 75-g oral glucose tolerance test was performed in all subjects after a 12-h overnight fast at baseline and after 3 months. Blood samples were collected before and 30, 60 and 120 minutes after the glucose load. All other parameters were quantified using routine laboratory methods.

**6.2.4.4 Amino acids**

Arginine, ornithine and citrulline were measured with modifications of previous described chromatographic methods (Schwarz, Roberts & Pasquali 2005). Briefly, after precipitation of EDTA plasma with perchloric acid following neutralization of the supernatant with sodium carbonate, the extracted amino acids were derivatized with o-phtalaldehyde and separated on a reversed phase column with gradient elution. Quantification was performed with ratios of fluorescence signals of the interesting amino acids to the internal standard norvaline in comparison to the appropriate calibration curves. Intra-assay and inter-assay CVs were below 10%. Amino acid analyses were done at baseline and 3 months after commencing intensified risk factor management. GABR was calculated by L-arginine divided by
(L-ornithine and L-citrulline). AOR was calculated by L-arginine divided by L-ornithine.

6.2.4.5 Progenitor cells
Circulating progenitor cells were quantified by flow cytometry as previously described (Werner et al. 2005).

6.2.5 Risk scores
People with type 2 diabetes are at increased risk for cardiovascular disease. Multivariate risk scores have, therefore, been used in many countries to predict CVD risk in individuals with diabetes. Various scores describe the risk for the general population, but few are specific to people with diabetes.

UKPDS Risk engine
The UKPDS Risk Engine 2.0 is a type 2 diabetes specific risk calculator based on 53,000 patients years of data from the UK Prospective Diabetes Study, which also provides an approximate 'margin of error' for each estimate. The UKPDS risk engine provides risk estimates and 95% confidence intervals, in individuals with type 2 diabetes not known to have heart disease, for:

- non-fatal and fatal coronary heart disease
- fatal coronary heart disease
- non-fatal and fatal stroke
- fatal stroke

These can be calculated for any given duration of type 2 diabetes based on current age, sex, ethnicity, smoking status, presence or absence of atrial fibrillation and levels of HbA1c, systolic blood pressure, total cholesterol and HDL cholesterol (Stevens et al. 2001).

PROCAM Risk Score
The PROCAM-algorithm estimates the risk for acute coronary events (myocardial infarction, sudden cardiac death) within 10 years. The calibrated risk score includes: age, LDL cholesterol, smoking, HDL cholesterol, systolic blood pressure, family history of premature myocardial infarction, diabetes mellitus, and triglycerides (Assmann, Cullen & Schulte 2002). A score below 10% is considered low, 10-20% intermediate, and >20% high 10-year risk of coronary events. The
PROCAM cohort of German men was used to develop a risk prediction model for hard coronary heart disease.

**Framingham Risk score**

The Framingham risk score is a multivariable risk function that predicts 10-year risk of developing cardiovascular disease events (coronary heart disease, stroke, peripheral artery disease or heart failure). The sex-specific scores incorporate age, total and HDL-cholesterol, systolic blood pressure, treatment for hypertension, smoking, and diabetic status.

A score below 10% is considered low, 10-20% intermediate, and >20% high 10-year risk of cardiovascular events (D'Agostino RB et al. 2008).

**Figure 5 Risk scores (overview)**

<table>
<thead>
<tr>
<th>Study</th>
<th>UKPDS Risk engine 2.0</th>
<th>PROCAM Risk Score</th>
<th>Framingham Risk Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Age</td>
<td>35-65</td>
<td>30-74</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Blood pressure systolic</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Treatment for hypertension</td>
<td></td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td></td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history of premature myocardial infarction</td>
<td>√</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td></td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>HbA1c</td>
<td></td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td></td>
<td></td>
<td>√</td>
</tr>
</tbody>
</table>
Van der Heijden et al. reported that the use of the Framingham function to predict the first CHD event is likely to overestimate individual’s absolute CHD risk (van der Heijden et al. 2009) in a general, pre-diabetic and diabetic population during 10 years of follow-up. In a stable chest pain population the ability of the Framingham Score to predict for CHD was similar and significantly better compared to the PROCAM score (Versteylen et al. 2011).

6.2.6 Statistical analyses
The distribution of the continuous variables was evaluated by a Kolmogorov-Smirnov test. Data are expressed as means ± standard deviation when normally distributed and as median (interquartile range) if non-normally distributed. The paired student’s t-test or the Wilcoxon signed-rank test were used for the before and after multifactorial treatment measurements, as appropriate. A p-value of less than 0.05 was considered as statistically significant. All statistical analyses were performed by using SPSS 19.0 software (SPSS Inc, Chicago, Ill).
Mann-Whitney-U test was performed to compare global arginine bioavailability ratio and arginine to ornithine ratio at baseline and 3 months thereafter. Chi square test was used to compare categorical variables. Correlations were made using a Pearson’s product moment correlation coefficient.

6.3 Results

6.3.1 Baseline characteristics
111 subjects were screened for the CARDIONOR-study between March 2008 and May 2010. 97 patients were assigned to receive multifactorial treatment, 94 of them completed visit 2 after 3 months and 82 patients finished the study after 2 years (15 patients dropped out, figure 6). Baseline characteristics of our study population are shown in table 5.
6.3.2 Risk factors

Systolic blood pressure and diastolic blood pressure significantly improve from baseline to 3 months and from baseline to 2 years (table 8). Further risk factors, such as, HbA1c, total cholesterol, LDL cholesterol and HDL cholesterol also significantly improve from baseline to 3 months and from baseline to 2 years. However, triglycerides significantly improves from baseline to 3 months (p=0.007), but there was no significant improvement from baseline to 2 years (p=0.292). Table 9 summarizes the changes of traditional and non-traditional risk factors from baseline to 3 months and to the visit after 2 years.
Table 5 Baseline Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female/male)</td>
<td>38/59</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60±8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>170±9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>92±16</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>113±9</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>109±12</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>32±5</td>
</tr>
<tr>
<td>Duration of diabetes mellitus (years)</td>
<td>7.7±6.8</td>
</tr>
<tr>
<td>Blood pressure systolic (mmHg)</td>
<td>152±18</td>
</tr>
<tr>
<td>Blood pressure diastolic (mmHg)</td>
<td>90±10</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>192±43</td>
</tr>
<tr>
<td>High density lipoproteins (mg/dL)</td>
<td>44±14</td>
</tr>
<tr>
<td>Low density lipoproteins (mg/dL)</td>
<td>108±42</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>189±102</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>67±12</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.3±1.1</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dL)</td>
<td>179±49</td>
</tr>
<tr>
<td>2 hours blood glucose (mg/dL)</td>
<td>332±90</td>
</tr>
<tr>
<td>C-peptides (ng/mL)</td>
<td>6.0±3.6</td>
</tr>
</tbody>
</table>

### 6.3.3 Risk scores

All calculated risk scores from the UKPDS risk engine 2.0 (table 6) significantly improve from Baseline to 3 months, but a significant rise can be reported from 3 months to 2 years. Nevertheless the risk for non-fatal coronary heart disease and fatal coronary heart disease significantly improves from baseline to 2 years. On the contrary, the risk for non-fatal stroke and fatal stroke significantly rises from baseline to 2 years (p<0.001).
### Table 6 UKPDS risk engine

<table>
<thead>
<tr>
<th>UKPDS risk engine</th>
<th>Baseline</th>
<th>3 months</th>
<th>2 years</th>
<th>p-value (Baseline-3 months)</th>
<th>p-value (Baseline-2 years)</th>
<th>p-value (3 months-2 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fatal coronary heart disease (%)</td>
<td>20.7 (13.0 – 32.4)</td>
<td>11.8 (7.5 – 17.4)</td>
<td>19.3 (12.3 – 29.4)</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fatal coronary heart (%)</td>
<td>13.3 (8.2 – 23.0)</td>
<td>6.5 (4.9 – 11.4)</td>
<td>12.5 (7.9 – 22.3)</td>
<td>&lt;0.001</td>
<td>0.016</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-fatal stroke (%)</td>
<td>8.1 (4.4 – 15.7)</td>
<td>6.9 (3.3 – 11.9)</td>
<td>10.2 (5.7 – 18.5)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fatal stroke (%)</td>
<td>1.4 (0.7 – 2.9)</td>
<td>0.9 (0.4 – 1.6)</td>
<td>1.5 (0.9 – 2.6)</td>
<td>&lt;0.001</td>
<td>0.026</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as medians (interquartile ranges).

All of the Framingham risk scores (please see appendix, table 23) significantly improve from Baseline visit to visit after 3 months. However, a significant regain in the risk for coronary heart disease (p=0.020) and myocardial infarction (p=0.024) from 3 months to 2 years can be determined.

The PROCAM Score in our CARDIONOR study significantly improves from baseline to 3 months (p<0.001), whereas a significant raise can be observed from 3 months to the study visit after 2 years (p<0.001).

### Table 7 PROCAM Risk score

<table>
<thead>
<tr>
<th>PROCAM Risk score</th>
<th>Baseline</th>
<th>3 months</th>
<th>2 years</th>
<th>p-value (Baseline-3 months)</th>
<th>p-value (Baseline-2 years)</th>
<th>p-value (3 months-2 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial infarction (%)</td>
<td>17.1 (9.5 – 28.0)</td>
<td>8.3 (5.6 – 15.4)</td>
<td>12.9 (8.3 – 18.1)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as medians (interquartile ranges).
6.3.4 Progenitor cells

No significant changes in the number of CD34+CD133+VEGFR2 could be observed from Baseline to 3 months [15.0 (7.0-27.8) vs. 16.5 (9.0 – 32.0) at 3 months; p=0.164]. Further, no significant associations with changes in cardiovascular risk factor (HbA1c, systolic blood pressure, diastolic blood pressure, HDL cholesterol, LDL cholesterol) from baseline to 3 months could be demonstrated. No significant association between CD34+CD133+VEGFR2 and RHI could be shown at baseline. Additionally, no significant association in the changes of the number of CD34+CD133+VEGFR2 and RHI from baseline to 3 months could be observed.

6.3.5 Amino acids

Global arginine bioavailability ratio was measured in a subset of 41 patients (25 men/16 women) with a mean age of 60±10 years. Baseline characteristics of these patients and treatment effects are shown in table 9. A significant improvement in HbA1c, blood pressure and lipid measurements was achieved. Intensified risk factor management significantly improved GABR (0.33±0.12 at baseline vs. 0.38±0.14 after 3 months; p=0.018) (figure 7) as well as AOR (0.39±0.15 at baseline vs. 0.46±0.19 after 3 months; p=0.039) (figure 8). However, a significant improvement was only seen in patients with short diabetes duration of up to 5 years (p=0.039) whereas in patients with longer diabetes duration (more than 5 years) improvement did not reach statistical significance.
Figure 7 Changes in GABR from Baseline to 3 months

Figure 8 Changes in AOR from Baseline to 3 months
<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline (n=41)</th>
<th>12 Weeks (n=41)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>25/16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>60±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>8±7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>170±9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>93±13</td>
<td>94±13</td>
<td>0.907</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>32.4±4.7</td>
<td>32.5±4.4</td>
<td>0.979</td>
</tr>
<tr>
<td>Blood pressure systolic (mmHg)</td>
<td>152±18</td>
<td>134±10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood pressure diastolic (mmHg)</td>
<td>90±9</td>
<td>78±7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Biochemical parameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting serum total cholesterol (mmol/L)</td>
<td>4.9±1.0</td>
<td>4.3±1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting serum triglycerides (mmol/L)</td>
<td>2.1±1.2</td>
<td>1.8±1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting serum HDL cholesterol (mmol/L)</td>
<td>1.2±0.4</td>
<td>1.3±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting serum LDL cholesterol (mmol/L)</td>
<td>2.7±1.1</td>
<td>2.0±1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c mmol/mol (%)</td>
<td>67±10 (8.3±1.2)</td>
<td>52±8 (6.9±1.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>9.8±2.4</td>
<td>7.2±1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GABR</td>
<td>0.33±0.12</td>
<td>0.38±0.14</td>
<td>0.018</td>
</tr>
<tr>
<td>AOR</td>
<td>0.39±0.15</td>
<td>0.46±0.19</td>
<td>0.039</td>
</tr>
<tr>
<td>Glucose-lowering treatment (no. of patients)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>18</td>
<td>32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sulfonylurea</td>
<td>10</td>
<td>16</td>
<td>0.032</td>
</tr>
<tr>
<td>Acarbose</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Dipeptidyl-peptidase 4 inhibitors</td>
<td>1</td>
<td>3</td>
<td>0.160</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>7</td>
<td>19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin</td>
<td>4</td>
<td>8</td>
<td>0.042</td>
</tr>
<tr>
<td>Other oral antidiabetics</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive treatment (no. of patients)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin II-receptor antagonist</td>
<td>5</td>
<td>8</td>
<td>0.183</td>
</tr>
</tbody>
</table>
The change of GABR ($r=-0.381; p=0.014$) as well as the change of AOR ($r=-0.398; p=0.010$), were inversely correlated with cIMT. In patients reaching at least 3 treatment targets after 3 months intensified therapy, a significant improvement in global arginine bioavailability ratio ($0.33\pm0.11$ at baseline vs. $0.40\pm0.11$ at 3 months; $p=0.031$) as well as arginine to ornithine ratio ($0.39\pm0.12$ at baseline vs. $0.48\pm0.14$ at 3 months; $p=0.050$) was observed while no significant change was observed in those reaching 2 or less treatment targets.

### 6.3.6 Surrogate measurements

**Reactive hyperaemia index**

RHI was calculated from the ratio of the digital pulse volume during reactive hyperemia at baseline in 97 patients and after 3 months in 94 patients (table 10). Multifactorial risk factor intervention reduces RHI from baseline to 3 months not significantly ($p=0.072$). However, there were no significant associations between ∆RHI and cardiovascular risk factors.

**Intima media thickness**

The mean carotid IMT significantly reduced from baseline to 2 year ($0.88\pm0.12$ mm vs. $0.86\pm0.13$ mm; $p=0.021$).

Mean cIMT correlates at baseline significantly with age ($r=0.365; p<0.001$), and duration of diabetes ($r=0.273; p=0.007$) as well as systolic blood pressure ($r=0.320; p=0.001$). Complementary, mean cIMT correlates significantly with
calculated cardiovascular risk scores (table 9). Changes from baseline to 2 year correlates significantly with changes in triglycerides ($r=0.292; p=0.012$).

<table>
<thead>
<tr>
<th>UKPDS risk engine</th>
<th>Non-fatal CHD</th>
<th>Fatal coronary heart</th>
<th>Non-fatal stroke</th>
<th>Fatal stroke</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cIMT</td>
<td>$r=0.417$</td>
<td>$r=0.461$</td>
<td>$r=0.503$</td>
<td>$r=0.504$</td>
</tr>
<tr>
<td></td>
<td>p$&lt;0.001$</td>
<td>p$&lt;0.001$</td>
<td>p$&lt;0.001$</td>
<td>p$&lt;0.001$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Framingham</th>
<th>Coronary Heart Disease</th>
<th>Myocardial Infarction</th>
<th>Stroke</th>
<th>Cardiovascular Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cIMT</td>
<td>$r=0.290$</td>
<td>$r=0.222$</td>
<td>$r=0.476$</td>
<td>$r=0.381$</td>
</tr>
<tr>
<td></td>
<td>p$=0.004$</td>
<td>p$=0.029$</td>
<td>p$&lt;0.001$</td>
<td>p$&lt;0.001$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PROCAM</th>
<th>Myocardial infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cIMT</td>
<td>$r=0.377$</td>
</tr>
</tbody>
</table>
Table 10 Traditional and non-traditional risk factors

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>3 months</th>
<th>2 years</th>
<th>p-value (baseline-3 months)</th>
<th>p-value (3 months -2 year)</th>
<th>p-value (baseline-2 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>traditional risk factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>191±43</td>
<td>166±43</td>
<td>176±50</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>0.007</td>
</tr>
<tr>
<td>Low density lipoproteins (mg/dL)</td>
<td>108±43</td>
<td>76±39</td>
<td>93±43</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.008</td>
</tr>
<tr>
<td>High density lipoproteins (mg/dL)</td>
<td>44±14</td>
<td>49±13</td>
<td>48±14</td>
<td>&lt;0.001</td>
<td>0.704</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>186±89</td>
<td>157±105</td>
<td>175±100</td>
<td>0.007</td>
<td>0.192</td>
<td>0.292</td>
</tr>
<tr>
<td>ApoA1 (mg/dL)</td>
<td>143±22</td>
<td>81±25</td>
<td>-</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ApoB (mg/dL)</td>
<td>94±23</td>
<td>146±23</td>
<td>-</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blood pressure systolic (mmHg)</td>
<td>154±17</td>
<td>135±12</td>
<td>139±16</td>
<td>&lt;0.001</td>
<td>0.053</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood pressure diastolic (mmHg)</td>
<td>90±9</td>
<td>82±8</td>
<td>86±16</td>
<td>&lt;0.001</td>
<td>0.025</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.0±0.3</td>
<td>1.0±0.2</td>
<td>-</td>
<td>0.903</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Value</td>
<td>Value</td>
<td>p-value</td>
<td>Value</td>
<td>Value</td>
<td>Value</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>---------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td>109±361</td>
<td>108±440</td>
<td>-</td>
<td>0.893</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.2±1.1</td>
<td>7.2±0.9</td>
<td>7.7±1.1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>66±12</td>
<td>54±14</td>
<td>61±12</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Fasting blood insulin (mg/dL)</td>
<td>11.4±9.7</td>
<td>10.0±8.3</td>
<td>-</td>
<td>0.159</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dL)</td>
<td>179±49</td>
<td>146±42</td>
<td>-</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>5.1±4.3</td>
<td>3.9±4.1</td>
<td>-</td>
<td>0.015</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.8±4.7</td>
<td>31.4±5.3</td>
<td>31.2±4.6</td>
<td>0.465</td>
<td>0.891</td>
<td>0.807</td>
</tr>
<tr>
<td>non traditional risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cIMT (mm)</td>
<td>0.88±0.12</td>
<td>-</td>
<td>0.86±0.13</td>
<td>-</td>
<td>-</td>
<td>0.021</td>
</tr>
<tr>
<td>Mean B-score</td>
<td>1.5 (0.5 – 2.5)</td>
<td>-</td>
<td>2 (1 – 3)</td>
<td>-</td>
<td>-</td>
<td>0.287</td>
</tr>
<tr>
<td>Reactive hyperaemia Index</td>
<td>1.70 (1.46 – 2.08)</td>
<td>1.59 (1.38 – 1.94)</td>
<td>-</td>
<td>0.072</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stiffness Index</td>
<td>14 (8 – 24)</td>
<td>14 (7 – 28)</td>
<td>-</td>
<td>0.899</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD34+CD133+VEG FR2</td>
<td>15.0 (7.0 – 27.8)</td>
<td>16.5 (9.0-32.0)</td>
<td>-</td>
<td>0.164</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### 6.3.7 Medication

Table 11 displays the changes in medication from baseline to 3 months and from baseline to 2 years.

**Table 11 Glucose lowering treatment and antihypertensive treatment**

<table>
<thead>
<tr>
<th></th>
<th>Baseline (97 patients)</th>
<th>3 months (94 patients)</th>
<th>2 years (82 patients)</th>
<th>p-value Day0-3mon</th>
<th>p-value Day0-2 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biguanides (%)</td>
<td>61.9</td>
<td>76.6</td>
<td>67.1</td>
<td>0.005</td>
<td>0.655</td>
</tr>
<tr>
<td>Acarbose (%)</td>
<td>3.1</td>
<td>3.2</td>
<td>1.2</td>
<td>1.000</td>
<td>0.317</td>
</tr>
<tr>
<td>Sulfanylureas (%)</td>
<td>24.7</td>
<td>29.8</td>
<td>19.5</td>
<td>0.157</td>
<td>0.285</td>
</tr>
<tr>
<td>Glitazones (%)</td>
<td>11.3</td>
<td>26.6</td>
<td>19.5</td>
<td>&lt;0.001</td>
<td>0.058</td>
</tr>
<tr>
<td>DPP-IV Inhibitors (%)</td>
<td>9.3</td>
<td>20.2</td>
<td>26.8</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>Insulin (%)</td>
<td>26.8</td>
<td>30.8</td>
<td>29.3</td>
<td>0.027</td>
<td>0.105</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>34.0</td>
<td>74.8</td>
<td>68.3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ezetrol (%)</td>
<td>4.1</td>
<td>4.3</td>
<td>1.2</td>
<td>1.000</td>
<td>0.157</td>
</tr>
<tr>
<td>ACE-Inhibitors (%)</td>
<td>46.4</td>
<td>62.8</td>
<td>46.3</td>
<td>&lt;0.001</td>
<td>1.000</td>
</tr>
<tr>
<td>Ca-antagonists (%)</td>
<td>21.6</td>
<td>48.9</td>
<td>46.3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Beta-blockers (%)</td>
<td>34.0</td>
<td>40.4</td>
<td>42.7</td>
<td>0.102</td>
<td>0.109</td>
</tr>
<tr>
<td>AT2-blockers (%)</td>
<td>21.6</td>
<td>28.7</td>
<td>29.3</td>
<td>0.034</td>
<td>0.132</td>
</tr>
<tr>
<td>Fibrats (%)</td>
<td>6.2</td>
<td>7.4</td>
<td>4.8</td>
<td>0.564</td>
<td>1.000</td>
</tr>
<tr>
<td>Other oral antidiabetics (%)</td>
<td>3.1</td>
<td>3.2</td>
<td>2.4</td>
<td>1.000</td>
<td>0.564</td>
</tr>
</tbody>
</table>
6.4 Discussion

T2DM is a strong risk factor for cardiovascular disease. Multifactorial interventions, targeting hypertension, hyperglycemia and hyperlipidemia have been shown to be effective in the STENO-2 study, in reducing the risk of non-fatal (Gaede et al. 2003) and fatal CVD (Gaede et al. 2008).

A subgroup analysis of 41 patients of our CARDIONOR cohort demonstrates that intensified risk factor intervention in patients with type 2 diabetes improves global arginine bioavailability ratio as well as the arginine to ornithine ratio. In addition both ratios were inversely associated with intima media thickness, another surrogate parameter for cardiovascular outcome. Sourij and colleagues recently reported in patients referred to coronary angiography, that both, global arginine bioavailability ratio and arginine to ornithine ratio are associated with biochemical markers of endothelial dysfunction and both are clearly diminished in subjects with type 2 diabetes mellitus (Sourij et al. 2011). The present study demonstrated that diabetes duration has a major impact on the extent of the benefit seen by multifactorial treatment with the largest effect in the first 5 years after diagnosis. This is also in line with the findings, that the ACCORD trial, including patients with type 2 diabetes with a median diabetes duration of 10 years did not show a beneficial effect of intensive antihyperglycemic therapy in comparison to a less strict “conventional” treatment regimen (Action to Control Cardiovascular Risk in Diabetes Study Group et al. 2008, Gerstein et al. 2000), while the UKPDS trial in newly diagnosed subjects with type 2 diabetes showed a superiority of an intensive glucose lowering treatment regimen on macrovascular and microvascular complications (Holman et al. 2008). Arginine bioavailability ratios were shown to be associated with incident cardiovascular events previously (Sourij et al. 2011, Tang et al. 2009) but our study adds the important finding, that intensified risk factor management improves these ratios. Whilst we investigated the impact of multiple risk factor intervention on the global arginine bioavailability ratios only, future studies need to assess the impact of single risk factor interventions (e.g. hyperglycaemia, blood pressure, LDL-cholesterol only) on these ratios.

However, further studies with a larger sample size in different patient populations need to be performed to clarify whether arginine bioavailability ratios are superior
to currently available biomarkers for cardiovascular risk prediction which will determine the applicability of these parameters in future clinical practice.

In another analysis we could determine that there is no significant change in the number of EPCs after 3 months of multifactorial risk factor intervention. The number of EPCs is a surrogate marker for cardiovascular risk factors and vascular function in healthy people (Hill et al. 2003, Werner et al. 2005, Fadini et al. 2007). Fadini and colleagues showed that low number of EPCs is associated with peripheral vascular disease in patients with type 2 diabetes (Fadini et al. 2005). Further a recent study reported, that type 2 diabetics with satisfactory glycaemic control have an increased number of circulating EPCs (Yue et al. 2011). The depletion of circulating EPC in patients with T2DM may result of a poor mobilization of EPCs from bone marrow, or of a shortened survival time (Churdchomjan et al. 2010).

The present study demonstrated that multifactorial risk factor intervention has no significant impact on the number of CD34+CD133+VEGFR2 in patients with T2DM. Former studies showed that oral hypoglycemic agents and insulin (Yue et al. 2011), and statins (Jaumdally et al. 2010) as well as eplerenone (Jung et al. 2012)(Jung et al. 2010) have a positive effect on endothelial progenitor cells. The results of our study are in line with a previous study that showed that circulating angiogenic cell phenotypes were not associated with measures of vascular function (Cheng et al. 2012).

One limitation of our study is that potentially the time of the intervention is too short. Comparable studies had a stable medication for at least 6 months (Yue et al. 2011). Another limitation is the lack of functional evaluation and characterization of circulating EPCs. In conclusion, this study could not confirm evidence from former studies (Yue et al. 2011, Jaumdally et al. 2010) and presents data that there is an trend towards an increase in EPCs, but the rise does not reach statistical significance ($p=0.164$).

In our third analysis we could demonstrate that a multifactorial risk factor intervention of 3 months not improve RHI ($p=0.072$). Further, we could not report a significant association between HbA1c and RHI, as previously showed by Gargiulo and colleagues (Gargiulo et al. 2011). Previous studies showed that ACE-inhibitors have beneficial impact on RHI (Iwatsubo et al. 1997, Okuro et al. 2006).
However, nearly half of the patients received ACE-inhibitors (46.3%) we could not report beneficial effect on RHI in our study population.

Finally, we could demonstrate that intensified risk factor intervention in patients with type 2 diabetes significantly improves mean cIMT (p=0.021) from baseline to 2 years. IMT increases progressively over time, influenced by age and cardiovascular risk factors (Mackinnon et al. 2004). A high progression rate of IMT is indicative for future vascular events (Lorenz et al. 2006) and treatment of risk factors reduces progression of IMT (Crouse et al. 2007, Hanefeld et al. 2004).

Consequently, IMT is one of the best validated surrogate endpoint for estimating future risk (Crouse 2006) and correlate with risk of future cardiovascular events (Hodis et al. 1998). Statins, glitazones and calcium channel antagonists have been shown to have a positive effect on IMT. The finding in our study is in line with several large studies: In the PLAC-2 study, a randomized, placebo-controlled study, a significant reduction in common carotid IMT was observed in the pravastatin group (Crouse et al. 1995). In the ACAPS-study, a double-blind, randomized trial, showed regression of mean maximum IMT after lovastatin treatment (Furberg et al. 1994). Minamikawa and colleagues reported that troglitazone improves cIMT after six months of treatment (Minamikawa et al. 1998). A comparison of pioglitazone with glimepiride showed a reduction in cIMT at 24 weeks (Langenfeld et al. 2005). Further, several trials showed a positive effect of treatment with ca-antagonists on IMT (Zanchetti et al. 1998, Simon et al. 2001, Pitt et al. 2000), too. All above mentioned trials investigated the association between a single cardiovascular risk factor (IMT) and a single treatment. However, our data show, for the first time to our knowledge, that multifactorial risk factor intervention significantly improves mean cIMT although in increase due to aging would be expected. Several trials showed that increased cIMT is associated with known cardiovascular risk factors (Brohall, Oden & Fagerberg 2006, Davis et al. 2001, Shah et al. 2009). That is in line with findings in our study, we could demonstrate that intima media thickness is significantly associated with age of patients (p=0.012), duration of diabetes (p<0.001) as well as systolic blood pressure (p=0.001). In contrast to our finding, Geroulakos (Geroulakos et al. 1994b) and Kanters (Kanters, Algra & Banga 1997) could not find significant associations between these risk factors. Additionally, in our study, IMT significantly correlates with cardiovascular risk scores (Framingham, UKPDs risk engine and
PROCAM risk score). Touboul and colleagues reported a significant correlation between Framingham risk score and IMT (Touboul et al. 2007), whereas Yanase and colleagues could present significant associations between IMT and Framingham risk score as well as PROCAM risk score (Touboul et al. 2007, Yanase et al. 2006).

Interestingly, we observed a significant rise from baseline to 2 year (p<0.001) in the risk of fatal and non-fatal stroke, even though a significant improvement of systolic blood pressure, HDL-cholesterol, HbA1c and total cholesterol could be reported in this period of time. The crucial factor for this seems to be the simultaneous increase in the patients’ age and diabetes duration.
7 Interventional Study (Lactobacillus casei Shirota)

7.1 Background and aims

The probiotics definition has been widely debated but in 2002 a FAO/WHO working group (Joint FAO/WHO Working Group 2002) defined probiotics as: “live microorganisms which when administered in adequate amounts confer a health benefit on the host.”

Probiotics, especially *bifidobacteria* and *lactobacilli* have been suggested to be associated with alleviation of lactose tolerance (Levri et al. 2005), antimutagenic effects (Chalova et al. 2008), anticarcinogenic effects (Liong 2008), prevention and cure of viral, bacterial and antibiotic diarrhoeas (Parvez et al. 2006, Guandalini 2006, Guandalini 2011), immunomodulation (Forsythe, Bienenstock 2010, Van Puyenbroeck et al. 2012), and blood cholesterol reduction (Ooi, Liong 2010).

![Figure 9 Effects of probiotics (especially *bifidobacteria* and *lactobacillus*)](image)

*Lactobacillus casei* Shirota is a representative probiotic strain consumed in a fermented milk product that has been produced since 1935 (Yakult 2012) and is today commercially available as a health food in a number of countries.

Obesity and insulin resistance are major risk factors for the development of the metabolic syndrome and type 2 diabetes mellitus, conditions contributing to accelerated atherosclerosis and increased mortality (Prospective Studies Collaboration et al. 2009, Thomas et al. 2007). The pathogenesis of obesity is complex and seen as interplay between individual phenotype and environmental factors.

More recently, altered gut microbiota has been suggested to be involved in the pathogenesis of obesity, since in rodents the ratio of Firmicutes to Bacteroides of the cecum microbiota was found to be significantly higher in obese mice than in
lean counterparts (Ley et al. 2005). This finding of an altered distal gut microbiota was then confirmed and extended to humans (Ley et al. 2006). Additionally, these investigations in humans demonstrated that as obese people lose weight, the composition of microflora shifted, and more closely resembled that of the lean individuals (Ley et al. 2006), overall suggesting that the microflora might be involved in the pathogenesis of obesity.

Furthermore Larsen et al. (Larsen et al. 2010) showed that the intestinal microflora is substantially altered in diabetic compared to healthy individuals and further studies showed an association of gut microflora with insulin resistance (Creely et al. 2007).

Obesity and the metabolic syndrome were shown to be associated with increased circulating markers of oxidative stress, low-grade inflammation and endothelial dysfunction and in turn these parameters were shown to play a key role in atherosclerosis and subsequently cardiovascular complications (Keaney et al. 2003). Bacterial lipopolysaccharide derived from the intestinal microbiota was shown to trigger this inflammatory processes (Cani et al. 2007), and therefore to cause insulin resistance and beta-cell dysfunction (Cani et al. 2007, Cani et al. 2009).

In several murine models, the oral administration of \textit{LcS} improves inflammatory disorders, including inflammatory bowel disease (Matsumoto et al. 2005, Matsumoto et al. 2009), arthritis (Kato, Endo-Tanaka & Yokokura 1998), type 1 diabetes mellitus (Matsuzaki et al. 1997a) and indomethacin-induced small intestinal injury (Watanabe et al. 2009). Matsuzaki et al. (Matsuzaki et al. 1997b) reported that oral administration of heat-killed \textit{LcS} suppresses the elevation of plasma glucose levels in an obese type 2 diabetic murine model (KK-Ay). In obesity endotoxin levels are elevated which leads to a significant increase in pro-inflammatory cytokine production in adipocytes via a toll-like receptor (TLR) mediated pathway, contributing to the pro-inflammatory state in obesity (Ruiz et al. 2007). Consequently, in mice antibiotics treatment is followed by an improvement in inflammation and glucose metabolism (Cani et al. 2008).

Therefore it has been suggested, that changes in gut microbiota by the supplementation of probiotic drinks might alter gut permeability, bacterial lipopolysaccharide load and consequently chronic low-grade inflammation
(Borchers et al. 2009) but interventional data in obese humans are lacking are sparse (Andreasen et al. 2010).

However, despite several mechanistic studies and encouraging results in animals it remains to be determined whether LcS affects insulin resistance resulting from obesity-associated inflammation. The aim of our study was to investigate the effect of LcS supplementation over 12 weeks on glucose tolerance and indices of insulin sensitivity and β-cell function as well as on markers of oxidative stress and inflammation in subjects with metabolic syndrome.

7.2 Methods

7.2.1 Subjects

Thirty five subjects were screened for the study between January and August 2010. Thirty adult patients with metabolic syndrome were identified from the outpatient clinic at the Division of Endocrinology and Metabolism at the Medical University of Graz. All patients gave written informed consent and the study protocol was approved by the Ethics Committee of the Medical University of Graz (20-037 ex 08/09) and performed according to the Declaration of Helsinki. Metabolic syndrome was defined by the National Cholesterol Education Program and the Adult Treatment Panel-III (NCEP-ATP-III) in subjects presenting at least three of the following five criteria: waist circumference ≥102 in men or ≥88 in women; HDL cholesterol <40 mg/dL (men) or <50 mg/dL (women) or drug treatment for low HDL cholesterol; triglycerides ≥150 mg/dL or drug treatment for elevated for high triglycerides; raised blood pressure (systolic >130 mmHg. diastolic >85mmHg) and raised fasting glucose (>100mg/dl) or previously known type 2 diabetes mellitus (Alberti et al. 2009).

Patients were treated with antibiotics within the previous 7 days, with current antihyperglycemic treatment, any immunomodulatory therapy 1 month prior to study entry, with elevated transaminases (>2xULN), concomitant use of supplements (pre-, pro-, or synbiotics), inflammatory bowel disease (Crohn’s disease, ulcerative colitis) or celiac disease or those with clinical signs of infectious diseases were excluded from participation.
7.2.2 Study design

We performed a single-centre, prospective, permuted-block randomized and controlled 12 weeks clinical trial. Patients were randomized to either receive food supplementation with a milk drink containing *Lactobacillus casei* Shirota (3 bottles a day, á 65ml. containing *Lactobacillus casei* Shirota at a concentration of $10^8$/ml, Yakult light®, Yakult Austria, Vienna) for twelve weeks or served as controls without the supplementation of the mild drink. Patients were randomized by using the online-software “randomizer” (Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Austria). Patients were advised to consume no other probiotic supplements during the study period. This was ensured by handing out a checklist containing all commercially available probiotics in Austria to the patients.

7.2.3 Study procedures

Table 12 Time schedule study visits (LcS)

<table>
<thead>
<tr>
<th>Day</th>
<th>Visit no.</th>
<th>-21 to -1</th>
<th>0</th>
<th>28</th>
<th>56</th>
<th>84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inclusion/exclusion criteria</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demographics /relevant medical history/current medical conditions</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior concomitant meds/therapies</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical examination/blood pressure, heart rate, respiratory rate</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight/ height</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food frequency questionnaire</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal ultrasound and Fibroscan</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood count, liver function tests, renal function tests, Lipids, glucose, CRP and hsCRP, glucose, insulin</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Oral glucose tolerance test (OGTT)</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary analysis for albumin and creatinine</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Gut permeability test (Saccharose/Lactulose/Mannitol)</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
At day 0 and at day 84 (12 weeks) patients were seen in the outpatients clinic for a detailed examination. Every two weeks the patients in the treatment group visited the outpatients clinic to receive the milk drink.

Standard anthropometric data (height, weight, waist circumference) were obtained from each subject. Blood pressure was measured after subjects with metabolic syndrome have been seated for at least 5 minutes with an automated sphygmomanometer Boso Medicus Uno (Bosch & Sohn GmbH, Juningen, Germany). The body mass index (BMI) was calculated as the weight (kilograms) divided by the square of height (meters). Waist circumference was measured in a standing position midway between the lower costal margin and the iliac crest.

Moreover, basal blood samples for metabolic (fasting glucose, insulin, HbA₁c [glycated hemoglobin], total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) determinations and inflammation determinations (vWF [von Willebrand factor], IL-6, IL-10, TNF, carbonyl protein, MDA-LDL, ox-LDL, CRP) were collected at 8.00-9.00 after an overnight fast.

Insulin was measured by ELISA (Siemens Healthcare Diagnostics, Eschborn, Germany) with intra- and interassay CV of 4.0 and 2.6%, respectively. Fasting glucose, triglycerides, total cholesterol, HDL cholesterol, and CRP were determined using Modular Analytics SWA (Roche, Basel, Switzerland).

Patients will be managed for their diabetes mellitus type 2 and the metabolic syndrome according to the current national guidelines (http://www.oedg.org/oedg_leitlinien.html). If patients had elevated liver function tests, a complete hepatological workup was performed prior to the study to exclude any underlying liver disease other than steatosis.
7.2.3.1 Insulin sensitivity and Beta-cell function

A frequently sampled 75-g OGTT (oral glucose tolerance test) was performed in all subjects after a 12-h overnight fast at baseline and at study end. Blood samples were collected before and 15, 30, 60, 120 and 180 minutes after the glucose load to determine plasma glucose and serum insulin to calculate the below described indices.

The area under the curve (AUC) for glucose during the OGTT was calculated using the trapezoidal rule. Insulin resistance was expressed by using a number of four different indexes: the Matsuda-Index ($IS_{OGTT}$) (Matsuda, DeFronzo 1999), the HOMA-IR (Homeostasis Model Assessment for Insulin Resistance) (Matthews et al. 1985), the QUICK-Index (Katz et al. 2000) and the insulin sensitivity index (ISI) (Stumvoll et al. 2001).

\[
IS_{OGTT} = \frac{10000}{\sqrt{(\text{glucose0} + \text{insulin0}) \cdot (\text{mean glucose} + \text{mean insulin})}}
\]

\[
\text{HOMA-IR} = \frac{\text{FPG (mmol/l)} \cdot \text{FSI (U/l)}}{22.5}
\]

\[
\text{QUICKI} = \frac{1}{\log(\text{insulin0}) + \log(\text{glucose0})}
\]

\[
\text{ISI} = 0.222 - 0.00333 \cdot \text{BMI} - 0.0000779 \cdot \text{Ins120} - 0.000422 \cdot \text{age}
\]

Beta-cell function was estimated in the fasting state with HOMA-$\beta$ and during the oral glucose tolerance test with the Stumvoll-Index and the ratio of the incremental insulin to glucose response over the first 30min during the OGTT.

\[
\text{HOMA-} \beta = \frac{20 \cdot \text{Insulin0}}{\text{Glucose0} - 3.5}
\]

1st phase = 1283 + 1.829 * Ins30 − 138.7 * Glc30 + 3.772 * Ins0

2nd phase = 286 + 0.416 * Ins30 − 25.94 * Gluc30 + 0.926 * Ins0

$\Delta$ Insulin (30) / $\Delta$ Glucose (30)
Glucose and insulin were measured by routine methods using commercially available kits.

**7.2.3.2 Inflammation and endothelial function**

*Enzyme-linked immunosorbent assays (ELISA) for MDA-LDL and ox-LDL*

Titres of oLaB were measured in serum with a commercial enzyme immunoassay (oLaB, Biomedica, Vienna, Austria) according to the method of Tatzber and Esterbauer (Bellomo 1995). The assay is based on the binding reaction of the 1:50 diluted samples to the previously oxidized LDL (by cupric ions) bound to the microtitre wells. Detection was carried out by binding a secondary, peroxidase-coupled anti-immunoglobulin G (IgG) antibody, which permitted colorimetric detection of this enzyme with tetramethylbenzidine as substrate. The detection of IgG antibodies is accomplished in diluted serum samples. MDA-LDL-IgM binds to MDA-LDL coated microtiter wells and are detected by a protein A - peroxidase conjugate using TMB as the substrate. The enzyme reaction is measured at 450 nm.

*Ex vivo cytokine stimulation in whole blood*

Ex vivo stimulation of cytokines was done after stimulation of 100µl heparinized whole blood with endotoxin. Blood was mixed with 1ml PBS containing 500ng LPS (E. coli O111:B4. Lot 089K4034, Sigma Aldrich, Schnelldorf, Germany). Samples were incubated at 37°C for 4 hours in a water bath. Afterwards cells were pelleted and cell free supernatant was stored at -80°C until further use.

*Cytokine detection*

Cytokines IL-6, IL-10, sVCAM-1, sICAM-1 and TNF-α were determined by flow cytometry. A FlowCytomix™ five-plex-assay (eBioscience, Vienna, Austria) of these cytokines was performed according to the manufacturers’ instructions. 25µl serum samples were mixed with 25µl bead mix and 50µl biotin-conjugate and after two hours incubation 50µl streptavidin-PE solution was added. After one hour incubation 200µl assay buffer were added for measurement by LSRII Flow Cytometer (BD Biosciences Europe. Heidelberg. Germany). Cytokines were quantified using the FlowCytomix Pro 2.1 Software (Bender MedSystems GmbH,
Vienna, Austria). Commercially available ELISA Kits were used for determination of von Willebrand Factor (Eubio, Vienna, Austria), LBP, sCD14, (Hycult Biotechnology, Uden, Netherlands) and carbonyl Proteins (Immundiagnostik, Bensheim, Germany) levels. A summarizing z score for inflammation and endothelial dysfunction, according to the Hoorn-study publication (van Hecke et al. 2005) was calculated: individual value minus the mean value for the study population)/standard deviation. The summarising score for inflammation was calculated as followed: (z-score for CRP + z-score for sICAM-1)/2.

7.2.4 Statistical analyses

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc. Chicago). Data are expressed as mean ± standard deviation (SD) unless otherwise stated. Statistical analysis was done with the Mann-Whitney-U test or the unpaired student’s t-test for the comparison of differences between groups and by the paired student’s t-test or the Wilcoxon signed-rank test for the before and after treatment measurements, as appropriate. Differences with a p-value below 0.05 were considered statistically significant.

7.3 Results

7.3.1 Baseline characteristics

Thirty five subjects were screened for the study between January and August 2010; thirty patients were finally included in the studies. 28 finished the study (2 dropped out due to withdrawal of informed consent; figure 10). Baseline characteristics of our insulin resistant population are shown in table 13. Subjects in the LcS group had a significantly higher height, weight and BMI than those in the control group at baseline whereas no significant difference in the other baseline characteristics including diet and physical activity between the two groups could be observed.
Table 13 Baseline Characteristics (LcS)

<table>
<thead>
<tr>
<th></th>
<th>LcS (n=13)</th>
<th>Control (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female/male)</td>
<td>4/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51±11</td>
<td>55±9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>175±8</td>
<td>169±8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>109±15</td>
<td>91±14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood pressure systolic (mmHg)</td>
<td>147±19</td>
<td>147±18</td>
</tr>
<tr>
<td>Blood pressure diastolic (mmHg)</td>
<td>95±12</td>
<td>94±18</td>
</tr>
<tr>
<td>Body mass index (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>35±5</td>
<td>32±4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>113±12</td>
<td>106±8</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.7±1.8</td>
<td>5.5±1.4</td>
</tr>
<tr>
<td>High density lipoproteins (mmol/l)</td>
<td>1.1±0.4</td>
<td>1.2±0.5</td>
</tr>
<tr>
<td>Low density lipoproteins (mmol/l)</td>
<td>3.3±1.3</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.5±1.9</td>
<td>1.9±1.2</td>
</tr>
<tr>
<td>Very low density lipoproteins (mmol/l)</td>
<td>1.2±0.9</td>
<td>0.9±0.6</td>
</tr>
<tr>
<td>HbA&lt;sub&gt;1c&lt;/sub&gt; (%)</td>
<td>5.6±0.3</td>
<td>6.0±0.6</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/l)</td>
<td>6±0.9</td>
<td>6.1±0.5</td>
</tr>
<tr>
<td>2 hours blood glucose (mmol/l)</td>
<td>7.9±2.2</td>
<td>8.4±2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>p=0.05 vs. probiotic group; <sup>b</sup>p<0.01 vs. probiotic group; <sup>c</sup>p=0.03 vs. probiotic group
7.3.2 Insulin sensitivity

Three months of probiotic supplementation significantly improved (0.058 ± 0.021 vs. 0.038 ± 0.025 at baseline; p<0.01) insulin sensitivity index (ISI). However, the changes from baseline to 3 months were not significantly different between the groups investigated (table 14). Further indices of insulin sensitivity including measurements in the fasting states as the HOMA-IR or more dynamic measurements during the OGTT as the ISOGTT and QUICKI did not show significant differences between the intervention groups either. By looking at different indices we assured, that both aspects, hepatic and peripheral insulin sensitivity were considered in our analysis.
7.3.3 Beta-cell function
The ratio of incremental insulin to incremental glucose responses over the first 30 minutes during the OGTT significantly decreases during the treatment in the LcS group, but no significant difference between the groups could be observed. Parameters of 1st and 2nd phase insulin secretion or the HOMA-B did not change from baseline to study end.
Table 14 Indices of insulin sensitivity and beta-cell function

<table>
<thead>
<tr>
<th></th>
<th>Lactobacillus casei Shirota</th>
<th>Control</th>
<th>p-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>3 months</td>
<td>∆</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.9 ± 5.3</td>
<td>34.8 ± 5.7</td>
<td>-0.2 ± 0.9</td>
</tr>
<tr>
<td>FPG</td>
<td>6.1 ± 0.9</td>
<td>5.9 ± 0.7</td>
<td>-0.2 ± 0.6</td>
</tr>
<tr>
<td>2h Glucose</td>
<td>7.9 ± 2.3</td>
<td>7.2 ± 2.9</td>
<td>-0.7 ± 2.6</td>
</tr>
<tr>
<td>Fasting</td>
<td>11.7 ± 9.9</td>
<td>11.9 ± 7.6</td>
<td>0.2 ± 6.6</td>
</tr>
<tr>
<td>AUC</td>
<td>20 ± 3.5</td>
<td>18.6 ± 3.9</td>
<td>1.4 ± 2.3</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.1 ± 2.7</td>
<td>3.2 ± 2.1</td>
<td>0.1 ± 1.9</td>
</tr>
<tr>
<td>ISOGTT</td>
<td>3.6 ± 1.8</td>
<td>4.6 ± 3.9</td>
<td>1.0 ± 3.3</td>
</tr>
<tr>
<td>ISI</td>
<td>0.038 ± 0.025</td>
<td>0.058 ± 0.021&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.020 ± 0.019</td>
</tr>
<tr>
<td>HOMA-B</td>
<td>103.0 ± 98.5</td>
<td>105.7 ± 71.2</td>
<td>1.9 ± 55.8</td>
</tr>
<tr>
<td>∆&lt;sub&gt;30&lt;/sub&gt;/ΔG&lt;sub&gt;30&lt;/sub&gt;</td>
<td>73.2 ± 64.6</td>
<td>47.7 ± 44.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-25.5 ± 39.2</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; phase</td>
<td>1297 ± 1077</td>
<td>1058 ± 777</td>
<td>-239 ± 565</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; phase</td>
<td>357 ± 244</td>
<td>300 ± 177</td>
<td>-23 ± 111</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.34 ± 0.04</td>
<td>0.34 ± 0.06</td>
<td>0.00 ± 0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>p≤0.05 vs. baseline; <sup>b</sup>p<0.01 vs. baseline; <sup>c</sup>p value reflects comparison of changes from 3 months to baseline between groups. FPG = fasting plasma glucose

Δ reflects difference between 3 months and baseline
7.3.4 Inflammation and oxidative stress

Three months of probiotic supplementation significantly improved carbonyl protein (375.5 [322.15-448.70] at baseline to 317.80 [231.90-365.00] at 3 months) and MDA-LDL IG (154 ± 68 at baseline vs. 130 ± 45 mU/mL at study end; p=0.028). Moreover TNF-α levels significantly decreased during LcS treatment from 15.11 [1.29-27.77] pg/mL to 0.26 [0.26 to15.38]. p=0.028. No significant changes in all other parameters assessing low-grade inflammation including sum scores could be observed (for summary see table 15).

The changes of weight (r=0.598. p=0.009) as well as changes of the BMI (r=0.589. p=0.008) from baseline to 12 weeks significantly correlated with changes of MDA-LDL IG.

7.3.5 Endothelial dysfunction parameters

SVCAM-1 measured in serum significantly improved after three months of probiotic supplementation (1614 ± 343 ng/mL at baseline to 1418 ± 265; p=0.010 at study end). Furthermore 3 months of probiotic supplementation significant improved sVCAM-1 after LPS stimulation (66 ± 14 vs. 55 ±14 ng/mL at 12 weeks; p=0.002). In addition the change from baseline to 3 months in sVCAM-1 measured in serum (-195 ± 232 in probiotic group vs. 30 ± 182 in control group; p=0.008) and sVCAM-1 after LPS stimulation (-11 ± 10 in probiotic group vs. -2 ± 8 in standard group; p=0.021) were significantly greater than the changes observed in the control group. No significant changes in all other parameters assessing endothelial function including sum scores as an estimate for endothelial function could be observed (for summary table 15).
<table>
<thead>
<tr>
<th>Table 15 Parameters of inflammation and endothelial dysfunction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LcS</strong></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
</tr>
<tr>
<td>Inflammation z score</td>
</tr>
<tr>
<td>von Willebrand factor (ng/mL) serum</td>
</tr>
<tr>
<td>von Willebrand factor (ng/mL) LPS</td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL) Serum</td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL) LPS</td>
</tr>
<tr>
<td>Interleukin-10 (pg/mL) Serum</td>
</tr>
<tr>
<td>Interleukin-10 (pg/mL) LPS</td>
</tr>
<tr>
<td>TNF – alpha (pg/mL) Serum</td>
</tr>
<tr>
<td>TNF – alpha (pg/mL) LPS</td>
</tr>
<tr>
<td>Carbonyl protein (pmol/mg)</td>
</tr>
</tbody>
</table>
|                            | MDA-LDL IG (mU/mL) | Ox-LDL (mU/mL) | Changes from baseline to 3 months:  
\* \( p=0.028; \)  
\$ \( p=0.010; \)  
\§ \( p=0.002; \)  
\° \( p=0.017; \)  
\; \( p=0.028 \)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-LDL IG (mU/mL)</td>
<td>154±68</td>
<td>130±45°</td>
<td>-20±20</td>
</tr>
<tr>
<td>Ox-LDL (mU/mL)</td>
<td>563±483</td>
<td>529±478</td>
<td>-32±74</td>
</tr>
</tbody>
</table>

**Endothelial function**

<table>
<thead>
<tr>
<th></th>
<th>Endothelial z score</th>
<th>SICAM-1 (ng/mL)</th>
<th>SICAM-1 (ng/mL)</th>
<th>sVCAM-1 (ng/mL)</th>
<th>sVCAM-1 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.186±0.610</td>
<td>884±572</td>
<td>32±10</td>
<td>1614±343</td>
<td>66±14</td>
</tr>
<tr>
<td></td>
<td>0.137±0.597</td>
<td>715±338</td>
<td>26±10</td>
<td>1418±265$</td>
<td>55±14$</td>
</tr>
</tbody>
</table>

Changes from baseline to 3 months:  
\* \( p=0.028; \)  
\$ \( p=0.010; \)  
\§ \( p=0.002; \)  
\° \( p=0.017; \)  
\; \( p=0.028 \)

Column with p values: p reflects comparison of changes from baseline to 3 months between groups.

Data are presented as means ± standard deviation or, in the case of a skewed distribution, as medians (interquartile ranges). TNF= tumor necrosis factor; LPS= lipopolysaccharide; HDL=high-density lipoprotein; LDL=low-density lipoprotein; MDA-LDL IG= malondialdehyde-modified LDL; ox-LDL= autoantibodies against oxidatively modified LDL.
7.4 Discussion
In this study we showed that \textit{LcS} supplementation overall failed to improve convincingly parameters of insulin sensitivity, beta-cell function, endothelial function or low grade inflammation.
Although one single estimate of insulin sensitivity, the ISI, improved from baseline to study end in the \textit{LcS} group, the changes from baseline to 3 months were not significantly different between the \textit{LcS} and the placebo group, and therefore this finding should not be over interpreted. By looking at different indices we assured, that both aspects, hepatic and peripheral insulin sensitivity were considered in our analysis.
The only parameter which improved significantly in the \textit{LcS} group was sVCAM-1, but this finding could be rather a play of chance than a real signal for an improvement in endothelial function or chronic inflammation given that all the other parameters assessed do not support such a conclusion. We found that in a high proportion of our patients high sensitive CRP, which is a well defined biomarker for low-grade inflammation, was elevated. CRP was found to be elevated in patients with morbid obesity but did not improve after moderate weight loss (Sola et al. 2009). We found no association between BMI and hsCRP levels in our study, however only a minor proportion of our patients had morbid obesity. Among the vast amount of bacteria described to alter gut flora and exert positive effects on the host, we have chosen to study \textit{LcS} for several reasons: Firstly the commercially available milk drink preparation delivers a high bacterial number on a relatively small volume. Furthermore \textit{Lactobacillus casei} Shirota has been proven to survive the passage through the stomach and is present in the lower intestinal tract and was shown to reduce gram negative bacteria, the source for lipopolisaccharides (LPS) (Shirota, Aso & Iwabuchi 1966).
As our study had a duration of 12 weeks only, we intended to investigate intermediate term effects of \textit{LcS} supplementation on glucose metabolism and chronic inflammation only. Both, obesity and insulin resistance have been shown to be associated with low grade inflammation and several inflammatory factors such as TNF-\textgreek{a} or IL-6 are related to impaired insulin action (Shirota, Aso & Iwabuchi 1966). High fat diet was shown to induce a low-grade endotoxemia in
mice and infusing endotoxin causes weight gain and insulin resistance (Cani et al. 2007) in mice. Changes in gut microbiota were suggested to decrease endotoxinaemia and subsequently chronic inflammation and insulin resistance. In mice treated with antibiotics, the change in gut microbiota protected them against diet-induced fat mass development, glucose intolerance and insulin resistance (Cani et al. 2008). A similar result was found in mice treated with a probiotic that increases the number of Bifidobacterium spp., which leads to improved glucose tolerance, insulin secretion and a decrease in inflammatory tone (Cani et al. 2007). Matsuzaki and coworkers (Matsuzaki et al. 1997b) showed that the oral administration of LcS effectively decreased the plasma glucose in KK-A^y^-mice and finally treatment of mice with a probiotic mixture (VSL#3) decreased hepatic insulin resistance, supporting the concept that intestinal bacteria induce endogenous signals that play a pathogenic role in hepatic insulin resistance (Li et al. 2003). Cani et al. finally defined microbiota-derived LPS as the crucial factor in the development of inflammation in metabolic disease (Cani et al. 2007). The lack of efficacy of LcS treatment in patients with metabolic syndrome in our study could be due to several reasons: i) LcS supplementation does not have beneficial effects in this group of subjects ii) our study was a pilot trial and not sufficiently powered to answer this question appropriately iii) we do not know if the dose of probiotic was correct. This dose was shown in previous studies with alcoholic liver cirrhosis to be effective on neutrophile function or cytokine response, but may be inadequately low in subjects with metabolic syndrome (Gruber et al. 2008). We did not analyze stool samples and can therefore not prove, that gut microbiota really changed in our patients iv) the duration of intervention could have been too short to influence the low-grade inflammatory process. In addition one limitation of the study is that the BMI by chance was significantly higher in the LcS group, due to the fact that 4 patients with a BMI above 40 kg/m^2 were randomized to this group. However, the parameters observed do not differ significantly between these morbidly obese and the non-morbidly obese subjects and therefore we do not think that this imbalance has a major impact on the study results. Another limitation of our study is the open label design and the lack of a milk-drink placebo. However, participants in the control group refrained from consuming
probiotic drinks and we believe that with regard to the parameter investigated the introduction of a probiotic-placebo group would not have changed the results significantly.

Our finding is in line with a recently published paper by Andreasen et al. showing that in a mixed participant group including type 2 diabetics, subjects with impaired glucose tolerance and healthy subjects, a four week treatment with *Lactobacillus acidophilus* NCFM does not affect systemic inflammation (Andreasen et al. 2010). Additionally, Andreasen et al. showed a beneficial effect of 4 weeks treatment with *Lactobacillus acidophilus* NCFM on insulin sensitivity. But there was no significant improvement in insulin sensitivity in the verum group from baseline to end of study while the insulin sensitivity in the placebo group significantly decreased. The significant difference observed between the groups in the study by Andreasen et al. might be therefore largely due to the worsening in the placebo group rather than a real improvement due to the treatment.

In conclusion, we could not show an effect of 12 weeks supplementation of LcS in subjects with metabolic syndrome either on insulin sensitivity and beta-cell function or on markers of endothelial function and inflammation. Further studies using LcS in other dosages (e.g. adjusted for body weight) or for a longer treatment period are needed to confirm our findings.
8 Vitamin D - Mortality in patients with T2DM and PCH – a cohort analysis

8.1 Background and aim
Vitamin D was shown to be associated with all-cause mortality in patients referred to coronary angiography (Thomas et al. 2012). In this analysis we aimed to elucidate whether the association of 25(OH) vitamin D levels with mortality is the same in various stages of glucometabolic disturbances.

8.2 Study population
The Ludwigshafen Risk and Cardiovascular Health (LURIC) study is a prospective cohort of patients referred for coronary angioplasty and is designed to evaluate determinants of cardiovascular health (Dobnig et al. 2008, Pilz et al. 2008, Winkelmann et al. 2001). In total 3,316 Caucasian patients, referred for coronary angiography were recruited between July 1997 and January 2000, at the Herzzentrum (Cardiac Center) Ludwigshafen in southwest Germany. Exclusion criteria were any acute illness other than acute coronary syndrome, any predominant non-cardiac chronic disease, and a history of malignant neoplasm(s) within the past 5 years. Written informed consent was obtained from each participant, and the study was approved by the institutional review board at the Ärztekammer Rheinland-Pfalz (Medical Association of Rheinland-Pfalz) (Pilz et al. 2010b).

8.2.1 Follow-up
Follow-up procedures (median 7.7 years) have been described in detail elsewhere (Dobnig et al. 2008, Pilz et al. 2008, Winkelmann et al. 2001). Briefly, information on mortality was obtained from local registries. Death certificates were used to classify the deceased into those who died from cardiovascular vs. non-cardiovascular causes. This classification was done independently by two experienced clinicians who were blinded to the study participants, except for information that was required to classify the cause of death. In the event of disagreement or uncertainty a classification decision was made by the LURIC study principal investigator (Pilz et al. 2010b).
In 2565 patients 25 (OH)-vitamin D levels were measured and information on glucometabolic state at baseline were available. A fasting venous blood sample was obtained in the morning before coronary angiography from supine subjects. Selected variables were measured after samples were snap frozen and stored at –80°C. A summary of methods and test kits used for variables relevant to this study has been previously reported (Winkelmann et al. 2001). Serum levels of 25(OH)D were assayed on a weekly basis using a radioimmunoassay (DiaSorin SA, Antony, France) with intra-assay and inter-assay coefficients of variation of 8.6% and 9.2%, respectively (Dobnig et al. 2008, Winkelmann et al. 2001).

Normal glucose tolerance (NGT) was defined as a fasting plasma glucose <126 mg/dl and a 2h plasma glucose <140 mg/dL. Post-challenge hyperglycemia (pcHG) was defined as a 2h plasma glucose value above >140mg/dL. Type 2 diabetes mellitus was defined either by a history of diabetes, current diabetes medication or a fasting plasma glucose ≥ 126 mg/dl.

Hazard ratios (HR) with 95% confidence intervals (CI) for the mortality categories were calculated using Cox proportional hazards regression models, which enabled adjustment for potential confounding parameters. In these analyses model 1 describes the crude association; model 2 was adjusted for age and sex; and model 3 further adjusted for high density lipoprotein cholesterol, low lipoprotein cholesterol, triglycerides, mean diastolic blood pressure and mean systolic blood pressure. HRs for 25(OH)D categories were calculated using the highest 25(OH)D group as the reference. Multivariate regression analysis was performed to identify predictors for changes in the 2h glucose OGTT. The covariates included age, HDL cholesterol, triglycerides, BMI, mean systolic blood pressure, 25-hydroxy vitamin D and waist circumference. The between groups differences were assessed by analysis of variance (ANOVA). In 2085 patients plasma glucose after 2h was measured. All data were analyzed using SPSS (SPSS 19.0; SPSS Inc, Chicago, Illionois). All statistical tests were 2-sided, and statistical significance was defined as p<0.05.
8.5 Results

8.5.1 Baseline characteristics

Characteristics of the selected LURIC sample are summarized in table 16.

Patients with conventional diabetes had the lowest mean 25(OH)-vitamin D levels (14.5±8.1 ng/mL) followed by the post-challenge hyperglycemia (PCH) group (17.4±9.4 ng/mL) and normal glucose tolerance group (NGT) (18.7±9.6 ng/mL). (T2DM vs. PCH p<0.001. DM vs. NGT p<0.001. PCH vs. NGT p=0.002).

Table 16 Baseline characteristics LURIC database

<table>
<thead>
<tr>
<th></th>
<th>NGT (n=955)</th>
<th>PCH (n=1034)</th>
<th>T2DM (n= 576)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57±11</td>
<td>63±10</td>
<td>67±8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>72.1</td>
<td>74.4</td>
<td>66.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8±3.8</td>
<td>28.0±3.9</td>
<td>28.6±4.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Active Smoker (%)</td>
<td>23.9</td>
<td>17.2</td>
<td>14.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>195±39</td>
<td>194±38</td>
<td>186±39</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>120±35</td>
<td>118±34</td>
<td>110±34</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>40±11</td>
<td>38±11</td>
<td>36±10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>153±106</td>
<td>176±115</td>
<td>198±129</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D</td>
<td>18.7±9.6</td>
<td>17.4±9.4</td>
<td>14.5±8.1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Continuous data are shown as mean ± SD and categoric data are shown as percentages.

After a median follow-up of 7.7 years, a total of 582 participants (22.7%) died, 242 (41.6%) in the T2DM group, 217 (37.3%) in the PCH group and 123 (21.1%) in the NGT group, respectively.
8.5.2 Cox regression

Hazard ratios with 95% confidence intervals for the mortality categories were calculated using Cox proportional hazard regression models. HRs for 25(OH)D categories were calculated using the highest 25(OH)D group as the reference.

In the HRs in the crude model were 1.30 ([0.78 – 2.15], p=ns) and 2.47 ([1.57 – 3.88], p<0.001) for the 2nd and 3rd tertile respectively in comparison to the 1st tertile (reference) in the normal glucose tolerance group. Even after the full adjustment for sex, age, HDL cholesterol, LDL cholesterol, triglycerides, mean diastolic blood pressure and mean systolic blood pressure the hazard ratio for the 3rd tertile remained statistically significant (2.27 [1.41 – 3.64], p<0.001). In the T2DM group the crude model the HRs were 1.22 ([0.88 – 1.71], p=ns) and 1.84 ([1.34 – 2.52], p<0.001) for the 2nd and 3rd tertile respectively in comparison to the 1st tertile. Even after adjustment for sex and age (model 2) as well as after full adjustment (model 3) the hazard ratio for the 3rd tertile remained statistically significant (1.94 [1.40 – 2.68], p<0.001) in model 2 and 1.84 ([1.33 – 2.56], p<0.001) in model 3. Further, in the PCH group, all calculated models remained statistically significant for 2nd and 3rd tertile, respectively. Detailed data on the analysis with various models of adjustments are shown in table 17.

Table 17 Cox regression

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Tertile (&gt;21.1 ng/mL)</td>
<td>1 (Reference)</td>
<td>1 (Reference)</td>
<td>1 (Reference)</td>
</tr>
<tr>
<td>2nd Tertile (13.2-21.1 ng/mL)</td>
<td>1.30 [0.78 - 2.15]</td>
<td>1.11 [0.67 - 1.84]</td>
<td>1.12 [0.67 – 1.87]</td>
</tr>
<tr>
<td>3rd Tertile (&lt;13.2 ng/mL)</td>
<td>2.47 [1.57 - 3.88]*</td>
<td>2.29 [1.44 - 3.66]*</td>
<td>2.27 [1.41 – 3.64]*</td>
</tr>
<tr>
<td>PCH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Tertile (&gt;20.2 ng/mL)</td>
<td>1 (Reference)</td>
<td>1 (Reference)</td>
<td>1 (Reference)</td>
</tr>
<tr>
<td>2nd Tertile (12.1-20.2 ng/mL)</td>
<td>1.70 [1.17 - 2.48]**</td>
<td>1.64 [1.13 - 2.39]***</td>
<td>1.64 [1.13 – 2.39]**</td>
</tr>
</tbody>
</table>
### Table 18 Multivariate regression

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B-Coefficient (multivariate)</th>
<th>P-Value (multivariate)</th>
<th>B-Coefficient (univariate)</th>
<th>P-Value (univariate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-hydroxy vitamin D (µg/L)</td>
<td>-0.087</td>
<td>&lt;0.001</td>
<td>-0.125</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.219</td>
<td>&lt;0.001</td>
<td>0.211</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>-0.096</td>
<td>&lt;0.001</td>
<td>-0.134</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>0.112</td>
<td>&lt;0.001</td>
<td>0.136</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean systolic blood pressure (mmHg)</td>
<td>0.038</td>
<td>0.103</td>
<td>0.118</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>0.089</td>
<td>0.006</td>
<td>0.145</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>0.029</td>
<td>0.375</td>
<td>0.162</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Dependent Variable: glucose 2h post OGTT (whole blood) (mg/dL)
8.6 Discussion

To the best of our knowledge this is the first study to show that higher levels of 25(OH)D are associated with a lower risk of all-cause mortality in subjects with normal glucose tolerance, post-challenge hyperglycemia and in patients with manifest type 2 diabetes mellitus.

Age, HDL cholesterol, triglycerides, 25(OH) vitamin D and BMI were identified as independent predictors of 2 hour plasma glucose in our patient cohort.

Not surprisingly age is predictive for 2 hour glucose, reflecting the increased risk for hyperglycemia with increasing age (Nathan et al. 2007). Higher BMI is a known risk factor for type 2 diabetes mellitus and post-challenge hyperglycemia (Sullivan et al. 2005) and low levels of HDL cholesterol are also a characteristic finding in prediabetes, insulin resistance and part of the metabolic syndrome definition (O'Brien, Nguyen & Zimmerman 1998, Haffner et al. 1990). High fasting triglyceride levels (above 150 mg/dL) are also known to be strongly associated with impaired glucose tolerance (Love-Osborne et al. 2006). Finally, low serum 25(OH)D levels are associated with prediabetes (Shankar, Sabanayagam & Kalidindi 2011) and a higher probability of future diabetes mellitus (Grimnes et al. 2010, Knekt et al. 2008).

Our analysis contributes to the limited longitudinal literature describing the association between vitamin D and mortality (Dobnig et al. 2008, Parker et al. 2010, Melamed et al. 2008, Ginde et al. 2009) and extends these findings to patients at various stages of disturbed glucose metabolism.

Nevertheless, a couple of limitations of our study should be noted. 25(OH)D level was only assessed at one time point, and this may not reflect long-term vitamin D condition. Further, a single measurement does not consider the significant seasonal variations in 25(OH)D levels. However, despite significant seasonal variation, vitamin D levels are reported to track over time in a comparable manner to other risk factors, such as blood lipids and blood pressure (Jorde et al. 2010).

In summary, 25(OH)D levels were associated with a reduction in all-cause mortality in subjects with normal glucose tolerance, with post-challenge hyperglycemia as well as in patients with T2DM. As the 25(OH)D level is also predictive for 2 hour glucose, these data suggest, in particular patients with post-challenge hyperglycemia vitamin D supplementation could reduce progression to
overt diabetes as well as reduce cardiovascular events and mortality. Due to the fact that intervention trials testing this hypothesis are currently lacking we perform a randomized, controlled and double-blind study with endothelial dysfunction. a common surrogate parameter, defined as primary outcome.
9 Interventional Study (Vitamin D)

9.1 Background and aim

Patients with type 2 diabetes are at high risk for macrovascular disease and events as well as microvascular complications concerning the eye, the kidney or the nerves (Stratton et al. 2000). Impaired glucose tolerance precedes manifest diabetes and about 10% per year of patients affected by this glucometabolic disturbance develop an overt diabetes mellitus type 2. While the prevalence of impaired glucose tolerance in a putative healthy population between 50 and 75 years is about 16% (Rathmann et al. 2003), we (Saely et al. 2008, Wascher et al. 2005) and in particular the Euro Heart survey on Diabetes and the Heart (Bartnik et al. 2004) clearly established that the proportion of patients with impaired glucose tolerance is up to 30% and furthermore of patients with isolated post-challenge diabetes up to 20% in cardiovascular high risk patients as those undergoing elective coronary angiography for suspected coronary artery disease. A well-known and validated surrogate parameter to access (Davignon, Ganz 2004) the risk for future cardiovascular events is endothelial dysfunction (Akcakoyun et al. 2008, Davignon, Ganz 2004, Yeboah et al. 2007). It was clearly shown that coronary patients with post-challenge hyperglycaemia face a worse endothelial function (Sourij, Zweiker & Wascher 2006). Besides the well-established effects of vitamin D, large observational trials reported an association between low vitamin D levels and hypertension (Judd et al. 2008), incident cardiovascular disease (Wang et al. 2008), myocardial infarction (Giovannucci et al. 2008), cardiovascular death (Dobnig et al. 2008) and total mortality (Autier, Gandini 2007). Several recent studies have shown that brachial artery flow mediated dilatation (FMD), a measure of endothelium-dependent dilatation and vascular endothelial function, is inversely related to serum vitamin D levels in patients with diabetes (Sugden et al. 2008) and chronic kidney disease (London et al. 2007) as well as in adults without clinical disease (Jablonski et al. 2011), although recent studies failed to confirm this finding in patients with type 2 diabetes (Witham et al. 2010) as well as in patients after myocardial infarction (Witham et al. 2012). In particular, vitamin D has been implicated to suppress the renin-angiotensin aldosterone system (RAAS) (Li et al. 2002). Inappropriate high
activation of the RAAS has been observed in vitamin d receptor (VDR) and 1α-hydroxylase knockout mice (Xiang et al. 2005). Increased activation of the RAAS, which is a main regulator of electrolyte and volume homeostasis, contributes to the development of endothelial dysfunction and arterial hypertension. In addition, activations of the RAAS are increasingly suggested to contribute to altered insulin signalling pathways that may subsequently induce impaired glucose tolerance, insulin resistance, atherosclerosis and cardiovascular disease (CVD). With this in line, we have recently demonstrated an independent association between an activated circulating RAAS and increased insulin resistance (own unpublished data). Therefore we pose the question whether vitamin D administration provides a sufficient way to decrease RAAS activity in patients with post-challenge hyperglycaemia. In patients with disturbances of glucose metabolism the prevalence of low levels of 25-hydroxyvitamin D is high (Suzuki et al. 2006). Recently, Gagnon and colleagues showed that low levels of vitamin D in the blood were associated with an increased risk of developing T2DM (Gagnon et al. 2011). Furthermore one prospective study in non-diabetic subjects showed that baseline levels of 25-OH vitamin D are inverse associated with the extend of future glycaemia and insulin resistance (Forouhi et al. 2008). In animal studies administration of calcium and vitamin D improved pancreatic beta-cell function and peripheral insulin sensitivity (Boucher 1998, Tanaka et al. 1984, Norman et al. 1980, Chiu et al. 2004). Suggested effects were modulation of intracellular calcium concentrations, stimulation of insulin gene transcription or insulin receptor expression (Palomer et al. 2008). In addition 25-OH vitamin D levels were shown to be inversely associated with blood pressure (Pilz et al. 2009) as another important cardiovascular risk factor. However, all these data mainly gained in cross-sectional investigations or prospective association studies have to be proven in prospective, randomised controlled trials. Interventional data from the past are extremely rare, and these studies were done with inadequate low levels of vitamin D supplementation.

The aim of our study was to investigate the impact of vitamin D in 25-OH vitamin D deficient patients with either post-challenge hyperglycaemia or early T2DM and coronary artery disease on endothelial dysfunction, a surrogate parameter for future cardiovascular events. In addition, effects of vitamin D on parameters of insulin sensitivity and beta-cell function will be investigated and
should give further information whether prevention of type 2 diabetes mellitus by vitamin supplementation in vitamin D deficient patients with impaired glucose tolerance seems to be possible.

9.2 Study design

We performed a unicentre, prospective, randomized, controlled and double-blind trial conducted at the Medical University of Graz, Austria. The detailed study design is provided below (table 19) according to the revised CONSORT statement (Moher, Schulz & Altman 2001, Schulz et al. 2010).

Table 19 Time schedule of regular study visits and outcome parameters

<table>
<thead>
<tr>
<th></th>
<th>Screening (Week-2)</th>
<th>V1 (Baseline)</th>
<th>V2 (Month 3) ±7 days</th>
<th>V3 (Month 12) ±14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Informed Consent</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In/exclusion criteria</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Randomization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concomitant medication</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Physical examination</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Vital signs</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Blood sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung function measurement</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>24 h blood pressure measurement</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>24 h urine collection</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Flow mediated dilatation (FMD)</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Biochemical endothelial function (vWF, hsCRP, sICAM-1, sVCAM-1)</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Oral glucose tolerance test (oGTT)</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Drug account</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Adverse event reporting</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>
9.2.1 Inclusion and exclusion criteria

Inclusion criteria

- Aged from 40-75 years
- Post-challenge hyperglycemia (2h value in oral glucose tolerance test above 7.7 mol/l. normal fasting glucose) or early diabetes
- Angiographically verified coronary artery disease (>50% stenosis)
- Serum 25-OH- vitamin D < 30 ng/ml
- Stable antihypertensive therapy in the last 3 month

Exclusion criteria

- Acute coronary syndrome or cerebrovascular event within the previous 1 month
- BMI > 40 kg/m²
- Serum creatinine >2.5 times the upper limit of normal
- GOT or GPT > 3 times the upper limit of normal
- Heart failure > NYHA class II
- Uncontrolled hypertension (>160/100 mmHg)
- New onset of statins. ACE-inhibitors or ARBs within the previous 4 weeks
- History of urolithiasis
- Hypercalcaemia
- Major psychiatric disorders
- Ongoing treatment with spironolactone, canrenoate, eplerenone, amiloride, triamteren and aliskiren
- Treatment with antipsychotic drugs
- Regular significant antioxidants. vitamins or protein supplementation
- Immunosuppressive therapy
- Glucocorticoid therapy
- Ongoing chemotherapy
- Pregnancy
- Any other disease with an estimated life expectancy below 1 year.
9.2.2 Ethical considerations

Our study protocol was approved by the local ethics committee (21-016 ex 09/10) and the Austrian Competent Authority (AGES-Austrian Agency for Health and Food Safety) and is registered at both the European Union Drug Regulating Authorities-Clinical Trials (Eudra-CT: 2009-015776-95) and Clinicaltrials.gov (NCT01183442). The study was conducted in accordance to the Declaration of Helsinki in its currently applicable version, the guidelines of the International Conference on Harmonization of Good Clinical Practice (ICH-GCP) and the applicable Austrian laws. All participants were required to give written informed consent. The study was monitored according to ICH-GCP.

9.2.3 Study procedures

All measurements were performed at the Medical University of Graz at the Department of Endocrinology and Metabolism after an overnight fast (water only) and a 24-hour abstention from vigorous physical activity. At baseline an anamnesis, physical exam and measurement of routine laboratory parameters, markers of inflammation, NT-proBNP, plasma aldosterone concentration, plasma renin concentration, measurement of endothelial function, a 24-hour blood pressure measurement, measurement of 24 hours urinary sodium, measurement of lung functions, potassium and aldosterone concentration and a frequently sampled oral glucose tolerance test were performed.

Endothelial dysfunction

Endothelial function was measured by FMD. This method was estimated as the percentage increase in vessel diameter from baseline conditions to maximum vessel diameter during hyperaemia and required the patients to be supine in a quiet room (Bots, Hofman & Grobbee 1994, Bots et al. 1997). A longitudinal section of the brachial artery was analysed. After baseline measurement, a cuff, which was placed below the transducer position, was inflated to supra-systolic pressure (50mmHg above systolic blood pressure) to produce ischemia in the forearm. The cuff was deflated after 5 minutes thus causing a reactive hyperaemia which in turn produced a shear stimulus that induced the endothelium to release NO, a vasodilatators (Bots et al. 2005).
9.2.4 Randomization

Eligibility of patients was determined at the screening visit. After enrollment participants were randomly assigned to placebo or vitamin D groups with an allocation ratio of 1:1. Randomization was performed at the Medical University of Graz using web-based software (http://www.randomizer.at). Patients were stratified according to gender. Study personal and participants were blinded to treatment assignment for the duration of the study.

9.2.5 Interventions

Patients in one group received 2800 IU of vitamin D (Oleovit®) orally every other day, whereas patients in another group received 2800 IU of placebo orally every other day. Regularly study visits were carried out after 3 and 12 months. Investigations on the respective study visits are shown in table 19. Medications was re-labelled and prepared by Pharmacy of the University Hospital Graz (Graz, Austria) and supplied in identical bottles with study number but no identification of group allocation. Allocation was therefore concealed from researcher and subjects.

9.2.6 Outcome parameters

Primary outcome were changes in endothelial dysfunction (flow mediated dilatation and biochemical analyses) during the treatment period of 12 months. Additionally secondary endpoints comprised changes in indices for insulin resistance and beta-cell function, changes in blood pressure, changes in markers of chronic inflammation, changes in levels of renin, and aldosterone and changes in levels of NT-proBNP.
9.2.7 Sample size and power calculation

Sample size was estimated using free available calculator http://www.quantitativeskills.com/sisa/calculations/samsize.htm. Assuming an improvement of endothelial dysfunction of 30% (Sugden et al. 2008) a total of 128 subjects would be needed to detect such a difference with an alpha of 0.05 and a power of 0.90. To account for a dropout rate of about 10% each group will consist of 70 patients.

9.2.8 Statistical analyses

Continuous variables were presented as mean and standard deviation or median and range, categorical variables as frequencies and percentages. Group comparison between the placebo and the vitamin-D group was performed by using independent t-tests or non-parametric tests and Chi-squared test for continuous and categorical variables, respectively. The test level of statistical significance of
differences between both treatment arms was defined as p=0.05 for all tests. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc, Chicago).

9.2.9 Premature study termination

The principle investigator stopped the investigations before the trial was complete due to troubles in recruiting enough patients at a sufficient rate and ethical reasons. Recent studies showed that supplementation with vitamin D did not improve markers of endothelial function in patients with a history of myocardial infarction (Witham et al. 2012) and did not improve endothelial function, arterial stiffness, blood pressure or reduce CRP (Gepner et al. 2012). These studies do not support the use of vitamin D supplementation to reduce cardiovascular disease risk.

9.3 Results

9.3.1 Screening

We pre-screened 1814 subjects in the department of cardiology and department of endocrinology and metabolism. 579 patients were eligible after prescreening, but 401 of them were not interested in participate the study. We screened 178 patients in the outpatient clinic. Finally, 22 patients were randomly assigned to placebo or vitamin D groups.

Figure 12 Screening procedures (VitD in CAD)
### Table 20 Screening results

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64±9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>172±9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>82±13</td>
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<tr>
<td>Blood pressure systolic (mmHg)</td>
<td>131±24</td>
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<tr>
<td>Blood pressure diastolic (mmHg)</td>
<td>80±12</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>180±43</td>
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<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>110±31</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>52±23</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>169±80</td>
</tr>
<tr>
<td>25(OH) - Vitamin D3 (ng/ml)</td>
<td>29.9±10.8</td>
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<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>98±16</td>
</tr>
<tr>
<td>Blood glucose 2h (mg/dl)</td>
<td>142±55</td>
</tr>
</tbody>
</table>

### 9.3.2 Results (main study)

#### Table 21 Baseline characteristics

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64±9</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>17/5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>173±8</td>
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<tr>
<td>Weight (kg)</td>
<td>86±14</td>
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<tr>
<td>Blood pressure systolic (mmHg)</td>
<td>144±19</td>
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<tr>
<td>Blood pressure diastolic (mmHg)</td>
<td>83±11</td>
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<td>Total cholesterol (mg/dL)</td>
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<td>LDL cholesterol (mg/dL)</td>
<td>94±28</td>
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<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>45±13</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>133±27</td>
</tr>
<tr>
<td>25(OH) - Vitamin D3 (ng/ml)</td>
<td>21.2±6.1</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>103±13</td>
</tr>
<tr>
<td>Blood glucose 2h (mg/dl)</td>
<td>162±44</td>
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</tbody>
</table>
9.3.2.1 Endothelial function

Three months of vitamin D supplementation did not change FMD significantly (2.7±2.2 % at baseline vs. 3.9±3.0 % after 3 months; p=0.160). Neither did change the NMD by vitamin D supplementation (9.1±3.7% at baseline vs. 9.3±4.6% after 3 months; p=0.896). The changes of FMD from baseline to 3 months were not significantly different between the groups investigated (table 22).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Treatment</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD (baseline)</td>
<td>Verum</td>
<td>2.7</td>
<td>2.2</td>
<td>0.762</td>
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<tr>
<td></td>
<td>placebo</td>
<td>3.9</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>NMD (baseline)</td>
<td>Verum</td>
<td>9.1</td>
<td>3.7</td>
<td>0.129</td>
</tr>
<tr>
<td></td>
<td>placebo</td>
<td>14.4</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>FMD (3 months)</td>
<td>Verum</td>
<td>3.9</td>
<td>3.0</td>
<td>0.759</td>
</tr>
<tr>
<td></td>
<td>placebo</td>
<td>4.4</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>NMD (3 months)</td>
<td>Verum</td>
<td>9.3</td>
<td>4.6</td>
<td>0.213</td>
</tr>
<tr>
<td></td>
<td>placebo</td>
<td>13.3</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Δ FMD</td>
<td>Verum</td>
<td>1.5</td>
<td>1.9</td>
<td>0.349</td>
</tr>
<tr>
<td></td>
<td>placebo</td>
<td>0.5</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Δ NMD</td>
<td>Verum</td>
<td>0.2</td>
<td>4.1</td>
<td>0.471</td>
</tr>
<tr>
<td></td>
<td>placebo</td>
<td>-1.1</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

*p-value reflects comparison between groups
Δ reflects difference between 3 months and baseline

9.4 Discussion

The main aim of our investigation was to clarify, whether vitamin D supplementation in coronary artery disease patients with vitamin D deficiency and post-challenge hyperglycemia or early T2DM has an impact on endothelial dysfunction.
As far as we know, our trial was (one of) the first ever to test the concept that vitamin d supplementation may influence endothelial dysfunction, a surrogate
parameter for future cardiovascular events, in patients with coronary artery disease, post-challenge hyperglycemia or early T2DM and vitamin D deficiency. Due to recruitment problems our study group had to amend the study protocol. We amended the inclusion of patients with early diabetes (untreated, dietary control, oral monotherapy) and an HbA1c <7.5% (58.47 mmol/mol) as well as patients with 25-OH vitamin D level below 30ng/ml after approximately 1 year of recruitment. These amendments were approved by the local ethics committee.

An improvement of endothelial dysfunction as a cardiovascular surrogate parameter could be translated in a reduced risk for future cardiovascular events, which is of major interest, since patients with post-challenge hyperglycemia face a significantly higher cardiovascular risk than patients with normal glucose tolerance (Sourij et al. 2010). The impact of vitamin D supplementation on endothelial function is conflicting. Two studies by the same study group have tested vitamin D supplementation in patients with T2DM (Sugden et al. 2008, Witham et al. 2010). The initial trial reported an improvement of endothelial function after a large dose (100.000 IU) of oral vitamin D$_2$ in patients with T2DM and vitamin D insufficiency. The follow-up trial showed no difference in FMD after a single oral dose of 100.000 or 200.000 IU vitamin D$_3$ versus placebo.

Very recent, a study of the same study group showed no improvement of markers of vascular function in patients with a history of myocardial infarction after vitamin D supplementation (Witham et al. 2012). Additionally, Gepner and colleagues reported that vitamin D supplementation (2500 IU or placebo daily) did not improve endothelial function, inflammation or arterial stiffness (Gepner et al. 2012). Based on recruitment barriers and due to these current study results the principal investigator of the trial decided to stop the trial prematurely. At this stage no significant improvement in FMD by vitamin D supplementation could be observed. Study duration of above mentioned trials varies from 8 weeks (Sugden et al. 2008) over 2 months (Witham et al. 2012) to 4 months (Gepner et al. 2012). Our results after 3 months show no clear trend in favour of vitamin D supplementation.
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## Appendix

### Table 23 Framingham Risk Score

<table>
<thead>
<tr>
<th>Framingham Risk score</th>
<th>Baseline</th>
<th>3 months</th>
<th>2 years</th>
<th>p-value (Baseline-3 months)</th>
<th>p-value (Baseline-2 years)</th>
<th>p-value (3 months-2 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary Heart Disease (%)</td>
<td>29.6 (14.6 – 24.9)</td>
<td>12.2 (9.5 – 16.2)</td>
<td>14.3 (11.1 – 18.4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.020</td>
</tr>
<tr>
<td>Myocardial Infarction (%)</td>
<td>12.1 (8.0 – 17.6)</td>
<td>6.6 (4.5 – 10.0)</td>
<td>8.2 (5.7 – 11.2)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.024</td>
</tr>
<tr>
<td>Stroke (%)</td>
<td>8.6 (4.9 – 12.0)</td>
<td>4.9 (3.3 – 7.3)</td>
<td>5.7 (3.6 – 8.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.398</td>
</tr>
<tr>
<td>Cardiovascular Disease (%)</td>
<td>33.7 (26.6 – 42.6)</td>
<td>23.1 (18.1 – 28.3)</td>
<td>26.5 (21.5 – 33.4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Data are presented as medians (interquartile ranges).
Figure 13 Drug therapy of diabetes

![Drug therapy of diabetes graph]

Figure 14 Drug therapy of hypertension

![Drug therapy of hypertension graph]