MicroRNAs in Cardiometabolic Disease. With a Focus on miR-483-5p.

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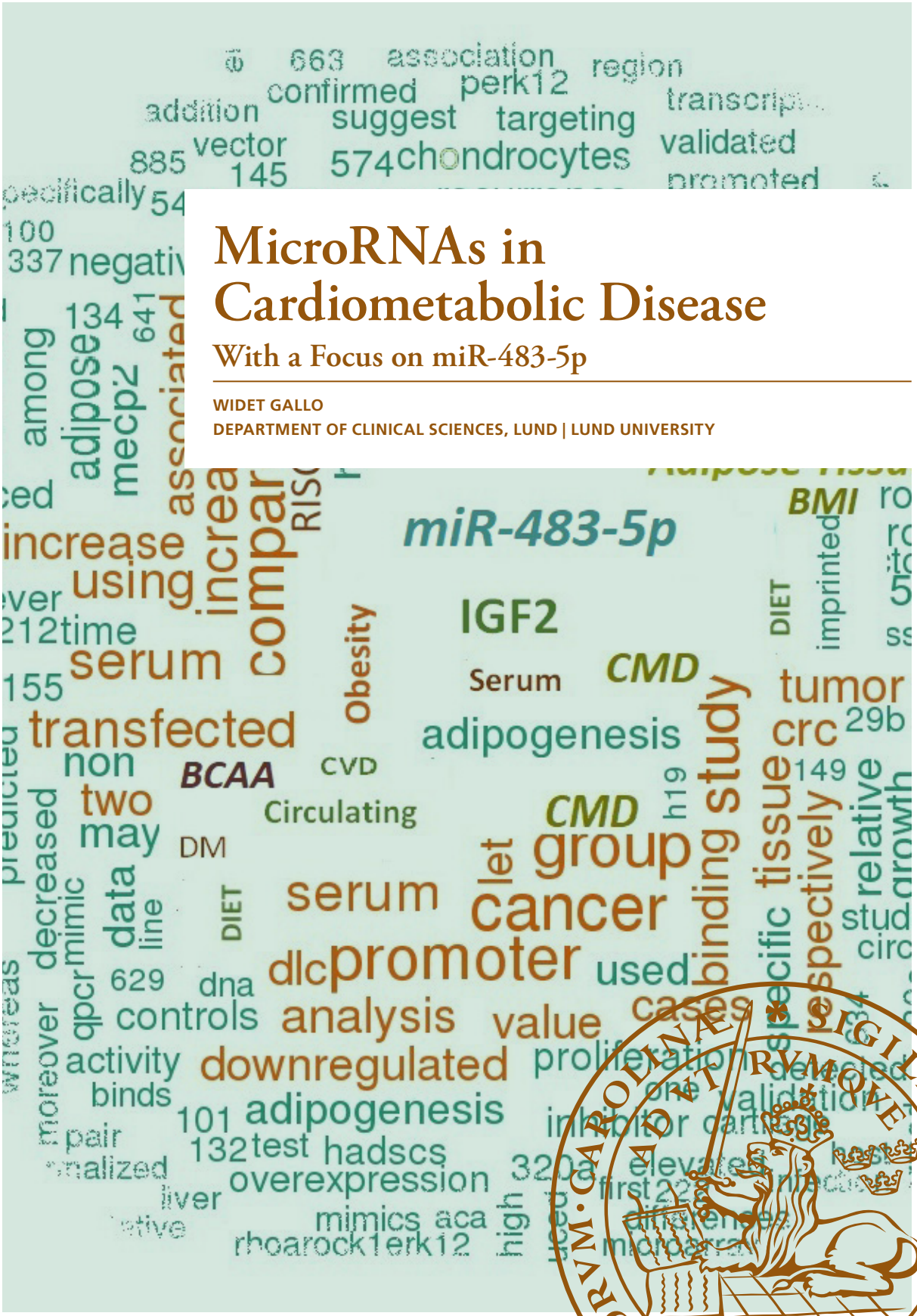

MicroRNAs in Cardiometabolic Disease

With a Focus on miR-483-5p

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LUND
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Abstract

Background
Cardiometabolic disease (CMD), comprising type 2 diabetes (T2DM) and cardiovascular disease (CVD) is a worldwide health concern whose pathophysiology starts many years before clinical manifestation. We hypothesized that alterations of microRNA levels can be traced in the circulation in healthy but CMD prone individuals.

Aims
Using two independent prospective cohort studies, our primary aim was to find consistent associations between serum levels of microRNAs and future risk of T2DM and CVD, as well as with presence of atherosclerosis. The secondary aim was to investigate whether such CMD-related microRNAs were correlated with life-style factors, in order to build hypotheses of how to alter their levels. Finally, by utilizing plasma metabolomics measurements, we test whether CMD-related microRNAs are correlated with alterations in certain metabolic pathways.

Methods
A cDNA synthesis and qPCR-based method was used to determine expressed microRNAs in serum from participants from the Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC) (N=553), and Malmö Offspring Study (MOS) (N=1223).

Results
Serum levels of circulating miR-483-5p were significantly associated with incident CMD and CMD-related risk factors in both paper-I and paper-II. Moreover, miR-483 5p was associated with carotid intima media thickness (IMT) in MDC-CC and associated with the number of carotid plaques in MOS. However, several other previously CMD-implicated microRNAs were not reproducibly associated with CMD neither in MDC-CC nor in MOS. In Paper-III, mir-483-5p was negatively related to physical activity. Positive correlations were found between miR-483 5p and the plasma levels of the branched-chain amino acids leucine and isoleucine and their catabolites propionylcarnitine and isovalerylcarnitine.

Conclusion
In this thesis, we find novel associations between miR-483-5p and future risk of cardiometabolic disease and its risk factors in two independent prospective cohorts. This highlights two potential future prospects for miR-483 5p. Either miR-483 5p could be used in a biomarker panel to improve risk stratification of T2DM or CVD or if the associations are causal, being targeted by pharmacological therapies aiming to lower the expression of miR-483 5p.

Key words: MicroRNAs, miR-483-5p, diabetes, cardiovascular disease, BMI, Branched-chain amino acids, lifestyle, obesity

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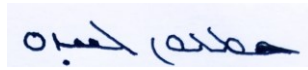
To my Father



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Abbreviations

AGO2	Argonaute
AHT	Antihypertensive treatment
BCAAs	Branched-chain amino acids
BCKD	Branched chain α-ketoacid dehydrogenase
BMI	Body mass index
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
cDNA	Complementary DNA
CI	Confidence interval
CMD	Cardiometabolic disorders
Ct	Threshold cycle
CV	Coefficient of variation
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DGCR8	Drosha and DiGeorge syndrome critical region 8
GLUT2	the glucose transporter-2
ERK1	Signal-regulated kinase
FAM	6-carboxyfluorescein
FPG	Fasting plasma glucose
GLP-1	Glucagon-like peptide-1
HbA1	Glycated hemoglobin
HCV	Hepatitis C virus
HDL	High-density lipoprotein
IFG	Impaired fasting glucose
IGF-II	Insulin-like growth factor-2
IGT	To impaired glucose tolerance
IMT	Intima media thickness
INS	Insulin

LDL	Low-density lipoprotein
LDL-C	Circulating low-density lipoprotein cholesterol
LNATM	Locked Nucleic Acids
MAPK3	Mitogen-activated protein kinase3
MDC	Malmö Diet and Cancer study
MDC-CC	Malmö Diet and Cancer Study-Cardiovascular Cohort
MGB	Minor groove binder
MI	Myocardial infarction
MOS	Malmö offspring study
mRNAs	Messenger-RNA
NAFLD	Non-Alcohol Fatty Liver Disease
NFQ	Nonfluorescent quencher
OGTT	Oral glucose tolerance test
OR	Odds ratio
PCI	Percutaneous coronary intervention
PG	Plasma glucose
pre-miRNAs	Precursor-microRNAs
pri-miRNA	Primary microRNA
PPARγ	Peroxisome proliferator-activated receptor gamma
Q	Quartile
qPCR	Quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNase-III	Ribonuclease-III
SRF	Serum response factor
RT	Reverse Transcription
SD	Standard deviation
SGLT-2	Sodium-glucose transport protein-2
SMCs	Smooth muscle cells
SOCS3	Suppressor of cytokine signalling3

T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TME	Tumor microenvironment
UA	Unstable angina
3′UTRs	3′-untranslated regions
VCAM-1	Vascular adhesion molecule-1
XPO5	Exportin-5

List of papers

I. **Widet Gallo**, Jonathan Lou S. Esguerra, Lena Eliasson, Olle Melander: miR-483-5p associates with obesity and insulin resistance and independently associates with new onset diabetes mellitus and cardiovascular disease. **PLoS ONE** 13(11): e0206974, October 2018.

II. **Widet Gallo**, Filip Ottosson, Cecilia Kennbäck, Amra Jujic, Jonathan Lou S. Esguerra, Lena Eliasson, Olle Melander: Replication Study Reveals miR-483-5p as an Important Target in Prevention of 1 Cardiometabolic Disease. **BMC Cardiovascular Disorders** (2021) 21:162, March 2021.

III. **Widet Gallo**, Filip Ottosson, Jonathan Lou S. Esguerra, Lena Eliasson, Ulrika Ericson, Olle Melander: Circulating miR-483-5p is related to decreased physical activity and diabetes related metabolites. **Manuscript.**

IV. **Widet Gallo**, Filip Ottosson, Cecilia Kennbäck, Amra Jujic, Jonathan Lou S. Esguerra, Lena Eliasson, Olle Melander: Prospective evaluation of circulating miR-126, miR-197 and miR-223 in relation to cardiometabolic diseases. **Manuscript.**

Papers not included in Thesis

I. Ottosson Filip, Smith Einar, **Gallo Widet**, Fernandez Céline, Melander Olle: Purine Metabolites and Carnitine Biosynthesis Intermediates Are Biomarkers for Incident Type 2 Diabetes. **JCEM**, 104 (10): 4921–4930, July 2019.

Introduction

Cardiometabolic Disease

Cardiometabolic disease, a global epidemic, comprising diabetes and cardiovascular disease, is the leading cause of death globally. Diabetes, which also is an established risk factor for cardiovascular disease (CVD), affects about 460 million peoples worldwide[1] and that can be classified into several categories, with type 1 diabetes accounting for up to 10 % whereas type 2 diabetes (T2DM) accounts for about 95% of all diagnosed patients[2].

Risk Factors

Obesity

Body mass index (BMI) is a measurement as a function of weight in kg divided by height in meters squared (kg/m2) where 25-29,9 kg/m2 describes overweight and ≥ 30 kg/m^2 obesity. An increased BMI, due to lack of physical activity with a combination of unhealthy diet and an excessive calorie consumption, leads to increased risk of metabolic diseases[3]. High BMI accounted for 4.0 million deaths worldwide in 2015 for which almost 70% were due to cardiovascular disease[4]. Half of all individuals in Sweden were overweight or obese in 2018[5]. Excessive energy intake and sedentary life-style results in weight gain and ectopic lipid accumulation. Depending on the fat distribution in the body, one can be metabolically healthy or unhealthy. Metabolically healthy obesity usually refers to fat in the gluteofemoral region whereas abdominal fat, especially visceral fat, is considered as metabolically unhealthy obesity[3]. Interestingly, lean women with low gluteofemoral fat mass were prone to have a higher risk of developing cardiovascular [3]. Change in lifestyle may prevent cardiometabolic disease. Specifically, a structured counselling regarding multifactorial lifestyle change that resulted in body-weight reduction, has been shown to prevent from development of diabetes in subjects with impaired glucose tolerance[6].

Dyslipidemia

Dyslipidemia is a term describing imbalance in lipid concentration in the blood. These lipids include triglycerides (TG), and lipoproteins such as low-density lipoprotein (LDL), also known as " bad cholesterol", high-density lipoprotein (HDL) also named "good cholesterol" and very low-density lipoprotein (VLDL)[7]. Lipoproteins act as transporters for TG and cholesterol in the blood, with HDL transporting cholesterol from the whole body to the liver and LDL transporting cholesterol back to the periphery. For identification of subjects with dyslipidemia, the following criteria are commonly recommended[8]; LDL-cholesterol > 4.1 mmol/L (160 mg/dL), triglycerides ≥ 1.7 mmol/L (150 mg/dL) and HDL-cholesterol for male < 1.0 mmol/L (40 mg/dL) and for female < 1.3 mmol/L (50 mg/dL). Elevated level of LDL-cholesterol was shown to be associated with CVD[9,10]. Moreover, reduction of LDL concentration has been shown to lead to reduced risk of CVD[11]. Although high concentration of HDL, the "good cholesterol", is considered to be associated with decreased risk of CVD, no studies have been able to show that HDL-raising therapies was able to protect against CVD[12].

Hypertension

The definition of hypertension, high blood pressure, is according to the World Health Organization, a systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg[13], compared to normal condition of SBP < 120 mmHg and/or DBP < 80 mmHg. Risk factors for hypertension are high intake of salt (sodium chloride), smoking, physical inactivity, overweight, diabetes, and genetic factors. Hypertension, which has been shown to be associated with increased risk of cardiovascular disease in prospective studies[14], is treated effectively with antihypertensive medication, which has been shown to prevent cardiovascular disease events[15].

Type 2 Diabetes

Pathophysiology, Diagnosis and Prevention

Diabetes Mellitus is characterized by elevated blood glucose, hyperglycemia, because of insulin resistance and/or impaired insulin secretion; the last due to defects in and/or destruction of pancreatic beta-cells[16,17]. The human islet cell is composed of different types of cells releasing different hormones. Glucagon-producing alfa-cells and insulin-producing beta-cells are though the cells that maintain blood glucose level. In case of low blood glucose, for instant during sleep or fasting, alfa-cells release glucagon, promoting glycogenolysis and gluconeogenesis in order to increase the level of blood glucose. Insulin attaches to its receptors on the muscle and adipose tissue, allowing the uptake of glucose into these organs and thereby lowering levels of blood glucose. In case of elevated level of exogenous glucose, the glucose transporter-2 (GLUT2) will allow the glucose enters the beta-cell, where it undergoes glycolysis, causing an increasing of Adenosine diphosphate (ATP) inside the cell. ATP has an inhibitory effect on the ATP-gated K+ channel in the cellular membrane when ATP-levels are low. ATP inhibits (potassium ions) K+ to leave the cell. In opposite condition, by elevated ATP, the K+ leave the cell which in turn cause depolarization of the cell membrane. This in turn will lead to inactivation of the voltage-dependent Ca+ channel, allowing Calcium ions (Ca+) entering the cell, which leads to insulin secretion[18].

In diabetic state, the insulin action is impaired because of lipid accumulation, which leads to increased plasma glucose and fatty acids. This can lead to Non-Alcohol Fatty Liver Disease (NAFLD) and Type 2 Diabetes Mellitus (T2DM)[19] (Figure 1). Diabetes diagnosis is based on fasting plasma glucose (FPG) with a concentration of ≥ 7.0 mmol/L, after a fast of at least 8 hours and at two different occasions or, a 2-hours plasma glucose (PG) test, after a 75 g oral glucose tolerance test (OGTT) and a concentration of ≥ 11.1 mmol/L or glycated hemoglobin (HbA1) level of ≥ 48 mmol/mol (6.5%) or random plasma glucose levels with a concentration of exceeding 11.1 mmol/L. Prediabetes is defined as having one of the following: (1) a FPG in the range previously referred to as impaired fasting glucose (IFG), i.e. between 5.6 mmol/L to 6.9 mmol/L; (2) After 75 g oral glucose tolerance test (OGTT) and a concentration of 7.8 mmol/L to impaired glucose tolerance (IGT) 11.0 mmol/L ; (3) HbA1 level of 5.7- 6.4% (39-47 mmol/mol)[2]. Prevention of diabetes in high-risk subjects, such as those having prediabetes/IGT may be achieved by lifestyle change or/or pharmacologically with metformin[20,21].

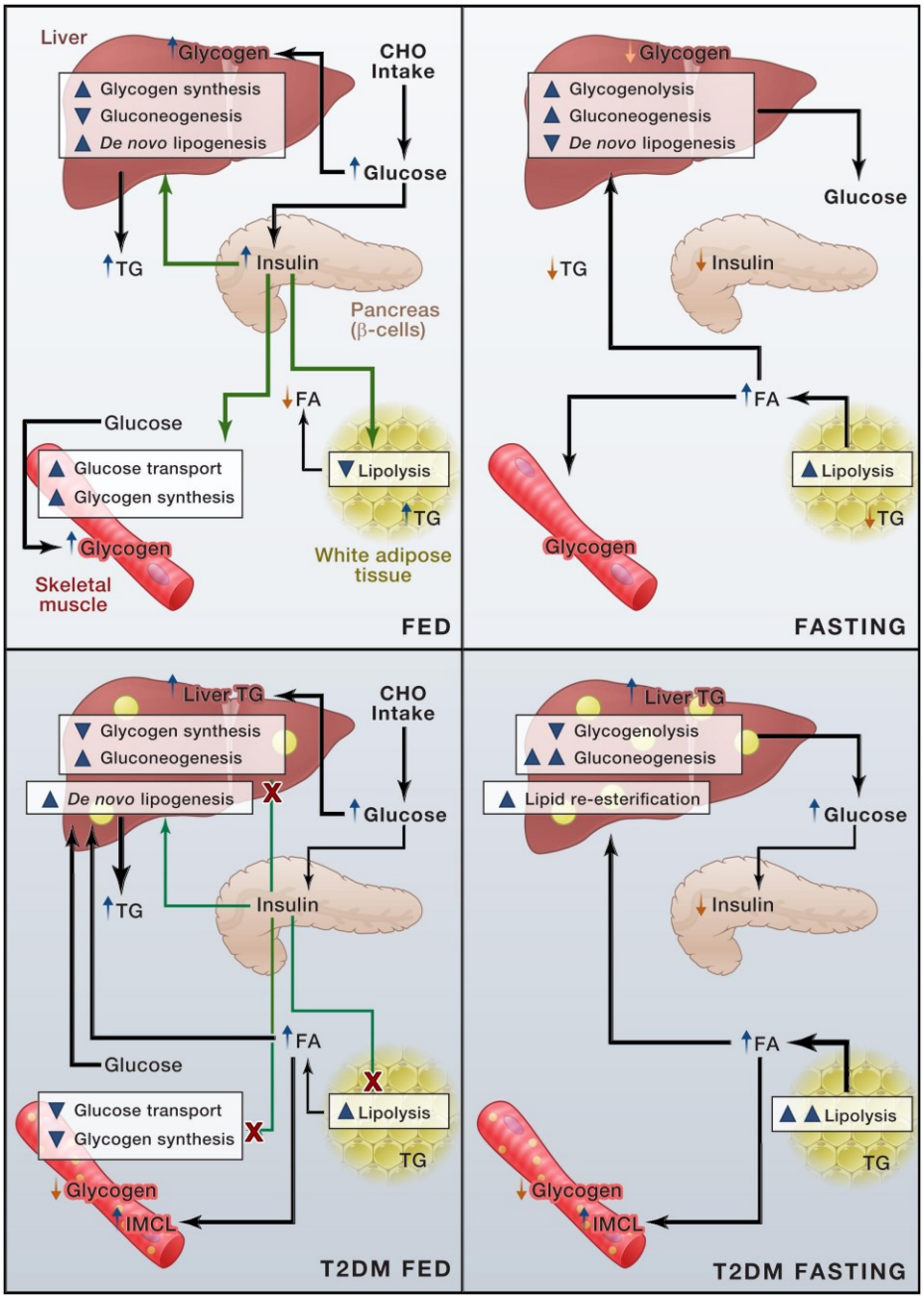

Figure 1 Mechanisms for Insulin Resistance in normal and diabetic state from referens[19] Carbohydrate (CHO), Triglycerides (TG), Fatty Acids (FA), Intramyocellular lipid (IMCL). (Figure 1 adapted from publisher, Reference[19])

Cardiovascular disease

Cardiovascular disease (CVD) encompasses several different vascular disorders that include stroke and coronary artery disease (CAD). CAD can result in myocardial infarction (MI). About 17.9 million people died in 2016 due to cardiovascular diseases, which represent approximately one third of global deaths. The majority of the deaths, about 85%, is a direct consequence of myocardial infarction or stroke[22].

Myocardial infarction (MI), unstable angina (UA) and sudden cardiac death present the acute coronary syndrome. MI is due to severe reduction of coronary blood flow which results in myocardial necrosis[23]. In case of reduction of blood flow in UA, coronary ischemia with pain in the chest occurs, without damaging the myocardium. To prevent the myocardium to suffer damage, coronary revascularization can be used. This can be made using coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI)[24].

Stroke can be ischemic, due to thrombosis or embolization, or hemorrhage. About 80% of all strokes are due to ischemic stroke whereas about 15% to hemorrhage[25].

CVD is commonly caused by atherosclerosis, which is a condition of thickening of the arterial wall with atherosclerotic plaques but also, though not frequently, by non-atherosclerotic causes. However, the focus in this thesis will be on atherosclerosis.

Pathophysiology, Diagnosis and Prevention

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease and thickening of large and medium-sized arterial walls[26]. Some markers for atherosclerosis are the intima media thickness (IMT) and plaques. The plaques are formed in the intima layer of the well. The IMT is divided into two common carotid arteries, one internal and one external, on each side of the neck. As the internal carotids supplies oxygen-rich blood to the brain, the external supplies blood to the face, tongue, scalp and neck[27]. Atherosclerosis occurs mainly at bifurcations and other sites with disturbed laminal flow[28]. Circulating low-density lipoprotein cholesterol (LDL-C), after entering the intima, becomes oxidized, which in turn activates endothelial cells to express adhesion molecules e.g., vascular adhesion molecule-1 (VCAM-1). This in turn recruits monocytes and T-cells [29]. Monocytes differentiate into macrophages after engulfing the oxidized LDL-C and converts to foam cells, which are lipid-containing macrophages. Excessive engulfing amount of LDL-C, the macrophages appear as foamy, hence the name [26,29,30].

As foam cells release their content with LDL, they attract more macrophages and smooth muscle cells (SMCs) to digest the released LDL[31], which leads to accumulation of foam cells, a condition called, fatty streak[26,29]. This is considered as the initiation of atherosclerotic plaque. A fibrous cap, consisting of connective tissue secreted by SMCs, is formed as a layer covering the atherosclerotic lesion, named atheromata, and which contains foam cells, T-cells, SMCs etc. The fibrous cap prevents rupture of the atheromata[32]. In case of macrophages death, lipids and other debris are released and a necrotic core is formed. Continued atherosclerotic lesion may lead to thinning of fibrous cap which in turn facilities the vulnerable plaque to rupture and cause thrombosis that may lead to myocardial infarction (MI) or stroke[32] (Figure 2). As medium and large-sized arteries are affected by atherosclerosis, organs, including the heart may be affected and lead to cardiovascular disease as myocardial infarction (MI), stroke and sudden cardiac death[32].

Prevention of cardiovascular disease can be achieved through e.g. lifestyle change[33], LDL-cholesterol lowering therapy and anti-hypertensive treatment[34]. During recent years, anti-diabetic therapy in form of sodium-glucose transport protein-2 (SGLT-2) inhibitors and Glucagon-like peptide-1 (GLP-1) analogues have also shown to protect from cardiovascular events in type 2 diabetes patients[35,36].

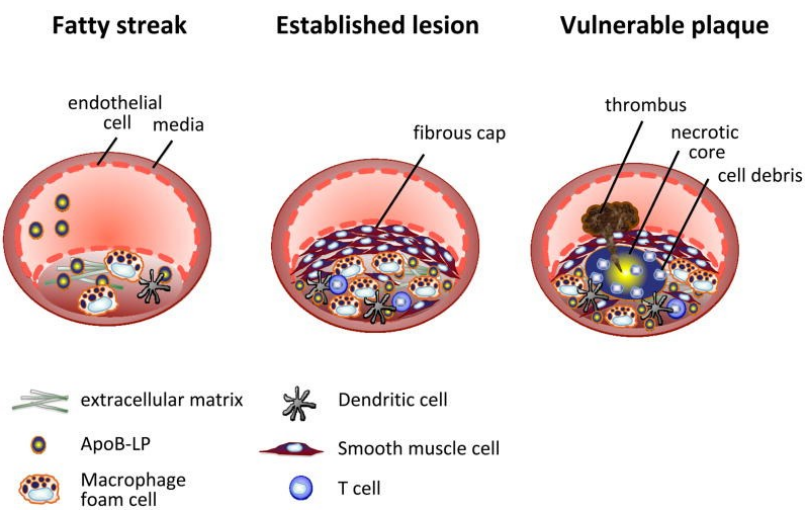

Figure 2. Progression of atherosclerosis (Figure 2 dapted from publisher, Reference [32])

MicroRNAs

The first small non-coding RNAs, microRNAs, were discovered in 1993 by Lee et al.[37] but it took 7 years to discover the second on by Reinhart et al.[38]. MicroRNAs, double-stranded and about 22 nucleotides long, are known to regulate protein expression, generally by interacting with the 3′-untranslated regions (3′UTRs) of target messenger-RNA (mRNAs)[39]. Circulating microRNAs are attractive biomarkers due to their accessibility since they can be found in different body fluids like serum, plasma, urine, and saliva,[40]. microRNAs are protected and transported attached to lipids, exosomes or protein[41] such as Argonaute2 (AGO2)[42]. In this thesis four microRNAs were identified as biomarkers of CVD and T2DM, miR-483-5p, miR-126, miR-223 and miR-197. However, emphasis will be given to miR-483-5p.

Accumulating evidence indicate the important role of microRNAs in endocrinology and their capacity of having hormone-like activity, i.e., communication between organs via the circulation. To accept the idea that microRNAs act like hormones, they should bind to receptors, known as miRceptors, which lead to an interplay between microRNAs and specific proteins as in cancer, but also other diseases. MiRceptors are referred to proteic receptors, that involve interaction between proteins and microRNAs. The miRceptors have been reported to be involved in tumor microenvironment (TME), which is consisting of different cell types around the cancer cells, and which actively facilitate the survival of cancer cells in different ways[43]. As an example of the capacity of microRNAs acting as endocrine molecules and having own receptors, Fabbri et al. have shown that tumor-secreted two microRNAs, miR-21 and miR-29a, bind to human miRceptors, Toll-like receptors 8 (TLR8) in immune cells, triggering inflammatory responses, leading to growth and metastasis of the tumor[44]. MicroRNAs may also act within organs and tissues, thus having paracrine-like or autocrine-like functions [45,46].

An important distinction for the interpretation of the current work is that microRNAs found in the circulation may either represent endocrine signaling (between organ signaling) or tissue microRNA leakage from paracrine or autocrine like “within organ signaling”.

Biogenesis of microRNAs

MicroRNAs originate from longer RNAs which form a hairpin-structure by folding back on itself, called primary microRNA (pri-miRNA)[47]. The biogenesis pathway of the canonical miRNA, which requires both Dicer and Drosha, two Ribonuclease-III (RNase-III) enzymes, starts in the nucleus, with transcription of the pri-miRNA, by RNA polymerase II. It is then recognized and cleaved by the microprocessor complex, composing of two proteins, Drosha and DiGeorge syndrome critical region 8 (DGCR8)[48] to generate the stem-loop-like precursor-microRNAs (pre-

miRNAs) which are about 70 nucleotides long. The pre-miRNAs enter the cytoplasm by binding to exportin-5 (XPO5)[49,50]. In the cytoplasm, the pre-miRNA is then cut by RNAse III Dicer[51] to finally generate the microRNA duplex. The miRNA duplex is subsequently processed to a complex called RNA-induced silencing complex (RISC) which even include AGO2. The duplex is untwisted by AGO2 into two strands, the guide- and a passenger-strand. The guide-strand or mature miRNA, within RISC, is hybridised to target mRNA with either a perfect match leading to mRNA degradation or an imperfect match leading to translational inhibition.[52,53] (Figure 3).

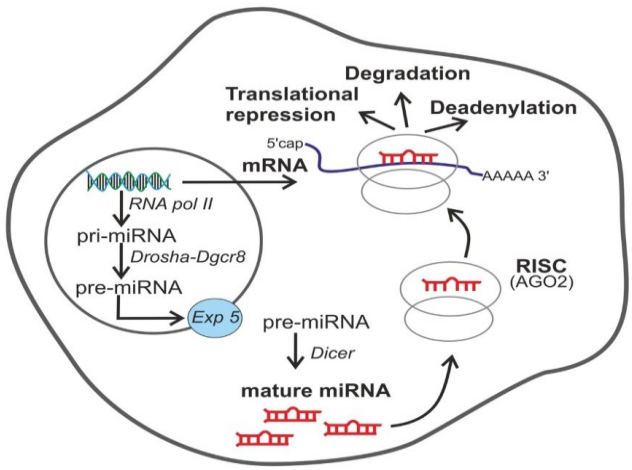

Figure 3. Biogenesis of microRNAs. Courtesy of Prof. Lena Eliasson

MicroRNAs as biomarkers in Cardiometabolic Disease

As circulating microRNAs have been shown to regulate gene expression post transcriptionally, they soon became attractive tools for prediction of numerous diseases including cardiometabolic disease. Several studies have been performed to profile and identify circulating microRNAs that might show altered pattern in e.g., diabetes[41,54,55], CVDs[56] and obesity[45,57]. In diabetes for instance, endothelial miR-126 has been shown to be down regulated[58-60], but positively associated with risk of myocardial infarction[61].

MicroRNAs, such as miR-223 and miR-197 have been reported to have lower levels in diabetes and in risk of myocardial infarction, [58,61].

MicroRNAs as therapeutics

MicroRNAs have been known to be involved in gene regulation for a while [53], which makes them becoming interesting tools for therapeutics. There are two types of microRNA-based therapeutics. The first one is miRNA mimics which are composed of double-stranded small RNA molecules. These molecules match the microRNA sequence which can restore the vanished or reduced microRNA-expression in different disorders. The second type are inhibitors, also called antimiRs. Different types of inhibitors are used in laboratory like miRNA sponges, antagomiRs, anti-miR, oligonucleotids and locked nucleic acids (LNA). AntagomiR, for example, is a modified antimiR by the Locked Nucleic Acids (LNA™) technology, which is thought to increase the effectiveness and stability to the complementary sequence of the target miRNA to inhibit its function[46,62,63]. Numerous microRNAs are now used in clinical trials. In hepatitis C virus (HCV) infection for example, the HCV genome is protected and upregulated by miR-122. Upregulating of the virus genome by miR-122 is inhibited by antimiR-122, which leads to reduction of HCV induced liver damage[64-66]. Further, antimiR-103/107 is tested in an ongoing trial in type 2 diabetes to increase insulin sensitivity[67]. Other therapeutics microRNAs in clinical trials that are ongoing, are nicely reported in review[62].

MiR-483-5p and Insulin-like growth factor-2 (IGF-II)

Insulin-like growth factor-2 (IGF-II), a gene located on the short arm of the 11th chromosome, 11p15.5, encodes a peptide consisting of 67 amino acids. IGF-II is a member of the insulin family. The insulin gene (INS) is located only 1.4 kilobases (kb) upstream of the 5´of the IGF-II gene. The IGF-II gene is maternally imprinted, which means that the gene only is paternally expressed. IGF-II has been shown to be involved in lipid metabolism and obesity[45,68]. The reverse transcription of IGF-II generates miR-483[69], which was identified with cloning of miR-483 from human liver[70]. Pri-mir-483 encodes two microRNAs, miR-483-3p and miR-483-5p[71]. The MIR-483 gene is located between exons 8 and 9[72] on chromosome 11. MIR-483 gene increases the transcription of three IGF-II promotors (P2, P3 and P4) and by that enhancing the expression of its host gene, IGF-II[73]. Both IGF-II and miR-48-5p have been reported to be co-expressed[74].

It is intriguing to mention that in mice, miR-483 has been shown to be higher expressed in pancreatic β-cells than α-cells. By targeting suppressor of cytokine signalling3 (SOCS3), overexpressed miR-483 increased the transcription of insulin in β-cells but decreased the transcription of glucagon in α-cells[75]. Furthermore, glucose intolerance and hyperglycemia, due to loss of miR-483, was shown in high-fat-diet fed, miR-483 knockout mice[76].

Metabolites

Metabolomics is the "omics" that is used for identification and quantification of biological small molecules (metabolites). Using metabolomics, metabolites can be measured in numerous biological compartments, including human serum and plasma[77] and gives us a picture of the metabolism in that biological compartment. As the metabolism is influenced by many factors, such as diet, physical activity, smoking, alcohol consumption etc, alterations may be reflected in metabolite levels.

It has been shown that some of metabolites, especially isoleucine, leucine and valine collectively named branched-chain amino acids (BCAAs), could be detected at higher levels in obese compared to lean individuals. The BCAAs were even shown to be associated with insulin resistance[78]. Several studies confirmed the association between BCAAs and diabetes which were elevated in diabetics compared to healthy individuals[79,80]. In the current thesis, we hypothesized that micro-RNAs may be involved in regulation of metabolomic alterations, which occur before derangement of known cardiometabolic risk factors and onset of overt cardiometabolic disease.

Aims

General Aims

The aim of this thesis was to identify circulating serum microRNAs in healthy subjects, that predict future onset cardiometabolic disease by profiling a panel of microRNAs, using quantitative polymerase chain reaction (q-PCR) based method. We also tested relationship between some microRNAs, which have been implicated by previous literature, and cardiometabolic disease.

Specific Aims for individual papers

I. To identify microRNAs that associate with risk of both diabetes and cardiovascular disease, by screening a subset of previously profiled microRNAs in serum.

II. To replicate the findings from the first paper by examine miR-483-5p in a new prospective cohort for relationship with diabetes, cardiovascular disease, and carotid atherosclerosis.

III. To investigate the relationship between miR-483-5p and lifestyle factors as well as with plasma metabolites.

IV. To investigate whether plasma level of microRNAs, implicated in cardiometabolic disorders by previous literature, are reproducibly associated with carotid atherosclerosis, incident diabetes and incident cardiovascular disease.

Methods

Malmö Diet and Cancer Study-Cardiovascular Cohort

The Malmö Diet and Cancer study (MDC), 1991-1996, is a population-based prospective cohort study in Malmö, Southern Sweden[81]. The main aim of this cohort study was to investigate the impact of diet on different kind of cancers. Men born 1923-1945 and women 1923-1950 were invited to participate in the study, which was a total of about 74,000 individuals, The recruitment was performed either through advertising in public places or by personal contact[82]. Subjects with language difficulties or mental incapacity were excluded from the study, contributing to decreased number of participants in which 68.905 were eligible. Finally, by the end of the baseline study, 28.098 subjects (11, 063 Male and 17,035 Female) were included in the cohort study, corresponding to a participation rate of 41%.

At baseline, all participants underwent physical examination and fasting blood-samples were drawn, collected in cryovials and stored at -80°C at the Malmo biological biobank[83]. Between 1991- 1994, half of MDC were randomly invited to participate in a sub-cohort study, the Malmö Diet and Cancer Study-Cardiovascular Cohort (MDC-CC), with the aim to study the epidemiology of carotid artery disease[84] and which resulted in inclusion of 6,103 participants. Twelve fasting individual serum samples from MDC-CC, healthy subjects at baseline, were selected to shape the pilot study. The study included four subjects that developed DM, four CVD and four healthy control during a follow up of ca 16 years. The next and larger case/control study consisted of 553 healthy subjects at the baseline, of whom 140 had developed DM and 169 CVD during the same follow up time as mentioned above. Data from this case/control study was analysed in paper I, II, III, IV.

Malmö Offspring Study

Malmö offspring study (MOS) is an ongoing population-based family study, launched in 2013, currently with 4300 participants consisting of children and grandchildren (18-60 years)[85] of the former and bigger cohort study, MDC-CC that

was initiated in 1991[81]. All subjects had to manage the Swedish language well to be able to participate. The invitation was performed by sending mail and were followed-up by phone calls. In April 2017, more children and grandchildren could be included in the study by expanding the geographical area to the region of Skåne, southern Sweden. Anthropometrics measurements, which include waist, weight, height, and hip circumference, were taken and fasting venous blood was drawn. 1221 serum samples were analysed for paper II and IV.

Physical Activity and Dietary Assessments

In MDC-CC a questionnaire including 17 different activities, adapted from the Minnesota Leisure Time Physical Activity Questionnaire, was used for assessment of physical activity[86,87]. Each duration of each physical activity was multiplied with an intensity factor, to create a score [88]. In MOS, depending on the participants' level of leisure time physical activity, they were assigned in to four categories. First: light exercise < 2 hours/week; second: light exercise > 2 hours/week: third: moderate exercise 1-2 per week and fourth: regular exercise > 3 times a week[89].

In MDC-CC, intake of the daily diet was measured by a diet history methodology, specially designed for MDC study. It was composed of a 7-day menu-book together with an interview about diet history for ca. one-hour and a dietary questionnaire consisting of 168-item about regular diet during the past year[90,91]. In MOS, a 4-day food record was used, by Riksmaten 2010, which was developed by the Swedish National Food Institute[92].

Measurements of carotid atherosclerosis

MDC-CC: the right carotid artery was examined by ultrasound, using a 7 MHz transducer Acuson Seqouia (Acuson, Mountain View, California). Scanning-images of the right carotid artery was obtained in a pre-defined window with three centimetres of the distal common carotid artery, the bifurcation area (bulb) and one centimetres of internal and external carotid arteries. Plaque-estimation was defined as intima media thickness (IMT) > 1.2 mm. The bulb maximum IMT, IMT-bulb, was measured off-line and calculated using a mean of three measurements, by Artery Measurement System (AMS)[93].

MOS: Ultrasound examinations were performed using Logiq E9 (GE Healthcare). Images of the left and right common carotid artery and the bifurcation were obtained in a predefined window and captured at end-diastole. Measurements of IMT-bulb were performed out off-line using the AMS program. A plaque was defined as a focal thickening of the intima layer with a height > 1.2 mm. All plaques visible in the bifurcation, and in the common, internal, and external were reported[94].

Clinical Measurements and Endpoint Definitions

All participants in both cohorts underwent a questionnaire about e.g., medical history, physical examination, and laboratory assessment. BMI was defined as the ratio of weight (kg) divided by squared height (m). The mercury-column sphygmomanometer was used to measure systolic and diastolic blood pressure in mmHg. Antihypertensive treatment (AHT) was defined as currently taking antihypertensive medication. Measurements of fasting glucose, HDL cholesterol, total cholesterol, and triglycerides were performed at the Department of Clinical Chemistry, Malmö University Hospital. The Friedewald equation was used to calculate LDL cholesterol. Smoking was defined as current smoker (within the past month). Insulin measurements were performed using Mercodia Insulin ELISA 188 (Mercodia, Uppsala, Sweden). Definition of Type2 Diabetes (T2D) was as a fasting plasma glucose concentration of >7 mmol/L or self-reported physician diagnosis of T2D or using antidiabetic medication or having been registered in local or Swedish diabetes registries[95].

Definition of cardiovascular disease events included coronary artery disease (CAD) or fatal or non-fatal stroke. CAD was defined as myocardial infarction (fatal and non-fatal) or death due to ischemic heart diseases. Myocardial infarction (MI) was defined based on International Classification of Diseases, 9th and 10th Revisions (ICD-9 and ICD10) code 410 and 121. Death due to ischemic heart disease was defined based on codes 412 and 414 (ICD9) or I22-I23 and I25 (ICD10). Stroke (fatal and non-fatal) was defined based on codes 430, 431, 434 and 436 (ICD9) and I60, I61, I63, and I64 (ICD10). Death from cardiovascular causes was defined based on ICD-9 codes 390-459 and ICD-10 codes C00-D48. From the Swedish Coronary Angiography and Angioplasty Registry (SCAAR) and from registry codes of the Swedish classification system of surgical procedures, the Coronary revascularizations could be identified[96]. For definition of CABG, following was used: codes 3065, 3066, 3068, 3080, 3092, 3105, 3127 or 3158 in the Op system or code FN in the KKÅ97 system.

Molecular Biology Methods

RNA isolation

The same RNA isolation method, miRNeasy 96 Total RNA isolation kit (Qiagen), was used for all human serum samples regardless cohort. A modified Qiagen protocol made by EXIQON was used. In this protocol the amount of Qiazol, Chloroform and water was modified. After thawing serum samples in ice, 250 µl

was centrifuged for 5 min at 1000g at 4˚C to remove debris, using 1.5 ml nuclease-free microcentrifuge tubes for pilot study consisting of 12 sample, and 96-well plates for two bigger sample set consisting of 553 and 1223 samples. 200 µl of the serum samples were transferred to new tubes/plates. A total of 750 µl Qiazol mix (1.25 µl 0.8 ug/ul MS2 RNA, Roche Cat. #10165948001added to 800 µl Qiazol) and after mixing, 200 µl Chloroform was added. A centrifugation of 12.000 g in 4˚C generated an aqueous phase which was transferred to new tubes/plates. 100% ethanol was added to aqueous phase which then was transferred to the RNeasy mini–Spin. After some washing steps, the mixture was further processed following the manufacturer's recommendations, and finally, the total RNA was eluted with 50 µl RNase-free water and stored at −80˚C.

Reverse transcription - quantitative polymerase chain reaction (RT-qPCR) and cDNA

MDC-CC

Complementary-DNA (cDNA) synthesis was performed starting with pre-amplification step by reverse transcription using the Megaplex RT Primers. Those are two pre-defined pools (Pool A and B) of about 380 stem-looped reverse transcription primers per pool that are used to synthesize single stranded cDNA from total RNA. The pre-amplification of the cDNA target to increases the amount of the cDNA for gene expression analysis with a set of two pools made of gene-specific forward and reverse primers for the use of very small amount material.

The TaqMan arrays consists of dried TaqMan primers and probes and are designed as a set of two 384-well microfluidic cards, Array A and B. This makes it possible to quantify up to 384 microRNAs and controls per card. The real-time PCR reaction is based on the 5′nuclease assay within TaqMan probes. TaqMan MGB probe contains a reporter dye (FAM or 6-carboxyfluorescein) attached to its 5′end, a nonfluorescent quencher (NFQ) and a minor groove binder (MGB), both attached to its 3′end. The fluorescence signal is inhibited by the Quencher dye because of its′ proximity to the Reporter dye. When Primers and probe anneal to the cDNA template, leading to the cleavage of the Quencher from the Reporter dye, the signal is emitted. For each cycle, the number of the cDNA template is doubling and so the fluorescence intensity.

To calculate the concentration, the threshold cycle (Ct) must be validated. The number of cycles needed for the fluorescence signal to pass the threshold, represent the definition of Ct. The relationship between the levels of Ct and the amount cDNA is inversely related, i.e., the higher the concentration of the target, the lower the Ct-value.

MOS

For cDNA synthesis, TaqMan Advanced NA Assays (Cat# A25576) protocol was performed. Poly(A) polymerase was used for adding a 3´adenosine tail to the miRNA to generate poly(A) tailing. Further, an adaptor ligation reaction at the 5´end occurred, where the adaptor works as forward-primer binding site for the Mir-Amp reaction to synthesize single strand cDNA. The process continued with Reverse Transcription (RT) reaction of microRNA where the RT primer bound to the 3´poly(A) tail and a miR-Amp reaction to increase the number of cDNA molecules. For performing qPCR, custom plated 384-well TaqMan plates (Thermofisher Scientific) with eight unique miRNA assays per plate was used. Real-time PCR was performed according to manufacturer's recommendation on ViiA 7 Real-Time PCR System (Thermofisher Scientific).

Normalization

To account for technical variability, cycle threshold (CT) values vs. internal controls had to be normalized. In pilot study (MDC-CC), qPCR-based panel with 753 human microRNAs in 12 serum samples where profiled. We set a cut-off of $Ct \leq 37$ in all 12 samples. To find the most suitable normalizer, stably expressed microRNAs with the lowest coefficient of variation (CV) were used. MiR-574-3p, miR-331 and miR-197 with CV of 3.0%, 3.2% and 3.7% respectively, were chosen and their geometric means were used for normalization. Based on this normalization strategy, 47 microRNAs were chosen for to screen for a larger cohort consisting of 553 serum samples from MDC-CC.

For the screening study with 553 serum samples, the GeNORM procedure[97] was applied for identification of endogenous controls. 15 miRNA assays expressed in all 553 samples were evaluated, of which two microRNAs, hsa-miR-17 and hsa-miR-106a, were shown to be the most stably expressed ones. The geometric mean of the selected microRNAs was used as endogenous controls for the relative quantification using the $2^{-\Delta\Delta Ct}$ method.

For MOS the most stable microRNA with less variation between samples was hsa-miR-17-5p and was selected to be used as normalizer. Log-transformed Ct-values from the selected samples were normalized against log-transformed miR-17-5p. Undetermined assays were coded as 40.

Statistical Analysis

In this thesis, logistic regression with age and sex adjusted odds ratio, expressed per one standard deviation increment of log transformed microRNA level (or per quartile) was applied in relation to binary outcomes.

Linear regression analysis was used to evaluate the relationship of miR-483-5p to continuous outcomes such as intima media thickness and the number of plaques in paper II, where beta- coefficient was expressed per one standard deviation (SD) increment of log transformed microRNA.

Adjusted for age and sex, partial Spearman´s correlation tests, were used to in paper III to test for correlation between miR-483-5p and physical activity in both MDC-CC and MOS.

Partial Spearman´s correlation tests were applied to test for correlation of miR-483-5p to both diet and plasma metabolites in both cohorts using R as statistical tool.

Cross sectionally, Pearson´s correlation test was applied for correlation between miR-483-5p and all classical risk factors in paper I and II.

Bonferroni correction was used in paper I, to correct the significant level for multiple comparisons in the correlation analyses between all 47 microRNAs and DM and CVD, respectively.

Results

This thesis is about identifying circulating serum microRNAs that might predict new onset cardiometabolic disorders (CMD). Specifically, one microRNA, miR-483-5p, was shown to be associated with increased risk for CMD in two Swedish cohorts, MDC-CC, and MOS. Since miR-483-5p also was positively related to obesity and insulin resistance, we investigated its role regarding metabolites and lifestyle, such as diet, physical activity, and smoking. The baseline characteristics of both cohorts are listed in Table 1.

Table 1: Baseline clinical characteristics of subjects in MDC-CC and MOS

	MDC-CC		MOS	
	n	mean (SD)	n	mean (SD)
Age, y	553	59.16 ± 5.8	1221	41.62 ± 13.6
Women, %	553	51.2	1221	51.8
BMI, kg/m2	553	27.06 ± 4.56	1221	26.04 ± 4.68
Waist circumference, cm	553	89 ± 14	1221	90 ± 14
Fasting glucose, mmol/L	553	5.37 ± 1.26	1219	5.48 ± 0.96
LDL cholesterol, mmol/L	553	4.27 ± 0.92	1217	3.21 ± 1.02
HDL cholesterol, mmol/L	553	1.29 ± 0.33	1218	1.6 ± 0.48
Triglycerides, mmol/L	553	1.47 ± 0.70	1208	1.17 ± 0.70
Systolic Blood Pressure, mmHG	553	147.69 ± 19.97	1165	120.62 ± 17.33
Diastolic Blood Pressure, mmHG	553	89.72 ± 9.57	1165	83.33 ± 10.71

Paper I

The main aim of this study was to identify circulating serum microRNAs in subjects that had developed diabetes and cardiovascular disease during a follow-up of 16 years. We used qPCR-based methods for profiling of 753 human microRNAs in 12 subjects of whom 47 were chosen to be further analysed in 553 incident diabetes and cardiovascular cases in MDC-CC as they were expressed in all 12 subjects. Cross sectional analysis showed that miR-483-5p to has a strong relationship to risk factors, such as BMI, waist, insulin, and triglycerides, and HDL (Table 2).

Table 2: Correlation between miR-483-5p and classical risk factors

		Age	Sex	BMI	Waist	Insulin	HDL	TG
miR-483-5p	Pearson Correlation	.041	-.013	.162**	.135**	.156**	-.099*	.110**
	Sig. (2-tailed)	.340	.766	.0001	.001	.0002	.020	.010
	N	553	553	553	553	548	553	553

Correlation of miR-483-5p to BMI, waist, insulin, triglycerides (TG) and HDL in MDC-CC, (n= 553)

After adjustment for age and sex, increasing quartile of miR-483-5p showed significant relationship with incident DM with a p for trend= 0.006 (Table 3) and with incident CVD, with a p for trend= 0.032 (Table 4). After full adjustment, higher quartile of miR-483-5p levels remained significantly associated with only incident CVD (p for trend= 0.037) (Table 4).

Each 1 SD increase of the miR-483-5p level (p= 0,001) was associated with a 48 % increased risk of future DM (Table 3) and a 40% increased risk of future CVD (0.001) with adjustment for age and sex. (Table 4). After adjustment for risk factors, the relationship between miR-483-5p and incident CVD remained virtually unchanged whereas the relationship with incident diabetes weakened (Tables 3-4).

Table 3: miR-483-5p and incident diabetes

	Continuous analysis (per SD increment)	P-value	*Quartile1*	*Quartile2*	*Quartile3*	*Quartile4*	P for trend
N / N events[a]	390 / 140		97 / 31	98 / 27	98 / 36	97 / 46	
OR (95% CI) (age and sex adjusted)	1.48 (1.18-1.84)	0.001	1.0 (ref)	0.84 (0.45-1.56)	1.24 (0.68-2.25)	2.11 (1.16-3.83)	0.006
OR (95% CI) (fully adjusted)*	1.28 (1.00-1.64)	0.049	1.0 (ref)	0.64 (0.33-1.27)	0.97 (0.51-1.82)	1.40 (0.73-2.68)	0.167

[a]Values for the variables are displayed as mean (SD, Standard Deviation); 95% confidence interval (CI) for odds ratio (OR).

*Adjusted for age, sex, BMI, HDL, TG, and insulin.

Table 4: miR-483-5p and incident CVD							
	Continuous analysis (per SD increment)	P-value	Quartile1	Quartile2	Quartile3	Quartile4	P for trend
N / N events[a]	428 / 169		71 / 36	68 / 39	65 / 42	55 / 52	
OR (95% CI) (age and sex adjusted)	1.40 (1.14-1.71)	0.001	1.0 (ref)	1.09 (0.62 - 1.93)	1.26 (0.72-2.21)	1.80 (1.03-3.14)	0.032
OR (95% CI) (fully adjusted)*	1.46 (1.18-1.84)	0.0005	1.0 (ref)	0.95 (0.52-1.72)	1.14 (0.63-2.07)	1.80 (1.00-3.24)	0.037

[a]Values for the variables are displayed as mean (SD, Standard Deviation); 95% confidence interval (CI) for odds ratio (OR).
*Adjusted for age, sex, SBP, AHT, current smoker, LDL, HDL, Diabetes Mellitus, TG, BMI, and insulin.

Paper II

As we in our first paper were able to show that miR-483-5p was associated with new onset cardiometabolic disorders, the aim of this study paper was to replicate these findings in a second Swedish cohort, MOS. We measured circulating miR-483-5p in 1223 healthy subjects at baseline exam, using a qPCR-based method. Cross sectional analysis was used to test the correlation between miR-483-5p and risk factors whereas logistic regression was applied for the association to diabetes and coronary heart disease (CAD). In this study, we also investigated whether miR-483-5p could relate to atherosclerosis, by measuring the intima media thickness of the carotid bulb with ultrasound (IMT-bulb) in both cohorts and the number of plaques in MOS, using linear regression.

During a follow-up of about 3.5 years, 12 subjects developed diabetes and 14 developed CAD. MiR-483-5p was significantly associated with incident DM and with incident CAD, in an age and sex adjusted logistic regression model with a p value of 0.032 for DM and 0.033 for CAD.

Like in paper 1, Pearson correlation analysis showed a significant positive correlation between miR-483-5p and triglycerides, BMI, and waist and negative correlation with HDL. No relationship with glucose was detected. As miR-483-5p was correlated to sex (higher in males than in females), we performed partial correlation analyses corrected for age and sex, which weakened the significance levels somewhat and made the relationship with HDL non-significant (Table 5).

Table 5: Correlation between miR-483-5p and diabetes risk factors in MOS

	Age	Sex	HDL	TG	BMI	Waist	Glucose
N	1221	1221	1218	1208	1221	1221	1219
Pearson Correlation	0.02	-0.100**	-0.085**	0.100**	0.099**	0.119**	0.023
P-value	0.40	0.0004	0.003	< 0.001	0.001	< 0.001	0.432
Partial Pearson Correlation			-.050	.076	0.078	0.082	0.006
P-value			0.083	0.008	0.007	0.004	0.841

Pearson Correlation: miR-483-5p correlated positively with TG, BM, Waist and negatively with HDL.
** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
Partial Correlation: miR-483-5p correlated positively with TG, BMI and Waist.

We found a significant association between miR-483-5p and IMT-bulb, a measure of atherosclerosis, in MDC-CC, but not in MOS. On the contrary, in MOS, miR-483-5p was related to the number of plaques (Table 6).

Table 6. Association between miR-483-5p and IMT-bulb and the number of Plaque respectively

	IMT-bulb (mm)				no.of Plaquesb	
miR-483-5p	MDC-CC (N=401)		MOS (N=485)		MOS (N=699)	
	beta	P-value	beta	P-value	beta	p-value
	0.196 (0.08-0.22)a	< 0.001	0.023 (-0.02-0.04)	0.56	0.09 (0.02-0.15)a	0.007

Age and sex adjusted
aBeta-coefficients are expressed per one SD increment of log-transformed miR-483-5p
bAbsence of plaques=0, 1 plaque=1, >1 plaques=2

Paper III

We previously reported our findings about the association of miR-483-5p with new onset T2D and CVD in two Swedish cohorts, MDC-CC, and MOS. In a very first attempt to approach relationships with therapeutically modifiable factors, we aimed in this study to investigate whether lifestyle factors such as diet, physical activity and smoking were related to miR-483-5p. Furthermore, we investigated if alterations of miR-483-5p might be reflected by changes in the plasma metabolome.

In age and sex adjusted model, circulating miR-483-5p showed significant negative associations with physical activity in both MDC-CC (r= -0.092, p=0.034) and MOS (r= -0.091, p=0.004) whereas no correlations were found between miR-483-5p and diet or smoking.

Table 7. Correlation of miR-483-5p with Metabolites in MDC-CC and MOS

	MDC-CC (n=467)		MOS (n=1210)	
Metabolites	rho	p-value	rho	p-value
Leucine	0.092	0.048	0.11	0.001
Isoleucine	0.13	0.007	0.007	0.01
propionylcarnitine	0.14	0.003	0.11	< 0.001
isovalerylcarnitine	0.12	0.007	0.12	<0.001
Beta-carotene	-0.11	0.018	-0.18	<0.001

Adjusted for age and sex

In both cohorts, in the age and sex adjusted model, a positive correlation was found between miR-483-5p and plasma levels of leucine, isoleucine, propionylcarnitine, isovalerylcarnitine but negative correlation with beta-carotene (Table 7).

Paper IV

A set of plasma microRNAs, miR-126, miR-197 and miR-223, were previously reported to be associated with incidence of cardiometabolic disease (addera ref for Meyer). Our aim here was to investigate if the serum levels miR-126, miR-197 and miR-223 were reproducibly associated with incident diabetes, incident cardiovascular disease and carotid atherosclerosis, measured as intima media thickness in the bulb (IMT), in two Swedish cohorts, MDC-CC (n= 553) and MOS (n=1221).

Table 8. miR-126 and incident diabetes in MDC-CC and MOS

	MDC-CC			MOS		
	N / N events	Continuous analysis (per SD increment)	P-value	N / N events	Continuous analysis (per SD increment)	P-value
OR (95% CI) (age and sex adjusted)	390/140	1.32 (1.07-1.63)	0.01	1177/12	1.58 (0.89–2.81)	0.12
OR (95% CI) (fully adjusted)	390/139*	1.26 (1.00-1.66)	0.05	1177/12	1.53 (0.85–2.73) **	0.15

* fully adjusted for Age, Sex, Body Mass Index, High Density Lipoprotein, Triglycerides, and Insulin
* fully adjusted for Age, Sex, Body Mass Index, High Density Lipoprotein, and Triglycerides

In MDC, an age and sex adjusted logistic regression showed a positive association between log-transformed levels miR-126 and incident diabetes. However, no association was longer detected after full adjustment of risk factors. The results were not replicated in MOS (Table 8).

In MDC-CC, logistic regression showed no association between miR-126, miR-197 and miR-223 and incident CVD. The associations remained non-significant when fully adjusted, with all risk factors included or in a third model where the three microRNAs were added to all risk factors simultaneously (Table 9). Linear

regression showed positive association between (per SD increment) miR-126 and IMT after age and sex adjustment and in the third model with all risk factors and all three microRNAs included. Even between miR-223 and IMT a positive association was found but only when all three microRNAs were added to risk factors. No association was found for miR-197 (Table 9).

Table 9. Risk Evaluation of three microRNAs in incident cardiovascular disease (CVD) and Intima Media Thickness (IMT) in MDC

	CVD			IMT		
	N/N	OR (95% CI)	P-value	N/N	Beta (95% CI)	P-value
miR-126	428/169*	1.12 (0.92-1.36)	0.25	401	0.08 (0,002–0.13) *	0.04
	428/167a	1.13 (0.92-1.38)	0.27	399	0.06 (-0.01-0.13) a	0.08
	428/167b	1.07 (0.82-1.35)	0.58	399	0.08 (0.01-0.15) b	0.02
miR-223	428/169*	1.03 (0.85-1.25)	0.73	401	0.06 (-0,002–0.13) *	0.06
	428/167a	1.04 (0.85-1.27)	0.7	399	0.06 (-0,003-0.13a	0.06
	428/167b	1.08 (0.88-1.32)	0.48	399	0,096 (0.03-0.17) b	0,007
miR-197	428/169*	1.13 (0.93-1.37)	0.23	401	0.02 (-0.04-0.09) *	0.49
	428/167a	1.17 (0.95-1.45)	0.15	399	0.02 (-0.04-0.09) a	0.52
	428/167b	1.14 (0.90-1.45)	0.27	399	0.03 (-0.04-0.10) b	0.35

*Adjusted for age and sex.

a Adjusted for age, sex, systolic blood pressure (SBP), Anti-hypertensive treatment AHT), current smoker, Low density lipoprotein (LDL), High density lipoprotein (HDL), Diabetes Mellitus (DM), triglycerides (TG), body mass index and insulin (BMI).

b Adjusted for age, sex, SBP, AHT, current smoker, LDL, HDL, DM, TG, BMI, insulin, miR-126-3p, miR-197-3p and miR-223-3p.

In MOS, miR-197 was not available and therefore evaluation included only miR-126 and miR-223 in the third model (all risk factors and both micro-RNAs). Here, no association was found between miR-126 and CVD and IMT, neither after age and sex adjustment, nor in fully adjusted model or when miR-126 and miR-223 were included simultaneously. (Table 10).

Table 10. Risk Evaluation of two microRNAs in incident cardiovascular disease (CVD) and Intima Media Thickness (IMT) in MOS

	CVD			IMT		
MicroRNAs	N/N	OR (95% CI)	P-value	N/N	Beta (95% CI)	P-value
miR-126*	1208/14	1.17 (0.70-1.97)	0.54	1221/483	0.01 (-0.02-0.04)	0.42
miR-126**	1208/08	1.41 (0.57-3.50)	0.46	1221/414	0.02 (-0.02-0.05)	0.33
miR-223*	1028/13	1.06 (0.61–1.82)	0.82	1221/452	0.01 (-0.03-0.38)	0.69
miR-223**	1208/06	1.08 (0.47-2.49)	0.85	1221/389	-0.01 (-0.04-0.03)	0.64

* Adjusted for age, sex.

** Adjusted for age, sex, systolic blood pressure, Anti-hypertensive treatment, current smoker, Low density lipoprotein, High density lipoprotein, Diabetes Mellitus, triglycerides, body mass index.

Discussion

Circulating microRNA in Cardiometabolic Disease

Cardiometabolic disease (CMD) is the leading cause of death worldwide, which increases the need for more effective biomarkers for predicting clinical outcomes long before onset of CMD. Lately, microRNAs have become more and more attractive tools for treatment and prediction of different diseases. By profiling circulating human serum microRNAs, this thesis is contributing to understand how microRNAs are related to cardiometabolic disease. The focus of the discussion will be on miR-483-5p and aims to put the results into a bigger context and speculate about future developments and implications.

Mir-483-5p and CMD

In paper I, miR-483-5p showed an association with incident diabetes and incident CVD, but also with cardiometabolic risk factors, such as BMI, waist circumference, insulin resistance, triglycerides, and HDL cholesterol in the prospective cohort, MDC-CC. These findings could later be replicated in paper II, where miR-483-5p was investigated in a second independent cohort, MOS. In paper II, positive associations were found between miR-483-5p and incident diabetes and incident coronary artery disease (CAD). MiR-483-5p was also related to intima media thickness and plaque, which are used to evaluate atherosclerosis. Our studies are the largest of their kind, but some evidence from smaller studies have also shown the relationship between miR-483-5p and cardiometabolic disease and its risk factors. Nunez Lopez et al. showed in a study consisting of 24 individuals with early diabetes, the baseline levels of miR-483-5p to be correlated with fasting glucose levels, baseline levels of HbA1c, among others[98]. Otherwise, prior literature on miR-483-5p as a predictor of cardiometabolic disease is scarce.

Hypothetical Tissue Source of miR-483-5p

Since no functional analysis were made for this thesis, the tissue-source for microRNAs, including miR-483-5p was not determined. In this thesis, we rather have a hypothesis of which tissue might be the source for miR-483-5p production based on the current literature on miR-483-5p. MIR-483, encoded within the IGF-

II gene, gives rise to two mature microRNAs, miR-483-3p and miR-483-5p. It has been shown that miR-483-5p is co-expressed with its host gene, IGF-II, thereby enhancing the expression of IGF-II. IGF-II is a polypeptide, consisting of 67 amino acids and located on chromosome 11 together with insulin and H19, a gene for long non-coding RNA (lncRNA) that is paternally imprinted. IGF-II is maternally imprinted, which means that only the paternal gene is expressed, and is involved in regulation of lipid metabolism and body weight[74].

Adipose tissue is thought to secrete microRNAs into the blood stream via extracellular vesicles and enables the crosstalk between itself and other tissues, such as liver, pancreas, muscle and cardiovascular system[45]. Since the tissue-source of miR-483-5p is unknown but given the replicable association with BMI and waist circumference, we hypothesize that it might be the adipose tissue. Adipose tissue is an interesting tissue with multiple functions as a storage place for triglycerides but also an endocrine tissue releasing different kinds of hormones. It comprised of white adipose tissue (WAT) to store energy, and brown adipose tissue (BAT) endocrine organ releasing adipokines. It is suggested that microRNAs are secreted from the adipose tissue and thereby acting in an endocrine fashion, cell-to-cell communicating with distant organs like liver and skeletal muscle to exert their function by releasing their content. This action is enabled by exosomes, small extracellular vesicles, < 200 nm, derived from multivesicular body[99]. However, what we observe in serum may also simply reflect paracrine and autocrine-like effects of miR-483-5p inside adipose tissue (and/ or other source tissues), with the variation of serum miR-483-5p concentration representing leakage, the degree of which is in equilibrium with the intracellular production of miR-483-5p. A third source affecting level of miR-483-5p in serum is tissue-damage or inflammation, i.e. that miR-483-5p serum concentration simply reflects degree of leakage from the source tissue due to different degree of permeability/leakage.

miR-483-5p and Lifestyle and Metabolite

In this section which includes paper III, the relationship between miR-483-5p and different lifestyle factors will be discussed. Higher serum levels of miR-483-5p showed a negative correlation to physical activity. Causal relationship could not be obtained due to the observational and cross-sectional nature of the study, because of temporal sequences cannot be established. Causality means change in exposure leads to change in outcome. However, confounders cause problems. The relationship between the exposure and outcome may not be causal because of a confounder. The relationship between miR-483-5p as and physical activity might be affected by several confounders, for example BMI, where individuals with higher level of physical activity tend to have higher BMI. MiR-483-5p was not related to any diet variables. However, a correlation was found between miR-483-5p and beta-carotene. Serum beta-carotene concentrations were shown to be associated with

fruits and vegetables in a study comprising 700 participants[100] Thus, beta-carotene is a suitable biomarker for intake of fruits and vegetables. This is not consistent with the lack of correlation between miR-483-5p and intake of fruit and vegetables. One can speculate that beta-carotene more reflects intake of specific vegetables and which miR-483-5p may be related to.

We hypothesized that metabolite level in plasma would represent a metabolic fingerprint that represent early dysmetabolic processes that are not yet possible to see in terms of BMI or waist or other clinical measures of metabolic risk. Speculatively, dysregulation of microRNA production and/or action would be an initial event that subsequently affects gene expression and altered enzyme production/activity in target tissues, which in turn lead to a metabolic signature that contributes to overt disease risk.

Branched-chain amino acids (BCAA), consisting of leucine, isoleucine, and valine, are essential amino acids that must be obtained from diet. These metabolites are strongly associated with diabetes[78]. In paper III, Leucine and isoleucine have shown positive associations with miR-483-5p. There are two primary steps in the catabolism of BCAAs. The first is performed by the branched-chain amino acid transaminase (BCAT) and the second (and irreversible) step is catalysed by the branched chain α-ketoacid dehydrogenase (BCKD)[101]. Mersey et al. reported miR-29b to inhibit the translation of BCKD[102], indicating the potential involvement of microRNAs in amino acid catabolism. Based on the consistent correlations between miR-483-5p and BCAA metabolites, we speculate that miR-483-5p may target either of the catabolic enzymes, BCAT or BCKD, in the BCAA catabolism. This could lead to increased levels of the BCAA in the circulation, explaining the positive correlation observed in paper III. This theory may seem inconsistent with simultaneously observing positive correlations between miR-483-5p and propionylcarnitine and isovalerylcarnitine, which are produced downstream of both enzymes in the BCAA catabolism. Given that miR-483-5p can inhibit BCAA catabolism, one would rather expect this correlation to be inverse. It should however be pointed out that BCAA catabolism occurs in several tissues (primarily adipose tissue, skeletal muscle and liver)[101], all of which may not be the target tissue for miR-483-5p. This theory is intriguing since BCAA have been suggested to be causally related to type 2 diabetes[103] and could serve as a mediator in the association between miR-483-5p and diabetes risk.

Replication study: miR-126. miR-223 and miR-197

Our aim with paper IV was to investigate whether Zampetakis et al CVD and diabetes associated miR-126 could be replicated in our two independent cohorts regarding prediction of both diabetes and CVD. The results from Zampetaki et al however, was not concordant with our findings. One of the most important observation in this study was opposite association of miR-126 regarding both

diabetes and cardiovascular disease. While Zampetaki showed miR-126 to be down-regulated in diabetes, we showed the opposite. However, this was only for one cohort, MDC. For the second cohort, MOS, no association could be found. Also, in relation to myocardial infarction miR-126 had a negative direction and no association in our study. However, both the direction and the association were changed to opposite when two more microRNAs (miR-197 and miR-223) were added to their analysis. We did not analyse myocardial infarction only, but incident CVD (coronary artery disease and/or myocardial infarction) as well as intima media thickness and the number of plaques, the latter as markers of atherosclerosis. Again miR-126 showed the opposite direction and was positively associated to inter media thickness only. We may only speculate about possible aspects of the non-concordant findings. Although both plasma, used in the Zampetaki paper, and serum (as used in our study) are widely used diagnostic methods, differences have been reported. Still, data normalization for microRNAs is a challenging as different normalization strategies lead to different results[104]. Lower plasma level of miR-126 has been detected in patients with established diabetes or diabetes-related disease[55,105,106]. Thus, miR-126 may possibly serve as a biomarker for manifested diabetes, but not as a predictor of future diabetes risk.

Risk stratification of CVD and diabetes mellitus development

Lessons from studies on prevention of e.g., CVD have shown that the effectiveness of interventions are correlated with the risk of getting CVD, i.e., the number of individuals that need to be treated during a certain time-period to prevent one CVD event, goes down with increasing risk of CVD[107]. Therefore, *secondary prevention*, i.e., therapy aiming at reducing risk for future CVD events in individuals who already have experienced at least one CVD event at start of therapy, is the prime focus of most pharmacological prevention strategies. On the other hand, once CVD is established, it is extremely difficult to reduce the absolute risk down to levels that equals that of the population without prior CVD. Also, from a more popular point of view, it appears logical to initiate therapy aimed at preventing CVD before the disease that the therapy is aimed to prevent is established. and might have resulted in a life-threatening myocardial infarction or stroke. These are the two main arguments for investing in research on primary prevention, i.e., prevention of future disease in the healthy population. Importantly, whether the intervention in question consists of lifestyle, drugs or surgery, population wide use has proven both unrealistic and impossible. Instead, within the healthy population (healthy in regards of not having had the disease that the therapy is meant to prevent), the challenge is to find those individuals with highest risk in order to maximise the efficiency of the intervention in relation to its costs and side effects. Such risk stratification is therefore fundamental for any current or future primary preventive therapy.

We approach improved risk stratification with attempts to identify circulating microRNAs that predict development of future diabetes and CVD. Current prediction of CVD is based on age, sex, cholesterol, blood pressure, diabetes status and assessment of smoking habits, factors which are then added into scores to calculate a 10-year risk of CVD[107]. For example, in Sweden, the SCORE algorithm is used. The problem with these "traditional risk factors" is that about 50% of individuals who actually develop CAD do not have presence of any of these risk factors, or only one of them, which is insufficient to place them in a high enough absolute risk category to motivate initiation of pharmacological primary prevention with e.g. statins[108].). On the other hand, a substantial number of individuals who are indeed classified into a high enough absolute risk category for motivation of life-long pharmacological prevention never develop events and are exposed to unnecessary costs and side effects.

Our finding that miR-483-5p predicts CVD independently of traditional risk factors in subjects free from CVD at baseline is a first important step towards improved risk stratification of CVD. However, it is important to underline that our findings are far from enough to lead to improvement of risk stratification in the clinical primary preventive setting. First, the association needs to be replicated in other populations, as our studies were confined to a South-Swedish population. Second, the participation rate in both MDC and MOS was between 40 and 50%, and we do not know what the association would look like in non-participants. Empirically, non-participants are less healthy (more smokers, higher prevalence of risk factors and low socioeconomic index) and would therefore be expected to have higher rates of CVD than those studied. Third, it is unlikely that one microRNA would radically improve risk stratification on top of the traditional risk factors as each of the currently used traditional risk factors alone are quite weak (for example mild elevation of blood pressure or cholesterol by itself only marginally increases the absolute CVD risk on top of age and sex). Thus, the future relevance of miR-483-5p remains to be elucidated. We speculate though, that if its association with CVD will be replicated in other populations, even if its association with CVD is modest, it might become part of a broader biomarker score that is composed of several other circulating microRNAs, metabolites and proteins derived from other studies. Such composite biomarker scores are likely to be able to add significant clinical gains in terms of assessing absolute 10-year risk when added on top of the traditional risk factors, and thus affect primary preventive therapeutic decisions.

Apart from risk stratification of CVD, we put particular emphasis on risk stratification of future diabetes development for several reasons. First, diabetes is as potent as risk factor for future CAD in CAD free diabetes patients as CAD itself is for a subsequent event in a non-diabetic individual[109]. Moreover, whereas the prevalence of most other modifiable risk factors such as hypertension, high cholesterol and smoking are declining, diabetes continues to increase in most parts of the world[110]. As sedentary lifestyle with less physical activity and unhealthy diet

resulting in obesity is a prime suspect behind this development, it is tempting to say that the solution is population-wide campaigns with the message "eat less and walk more". However, reality shows that such campaigns have extremely limited effects as the knowledge around physical activity, poor diet, obesity, and diabetes development have been there for much more than 30 years, but during that time obesity and diabetes have not decreased, instead they have increased continuously. Therefore, we believe that biomarkers for risk stratification of future diabetes development are needed in order to identify individuals with high diabetes risk and focus primary preventive actions. Instead of population wide campaigns, one can then focus resources on intensive lifestyle interventions specifically at the high-risk individuals. It remains to be shown if this is the case, however, the idea would be that a person with knowledge of a very high level of a composite biomarker score (see above), might be more receptive for intensive lifestyle change, as compared to without knowledge of that "biomarker-associated" risk.

Finally, although pharmacological primary prevention of diabetes has been suggested as an option in previous studies mainly pointing out metformin as such a therapy, evidence supporting broad use of metformin in diabetes prevention is frail[21]. There are indications that this might change in the future though. For example, newer anti-diabetic drugs such as GLP-1 analogues and SGLT-2 are accumulating evidence to beneficially affect not body weight and composition, several cardiovascular risk factors other than glucose and actual cardiovascular risk, possibly even in non-diabetic individuals[111]. Still, these newer drugs also have large costs and side effects, and the possible use of them in pharmacological primary prevention of diabetes will for sure be dependent on biomarkers that can help finding those at highest risk for diabetes and CVD development to maximize their cost-benefit ratio.

MicroRNAs as Therapeutics

As known, microRNAs bind to mRNA either to inhibit the translation or to degrade the mRNA. Based on this knowledge, gene expression and protein-synthesis can be altered by manipulation of microRNAs. AntimiRs, such as anti-miRNA oligonucleotides (AMO), locked nucleic acids (LNA)[64], sponge[112] and CRISPR/cas9[113], can be used to reduce or eliminate microRNA binding to target mRNA. microRNAs that are used as therapeutics in several disease in preclinical models are previously reviewed[62]. However, the delivery of microRNA therapeutics is a challenge. Several delivery systems are provided, considerations must be taken for probable toxicity, immunogenicity, low delivery etc. A promising delivery system based on extracellular vesicles, specially exosomes, are considered as good microRNA carrier with low cytotoxicity and specificity[114].

Theoretically, miR-483-5p could, in future be a candidate for therapeutics, possibly in the prevention if CVD and diabetes. The process would probably, in this case,

include antimiRs that inhibit the pre-miR-483-5p, preferably locked nucleic acid (LNA) due its increased affinity and specificity. Such a development would first require far more mechanistic studies including elucidation of the relevant organs/tissues of production and targets, where miR-483-5p acts locally or via "long distance calling" (paracrine endocrine effects), followed by miR-483-5p blockade and measurement of metabolic effects in cellular experiments testing, followed by animal models appropriate for the study of diabetes and atherosclerosis.

Methodological Strength and Limitations

The size of subjects included in both cohorts is one of our largest strengths. MDC-CC is population-based cohort study with 6,103 participants and follow-up of 16 years. A sub-set of 553 sample from this cohort with a mean age of 59 was used in this thesis and which is considered one of the biggest cohorts when investigating miR483-5p. As far as we know, the size of the MDC sample used is the among the largest thus in terms of examination of healthy individuals and future risk of CVD and diabetes.

MOS is population-based family cohort with almost 4000 samples and a follow up of about 4 years and a mean age of 42. For this thesis, 1223 cases were available, which also is considered as one of the biggest cohorts for analysing miR-483-5p. We do however acknowledge the low number of events in MOS, which is a consequence of lower age and shorter follow-up compared to MDC, as a major limitation. However, despite the low number of incident cases in MOS, we were to replicate the findings from the first paper. The low number of events certainly emphasizes the need of further replication.

In the first study, 47 consistently expressed microRNA from the pilot study with 12 samples was chosen. All other microRNAs with at least undetermined value were excluded for this thesis. This might however cause a loss of important microRNAs for each disease, respectively. To explain why we applied this strategy, we considered it more likely that "unmeasurable" micro-RNAs would be irrelevant or subject to technical measurement errors than representing an "on-off" signal with relevance for disease development. We rather believe that variation within the normal will contain most of the relevant predictive information. Thus, we focused on the 47 microRNAs with consistent expression in serum and excluded those who were not detectable on all 12 pilot samples.

Finally, the current thesis does not include any functional experiments, which is a weakness in terms of assessing likelihood of biological relevance of miR-483-5p. It is our hope and belief that, apart from providing stimulation for other groups to

replicate the epidemiological findings, that our results would also stimulate initiation of mechanistic studies linking miR-483-5p to cardiometabolic disease.

Conclusions

We have investigated microRNAs, in particular miR-483-5p, in relation to Cardiometabolic disease (CMD), comprising type 2 diabetes and cardiovascular disease in a Swedish population-based prospective study. MiR-483-5p was further studied in relation to lifestyle and metabolites. Elevated circulating levels of miR-483-5p were shown to be related to incident CMD, risk factors for CMD, lifestyle and metabolites. Importantly, those findings were reproducible in a second Swedish prospective study. Our conclusions are as follows:

- We found a novel potential biomarker for prediction of future risk of cardiometabolic disease. Although we are aware that only miR-483-5p might not be enough to improve risk stratification of CMD, which is the main reason to find high risk individuals, it may be included in a multi-biomarker panel.
- miR-483-5p is a potential therapeutics candidate as its expression could be decreased, which may consequently improve health status.
- We could not confirm the relationship between other microRNAs, previously reported to be associated with incident CMD, in particular miR-126. Further and larger studies are needed to investigate miR-126 in CMD.

Populärvetenskaplig Sammanfattning

Kardiometabola sjukdomar, är ett samlingsnamn för typ-2 diabetes och hjärt-kärlsjukdomar. Brist på fysisk aktivitet i kombination med överskott av mat och näring, ökar risken för insjuknande av kardiometabola sjukdomar. Diabetes sker när antingen bukspottkörteln inte producerar tillräckligt med insulin eller när ens kropp inte effektivt kan ta upp socker (glukos) från blodet med hjälp av insulinet. I båda fallen misslyckas glukosen att tas upp av muskel- och fettvävnad, vilket leder till förhöjda nivåer av glukos i blodet. Diagnostiken av diabetes sker genom att mäta upp ett högt blodsockervärde. Problemet med detta är att diabetes förmodligen har varit i gång långt innan diagnosen ställs och att skador på vävnader och organ redan kan ha skett. Diabetes räknas också som en av de starkaste riskfaktorerna för insjuknande i hjärt-kärlsjukdomar. Enligt Världshälsoorganisationen (WHO), hade 422 miljoner människor diabetes år 2014. Drygt 19 millioner dör i hjärt-kärlsjukdomar årligen. Därför är det viktigt att hitta nya och bättre biomarkörer som kan användas för att upptäcka riskindivider innan sjukdomen bryter ut.

MicroRNA reglerar uttrycket av gener för att kontrollera hur mycket av protein som kommer bildas. Studier har visat att uttrycket av microRNA i olika vävnader ändras vid olika sjukdomar. MicroRNA kan redan idag användas som verktyg för både behandling och prediktion av vissa sjukdomar. Uppreglering av genomet för hepatit-C virus sker med miR-122. För att inhibera uppregleringen och därmed minska på viruset, som skadar levern, används en s.k. antimiR-122 för att stoppa aktiviteten avav just miR-122. Eftersom många microRNA finns cirkulerande i blodet och kan extraheras och mätas relativt lätt är de också mycket attraktiva som biomarkörer, dvs mätbara markörer för sjukdomar och sjukdomsrisk.

Avsikten med denna avhandling, som består av fyra arbeten, är att förstå vilken betydelse som microRNA har för uppkomsten av diabetes och hjärt-kärlsjukdomar.

I den här avhandlingen studerade vi cirkulerande serum microRNA, och dess relation till diabetes och hjärt-kärlsjukdomar i två prospektiva befolkningsbaserade studier från Malmö, bestående av 553 respektive 1223 individer. Till detta användes serum från friska individer som senare i livet utvecklade diabetes eller hjärt-kärlsjukdomar under en uppföljning av flera år men också de som inte utvecklade sjukdomarna, dvs. kontrollgruppen. Vi studerade dels kopplingen av miR-483-5p till kardiometabola sjukdomar och dess riskfaktorer, men också samband med livsstilsfaktorer. Vidare undersökte vi om olika MicroRNA, som sen tidigare visat

relation till risk för kardiometabola sjukdomar, uppvisade liknande samband i våra två befolkningsmaterial. Vi visade att miR-483-5p fanns i högre koncentration i serum både hos individer som senare insjuknade i diabetes och hos de som senare utvecklade hjärt-kärlsjukdomar, jämfört med i kontrollgruppen. Vi kunde även se att miR-483-5p samvarierade med kardiometabola riskfaktorer som till exempel övervikt, midjeomfång, kolesterolnivå och insulinresistens.

Det är, sen tidigare känt, att ogynnsam livsstil, som till exempel stillasittande, rökning och övervikt kan leda till kardiometabola sjukdomar. Av denna anledning studerade vi vidare om miR-483-5p är relaterat till livsstil. Vi visade att förhöjda nivåer av miR-483-5p var kopplat till låg grad av fysik aktivitet. Detta betyder inte att låg fysisk aktivitet leder till ökad nivå av miR-483-5p men är ett intressant fynd för att i framtida studier närmare undersöka om fysisk aktivitet kan sänka nivåerna av miR-483-5p.

Vidare studerade vi miR-483-5p och dess korrelation till så kallade metaboliter i blodbanan. Dessa är små molekyler som tex kommer från nedbruten föda eller vävnader och sammansättningen av blodmetaboliter kan sägas beskriva vår ämnesomsättning. Vissa av metaboliterna har visat stark koppling till diabetes och brukar därför ofta kallas för diabetesrelaterade metaboliter. I den här delen av avhandlingen studerade vi huruvida miR-483-5p kunde kopplas till dessa metaboliter. Vi kunde då visa att förhöjda nivåer av miR-483-5p var positivt relaterade till vissa av de diabetesrelaterade metaboliterna, såsom exempelvis s.k. grenade aminosyror.

En teori som vi har är att miR-483-5p utsöndras från fettvävnaden till olika organ som till exempel muskler och därmed inhiberar blodsockerupptaget i dessa organ. Om miR-483-5p påverkar metaboliterna eller inte återstår att se, men det finns andra studier som visar att MicroRNA kan påverka metaboliterna och ämnesomsättningen. Detta öppnar för nya potentiella möjligheter att använda det här MicroRNA:t som ett verktyg för att dels upptäcka individer innan de insjuknar och uppmuntra dem till bättre livsstil, dels att i framtiden behandla de som redan är sjuka genom att sänka nivåerna av miR-483-5p.

I den andra delen av avhandlingen studerade bland annat ett microRNA, miR-126, som tidigare hade rapporterats vara relaterad till kardiometabola sjukdomar. Vår studie kunde inte bekräfta de tidigare fynden. Men för att kunna bekräfta eller dementera relationen mellan miR-126 och kardiometabola sjukdomar, krävs det flera undersökningar och helst i större studier.

De viktigaste slutsatserna i den här avhandlingen är att hög nivå av miR-483-5p i serum är kopplat till ökad risk för att senare i livet insjukna i kardiometabola sjukdomar. Resultaten öppnar för nya möjligheter att öka vår förståelse av miR-483-5p vid kardiometabola sjukdomar och eventuellt hur miR-483-5p kan utgöra ett mål för förebyggande behandling mot kardiometabola sjukdomar.

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References

1. Federation ID. IDF Diabetes Atlas, 9th edn. Brussels, Belgium: 2019. 2019.
2. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2020. *Diabetes Care.* 2020;43(Supplement 1):S14-S31.
3. Stefan N. Causes, consequences, and treatment of metabolically unhealthy fat distribution. *Lancet Diabetes Endocrinol.* 2020;8(7):616-627.
4. Health Effects of Overweight and Obesity in 195 Countries over 25 Years. *New England Journal of Medicine.* 2017;377(1):13-27.
5. The public health agency of sweden, Obesity. 2018.
6. Tuomilehto J, Lindström J, Eriksson JG, et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med.* 2001;344(18):1343-1350.
7. Fredrickson DS, Lees RS. A System for Phenotyping Hyperlipoproteinemia. *Circulation.* 1965;31:321-327.
8. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation.* 2002;106(25):3143-3421.
9. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55 000 vascular deaths. *The Lancet.* 2007;370(9602):1829-1839.
10. Sharrett AR, Ballantyne CM, Coady SA, et al. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. *Circulation.* 2001;104(10):1108-1113.
11. Cholesterol Treatment Trialists C. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. *The Lancet.* 2012;380(9841):581-590.
12. Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet.* 2012;380(9841):572-580.
13. Organization WH. A global brief on hypertension, https://www.who.int/cardiovascular_diseases/publications/global_brief_hypertension/en/. (2013).

14. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet.* 2002;360(9349):1903-1913.
15. Turnbull F. Effects of different blood-pressure-lowering regimens on major cardiovascular events: results of prospectively-designed overviews of randomised trials. *Lancet.* 2003;362(9395):1527-1535.
16. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature.* 2001;414(6865):799-806.
17. Withers DJ, Gutierrez JS, Towery H, et al. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature.* 1998;391(6670):900-904.
18. Röder PV, Wu B, Liu Y, Han W. Pancreatic regulation of glucose homeostasis. *Experimental & Molecular Medicine.* 2016;48(3):e219-e219.
19. Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. *Cell.* 2012;148(5):852-871.
20. The Diabetes Prevention Program. Design and methods for a clinical trial in the prevention of type 2 diabetes. *Diabetes Care.* 1999;22(4):623-634.
21. Diabetes Prevention Program Research G. Long-term effects of lifestyle intervention or metformin on diabetes development and microvascular complications over 15-year follow-up: the Diabetes Prevention Program Outcomes Study. *Lancet Diabetes Endocrinol.* 2015;3(11):866-875.
22. World Health Organization. Cardiovascular diseases (CVDs). 2020.
23. Mendis S, Thygesen K, Kuulasmaa K, et al. World Health Organization definition of myocardial infarction: 2008-09 revision. *Int J Epidemiol.* 2011;40(1):139-146.
24. Kumar A, Cannon CP. Acute coronary syndromes: diagnosis and management, part I. *Mayo Clin Proc.* 2009;84(10):917-938.
25. Sacco RL, Kasner SE, Broderick JP, et al. An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke.* 2013;44(7):2064-2089.
26. Falk E. Pathogenesis of atherosclerosis. *J Am Coll Cardiol.* 2006;47(8 Suppl):C7-12.
27. Santos AMF, dos Santos RM, Castro PMAC, Azevedo E, Sousa L, Tavares JMRS. A novel automatic algorithm for the segmentation of the lumen of the carotid artery in ultrasound B-mode images. *Expert Systems with Applications.* 2013;40(16):6570-6579.
28. Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. Role of Endothelial Shear Stress in the Natural History of Coronary Atherosclerosis and Vascular Remodeling. *Journal of the American College of Cardiology.* 2007;49(25):2379-2393.
29. Hansson GK. Inflammation, Atherosclerosis, and Coronary Artery Disease. *New England Journal of Medicine.* 2005;352(16):1685-1695.

30. Bergheanu SC, Bodde MC, Jukema JW. Pathophysiology and treatment of atherosclerosis : Current view and future perspective on lipoprotein modification treatment. *Neth Heart J.* 2017;25(4):231-242.
31. Doran AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology.* 2008;28(5):812-819.
32. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell.* 2011;145(3):341-355.
33. Estruch R, Ros E, Salas-Salvadó J, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. *New England Journal of Medicine.* 2018;378(25):e34.
34. Williams B, Mancia G, Spiering W, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH). *European Heart Journal.* 2018;39(33):3021-3104.
35. Marso SP, Bain SC, Consoli A, et al. Semaglutide and Cardiovascular Outcomes in Patients with Type 2 Diabetes. *New England Journal of Medicine.* 2016;375(19):1834-1844.
36. Zinman B, Wanner C, Lachin JM, et al. Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *New England Journal of Medicine.* 2015;373(22):2117-2128.
37. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell.* 1993;75(5):843-854.
38. Reinhart BJ, Slack FJ, Basson M, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature.* 2000;403(6772):901-906.
39. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science.* 2001;294(5543):853-858.
40. Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. *Clin Chem.* 2010;56(11):1733-1741.
41. Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. *Nat Rev Endocrinol.* 2013;9(9):513-521.
42. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Research.* 2011;39(16):7223-7233.
43. Fabbri M. MicroRNAs and miRceptors: a new mechanism of action for intercellular communication. *Philos Trans R Soc Lond B Biol Sci.* 2018;373(1737).
44. Fabbri M, Paone A, Calore F, et al. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proceedings of the National Academy of Sciences.* 2012;109(31):E2110-E2116.

45. Ji C, Guo X. The clinical potential of circulating microRNAs in obesity. *Nature Reviews Endocrinology.* 2019;15(12):731-743.
46. Vienberg S, Geiger J, Madsen S, Dalgaard LT. MicroRNAs in metabolism. *Acta Physiologica.* 2017;219(2):346-361.
47. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004;116(2):281-297.
48. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.* 2004;18(24):3016-3027.
49. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear Export of MicroRNA Precursors. *Science.* 2004;303(5654):95-98.
50. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development.* 2003;17(24):3011-3016.
51. Hutvágner G, McLachlan J, Pasquinelli AE, Bálint É, Tuschl T, Zamore PD. A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the let-7 Small Temporal RNA. *Science.* 2001;293(5531):834-838.
52. Roberts TC. The microRNA Machinery. *Adv Exp Med Biol.* 2015;887:15-30.
53. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-233.
54. Kong L, Zhu J, Han W, et al. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. *Acta Diabetol.* 2011;48(1):61-69.
55. Ortega FJ, Mercader JM, Moreno-Navarrete JM, et al. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care.* 2014;37(5):1375-1383.
56. Zhou S-s, Jin J-p, Wang J-q, et al. miRNAS in cardiovascular diseases: potential biomarkers, therapeutic targets and challenges. *Acta Pharmacologica Sinica.* 2018;39(7):1073-1084.
57. Hess AL, Larsen LH, Udesen PB, Sanz Y, Larsen TM, Dalgaard LT. Levels of Circulating miR-122 are Associated with Weight Loss and Metabolic Syndrome. *Obesity.* 2020;28(3):493-501.
58. Zampetaki A, Kiechl S, Drozdov I, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res.* 2010;107(6):810-817.
59. Zhang T, Li L, Shang Q, Lv C, Wang C, Su B. Circulating miR-126 is a potential biomarker to predict the onset of type 2 diabetes mellitus in susceptible individuals. *Biochem Biophys Res Commun.* 2015;463(1-2):60-63.
60. Liu Y, Gao G, Yang C, et al. The role of circulating microRNA-126 (miR-126): a novel biomarker for screening prediabetes and newly diagnosed type 2 diabetes mellitus. *Int J Mol Sci.* 2014;15(6):10567-10577.

61. Zampetaki A, Willeit P, Tilling L, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol.* 2012;60(4):290-299.
62. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov.* 2017;16(3):203-222.
63. Stenvang J, Kauppinen S. MicroRNAs as targets for antisense-based therapeutics. *Expert Opinion on Biological Therapy.* 2008;8(1):59-81.
64. Elmén J, Lindow M, Schütz S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature.* 2008;452(7189):896-899.
65. Elmén J, Lindow M, Silahtaroglu A, et al. Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic acids research.* 2008;36(4):1153-1162.
66. Luna Joseph M, Scheel Troels KH, Danino T, et al. Hepatitis C Virus RNA Functionally Sequesters miR-122. *Cell.* 2015;160(6):1099-1110.
67. Trajkovski M, Hausser J, Soutschek J, et al. MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature.* 2011;474(7353):649-653.
68. Jones BK, Levorse J, Tilghman SM. Deletion of a nuclease-sensitive region between the Igf2 and H19 genes leads to Igf2 misregulation and increased adiposity. *Human Molecular Genetics.* 2001;10(8):807-814.
69. Holly JMP, Biernacka K, Perks CM. The Neglected Insulin: IGF-II, a Metabolic Regulator with Implications for Diabetes, Obesity, and Cancer. *Cells.* 2019;8(10):1207.
70. Fu H, Tie Y, Xu C, et al. Identification of human fetal liver miRNAs by a novel method. *FEBS Letters.* 2005;579(17):3849-3854.
71. Landgraf P, Rusu M, Sheridan R, et al. A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing. *Cell.* 2007;129(7):1401-1414.
72. Rotwein P. The complex genetics of human insulin-like growth factor 2 are not reflected in public databases. *Journal of Biological Chemistry.* 2018;293(12):4324-4333.
73. Liu M, Roth A, Yu M, et al. The IGF2 intronic miR-483 selectively enhances transcription from IGF2 fetal promoters and enhances tumorigenesis. *Genes Dev.* 2013;27(23):2543-2548.
74. Ma N, Wang X, Qiao Y, et al. Coexpression of an intronic microRNA and its host gene reveals a potential role for miR-483-5p as an IGF2 partner. *Mol Cell Endocrinol.* 2011;333(1):96-101.
75. Mohan R, Mao Y, Zhang S, et al. Differentially Expressed MicroRNA-483 Confers Distinct Functions in Pancreatic β- and α-Cells*. *Journal of Biological Chemistry.* 2015;290(32):19955-19966.
76. Wang Z, Mohan R, Chen X, et al. microRNA-483 Protects Pancreatic β-Cells by Targeting ALDH1A3. *Endocrinology.* 2021;162(5).
77. Wishart DS. Current Progress in computational metabolomics. *Briefings in Bioinformatics.* 2007;8(5):279-293.

78. Newgard CB, An J, Bain JR, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab.* 2009;9(4):311-326.
79. Suhre K, Meisinger C, Döring A, et al. Metabolic Footprint of Diabetes: A Multiplatform Metabolomics Study in an Epidemiological Setting. *PLOS ONE.* 2010;5(11):e13953.
80. Chen T, Ni Y, Ma X, et al. Branched-chain and aromatic amino acid profiles and diabetes risk in Chinese populations. *Scientific Reports.* 2016;6(1):20594.
81. Berglund G, Elmstahl S, Janzon L, Larsson SA. The Malmo Diet and Cancer Study. Design and feasibility. *J Intern Med.* 1993;233(1):45-51.
82. Manjer J, Elmståhl S, Janzon L, Berglund G. Invitation to a population-based cohort study: differences between subjects recruited using various strategies. *Scand J Public Health.* 2002;30(2):103-112.
83. Pero RW, Olsson A, Berglund G, Janzon L, Larsson SA, Elmstahl S. The Malmo biological bank. *J Intern Med.* 1993;233(1):63-67.
84. Hamrefors V, Hedblad B, Engstrom G, Almgren P, Sjogren M, Melander O. A myocardial infarction genetic risk score is associated with markers of carotid atherosclerosis. *J Intern Med.* 2012;271(3):271-281.
85. Brunkwall L. JD, Ericson U., Hellstrand S., Östling G., Melander O., Engström G., Nilsson J., Ohlsson B., Klinge B., Orho-Melander M., Persson M., and Peter M Nilsson PM. The Malmö Offspring Study (MOS). *UNPUBLISHED DATA.* 2020.
86. Taylor HL, Jacobs DR, Jr., Schucker B, Knudsen J, Leon AS, Debacker G. A questionnaire for the assessment of leisure time physical activities. *J Chronic Dis.* 1978;31(12):741-755.
87. Richardson MT, Leon AS, Jacobs DR, Jr., Ainsworth BE, Serfass R. Comprehensive evaluation of the Minnesota Leisure Time Physical Activity Questionnaire. *J Clin Epidemiol.* 1994;47(3):271-281.
88. Li C, Aronsson CA, Hedblad B, Gullberg B, Wirfält E, Berglund G. Ability of physical activity measurements to assess health-related risks. *European Journal of Clinical Nutrition.* 2009;63(12):1448-1451.
89. Ericson U, Brunkwall L, Hellstrand S, Nilsson PM, Orho-Melander M. A Health-Conscious Food Pattern Is Associated with Prediabetes and Gut Microbiota in the Malmö Offspring Study. *The Journal of Nutrition.* 2019;150(4):861-872.
90. Wirfält E, Mattisson I, Johansson U, Gullberg B, Wallström P, Berglund G. A methodological report from the Malmö Diet and Cancer study: development and evaluation of altered routines in dietary data processing. *Nutrition Journal.* 2002;1(1):3.
91. Callmer E, Riboli E, Saracci R, Akesson B, Lindgärde F. Dietary assessment methods evaluated in the Malmö food study. *Journal of internal medicine.* 1993;233(1):53-57.
92. Nybacka S, Lindroos AK, Wirfält E, et al. Carotenoids and alkylresorcinols as objective biomarkers of diet quality when assessing the validity of a web-

based food record tool and a food frequency questionnaire in a middle-aged population. *BMC Nutrition.* 2016;2(53).
93. Rosvall M, Persson M, Ostling G, et al. Risk factors for the progression of carotid intima-media thickness over a 16-year follow-up period: the Malmo Diet and Cancer Study. *Atherosclerosis.* 2015;239(2):615-621.
94. Brunkwall L, Jönsson D, Ericson U, et al. The Malmö Offspring Study (MOS): design, methods and first results. *European Journal of Epidemiology.* 2020.
95. Enhörning S, Sjögren M, Hedblad B, Nilsson PM, Struck J, Melander O. Genetic vasopressin 1b receptor variance in overweight and diabetes mellitus. *Eur J Endocrinol.* 2016;174(1):69-75.
96. Ludvigsson JF, Andersson E, Ekbom A, et al. External review and validation of the Swedish national inpatient register. *BMC Public Health.* 2011;11(1):450.
97. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology.* 2002;3(7):research0034.0031.
98. Nunez Lopez YO, Retnakaran R, Zinman B, Pratley RE, Seyhan AA. Predicting and understanding the response to short-term intensive insulin therapy in people with early type 2 diabetes. *Molecular Metabolism.* 2019;20:63-78.
99. Mori MA, Ludwig RG, Garcia-Martin R, Brandão BB, Kahn CR. Extracellular miRNAs: From Biomarkers to Mediators of Physiology and Disease. *Cell Metabolism.* 2019;30(4):656-673.
100. Drewnowski A, Rock CL, Henderson SA, et al. Serum beta-carotene and vitamin C as biomarkers of vegetable and fruit intakes in a community-based sample of French adults. *The American Journal of Clinical Nutrition.* 1997;65(6):1796-1802.
101. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. *Cell metabolism.* 2012;15(5):606-614.
102. Mersey BD, Jin P, Danner DJ. Human microRNA (miR29b) expression controls the amount of branched chain α-ketoacid dehydrogenase complex in a cell. *Human Molecular Genetics.* 2005;14(22):3371-3377.
103. Lotta LA, Scott RA, Sharp SJ, et al. Genetic Predisposition to an Impaired Metabolism of the Branched-Chain Amino Acids and Risk of Type 2 Diabetes: A Mendelian Randomisation Analysis. *PLOS Medicine.* 2016;13(11):e1002179.
104. Faraldi M, Gomarasca M, Sansoni V, Perego S, Banfi G, Lombardi G. Normalization strategies differently affect circulating miRNA profile associated with the training status. *Scientific Reports.* 2019;9(1):1584.
105. Al-Kafaji G, Al-Mahroos G, Al-Muhtaresh HA, Skrypnyk C, Sabry MA, Ramadan AR. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: A potential blood-based biomarker. *Exp Ther Med.* 2016;12(2):815-822.

106. Wang LP, Gao YZ, Song B, et al. MicroRNAs in the Progress of Diabetic Nephropathy: A Systematic Review and Meta-Analysis. *Evid Based Complement Alternat Med.* 2019;2019:3513179.
107. Piepoli MF, Hoes AW, Agewall S, et al. 2016 European Guidelines on cardiovascular disease prevention in clinical practice: The Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts)Developed with the special contribution of the European Association for Cardiovascular Prevention & Rehabilitation (EACPR). *European Heart Journal.* 2016;37(29):2315-2381.
108. Khot UN, Khot MB, Bajzer CT, et al. Prevalence of Conventional Risk Factors in Patients With Coronary Heart Disease. *JAMA.* 2003;290(7):898-904.
109. Laakso M. Cardiovascular disease in type 2 diabetes from population to man to mechanisms: the Kelly West Award Lecture 2008. *Diabetes Care.* 2010;33(2):442-449.
110. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. *Nature.* 2001;414(6865):782-787.
111. Marso SP, Daniels GH, Brown-Frandsen K, et al. Liraglutide and Cardiovascular Outcomes in Type 2 Diabetes. *New England Journal of Medicine.* 2016;375(4):311-322.
112. Zhang Y, Wang Z, Gemeinhart RA. Progress in microRNA delivery. *J Control Release.* 2013;172(3):962-974.
113. Chang H, Yi B, Ma R, Zhang X, Zhao H, Xi Y. CRISPR/cas9, a novel genomic tool to knock down microRNA in vitro and in vivo. *Scientific Reports.* 2016;6(1):22312.
114. Dasgupta I, Chatterjee A. Recent Advances in miRNA Delivery Systems. *Methods and Protocols.* 2021;4(1):10.

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MicroRNAs in Cardiometabolic Disease

Widet Gallo obtained her Master´s in Biomedical Laboratory Science from Lund University, Sweden, 2006. The main aim of her doctoral thesis is to study the connection between serum circulating ,miR-483-5p and Cardiometabolic Disease in prospective cohort studies.

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