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ARTERIAL AND MOLECULAR BIOMARKERS FOR IMPROVED CARDIOVASCULAR DISEASE AND DIABETES RISK ASSESSMENT COMPARATIVE STUDY

DOCTORAL DISSERTATION

Biomedical sciences, Medicine (06B)

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The Thesis was developed in 2013-2016 in the Clinic of Cardiovascular diseases of Vilnius University Faculty of Medicine.

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ARTERINIŲ IR MOLEKULINIŲ ŠIRDIES IR KRAUJAGYSLIŲ LIGŲ BEI CUKRINIO DIABETO RIZIKOS BIOŽYMENŲ PALYGINAMASIS TYRIMAS

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ABBREVIATIONS

ACEI – Angiotensin-converting enzyme inhibitors

ACS – Acute coronary syndrome

AIx – Aortic augmentation index

AIx@75 – Aortic augmentation index adjusted for a heart rate of 75 beats per minute

Ao_PP – Aortic pulse pressure

AoPWV – Aortic pulse wave velocity

AMI – Acute myocardial infarction

AP – Angina pectoris

ARB – Angiotensin receptor blocker

ARIC – Atherosclerosis risk in communities

ASO – Arteriosclerosis obliterans

AUC – Area under the curve

BIC – Bayesian information criterion

BP – Blood pressure

BMI – Body mass index

CAD – Coronary artery disease

CAVI – Cardio-ankle vascular index

CHD – Coronary heart disease

CHF – Congestive heart failure

CI – Confidence interval

CIMT – Carotid intima-media thickness

CMR – Cardiovascular magnetic resonance

CV – Cardiovascular

CVD – Cardiovascular disease(s)

CVPU – Cardiovascular prevention units

DBP – Diastolic blood pressure

DCM – Dilated cardiomyopathy

ECG – Electrocardiogram

EFG – Event-free group

EG – Event group

FMD – Flow-mediated dilatation

FPG – Fasting plasma glucose

FRS – Framingham risk score

HbA_{1c} – Glycosylated hemoglobin

HCMV – Human cytomegalovirus

HDL – High-density lipoprotein

HDL-C – High-density lipoprotein cholesterol

HF – Heart failure

HR-REF – Heart failure with reduced ventricular ejection fraction

HOMA-IR – Homeostasis model assessment insulin resistance

H-L – Hosmer-Lemeshow

hs-CRP – High-sensitivity C-reactive protein

IAS-AGLA – Working Group on Lipids and Atherosclerosis (AGLA) of the Swiss Society of Cardiology (SGK) published guidelines for the prevention of atherosclerosis

ICM – Ischemic cardiomyopathy

IFG – Impaired fasting glucose

IGT – Impaired glucose tolerance

ISI – Insulin sensitivity index

LAS – Large artery stroke

LDL – Low-density lipoprotein

LDL-C – Low-density lipoprotein cholesterol

LitHiR – Lithuanian High Cardiovascular Risk

LV – Left ventricle

MAP – Mean arterial pressure

MeanBP_Ao – Mean aortic blood pressure

MetS – Metabolic syndrome

MI – Myocardial infarction

miR – Micro RNA

MMI – Myocardial mass index

NCEP ATPIII – National Cholesterol Education Program Adult Treatment Panel III

NICM – Non-ischemic cardiomyopathy

NSTEMI – Non-ST-segment elevation myocardial infarction

NYHA – New York Heart Association

OGTT – Oral glucose tolerance test

OR – Odds ratios

PAD – Peripheral arterial disease

PHCI – Primary healthcare institutions

PROCAM – Prospective cardiovascular Münster

PWV – Pulse wave velocity

qRT-PCR – Real-time quantitative reverse transcriptase polymerase chain reaction

QUICKI – Quantitative insulin-sensitivity check index

RAS – Renin–angiotensin system

ROC – Receiver operating characteristic

RRS – Reynolds risk score

RT-PCR – Reverse transcriptase polymerase chain reaction

SA – Stable angina

SBP – Systolic blood pressure

SCORE – Systemic Coronary Risk Evaluation

SD – Standard deviation

SEM – Standard error of the mean

STEMI – ST-segment elevation myocardial infarction

TC – Total cholesterol

TG – Triglycerides

T2DM – Type 2 diabetes mellitus

UA – Unstable angina

WC – Waist circumference

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1. INTRODUCTION

Cardiovascular disease (CVD), including coronary heart disease (CHD), stroke and peripheral arterial disease (PAD), is the most important cause of premature death worldwide as well as disability-adjusted life years in Europe [1,2]. About 600,000 people die each year of CVD in the USA – that is 1 in every 4 deaths [3]. CHD is the most common type of CVD and is responsible for the death of almost 380,000 people annually [4]. By the year 2020, it is expected that almost 25 million deaths worldwide will be caused by CVD each year [5]. The structure of the causes of death among the Lithuanian population has remained unchanged for many years. It is important to note that in 2013, over half (56.3%) of all deaths in Lithuania were caused by CVD [6]. Although the cost of CVD in the European Union costs 192 billion Euros annually [7], in the USA, CHD alone reaches a cost of 88 billion Euros ($108.9 billion) annually [8].

There are several risk factors for CVD. The INTERHEART study assessed the importance of risk factors for coronary artery disease (CAD) worldwide [9]. Nine measured and potentially modifiable risk factors accounted for over 90% of the proportion of the risk for acute myocardial infarction (AMI). Smoking, history of hypertension or diabetes, waist hip ratio, dietary pattern, physical activity, alcohol consumption, blood lipoproteins and psychosocial factors were identified as the key risk factors. The effect of these risk factors was consistent in men and women across different geographic regions and by ethnic group. The British Regional Heart Study also found that smoking, blood pressure (BP), and cholesterol accounted for 90% of the attributable risk for CHD [10]. There was a need to consider the likely impact of all risk factors in a reproducible and quantifiable manner before making clinical management decisions.

Over the past 30 years, several CVD risk-scoring systems have been developed; however, the majority of these are based on the American population from the 1970s and 1980s when lifestyle choices, eating habits, and stress levels, were quite different from what they are now. Today, almost one quarter of the world's adult population have metabolic syndrome (MetS), which increases the risk of CVD by two-fold and the risk of developing type 2 diabetes mellitus (T2DM) by five-fold. Additionally, the ever-growing population is too often being assessed using the metabolic syndrome score, which is based on the number of risk factors, without considering the

synergistic effects between the components. However, evidence for this group and relevant risk-assessment methods, and their sensitivity and specificity, is lacking.

Despite previous studies of CVD risk scoring systems across various patient types, there remains a lack of personalization and specificity. Advancing risk assessment to include subclinical atherosclerosis will accelerate patients' access to preventive strategies that may delay or avert the onset of CVD outcomes such as T2DM.

Knowing, that the early subclinical changes start sometimes as early, as in their twenties, means of detecting any early changes in endothelial function, arterial stiffness or arterial structure, some of which are directly related to CVD outcomes, might be of huge significance, enabling us not only to highlight subjects at a very early stage of a disease, but in addition, monitor the effect of the preventative measures, applied for each subject. This is especially important when the classical CVD risk-assessment tool, based on risk factors, fail.

Besides the need for early detection of a disease, with increasing list of preventative means and different pharmaceutical options, the ability to follow and adjust a patient's risk of developing the disease or its outcomes, should not be understated. Therefore, the hypothesis which were raised as part of this doctoral thesis were:

1. Widely used CVD risk-assessment tools are inaccurate for patients with MetS
2. Arterial stiffness is related to CVD outcomes in patients with MetS and could be used for further personalizing risk assessment
3. T2DM onset in patients with MetS can be predicted with high accuracy using a combination of biochemical biomarkers, and is related to aortic pulse wave velocity (AoPWV)
4. MicroRNAs (miRs) could be a reproducible, standardized CVD biomarker, indicating early atherosclerosis

Goals:

1. Assess the validated or widely used CV risk-assessment tools in a group of patients with MetS
2. Assess additive value of arterial and biochemical biomarkers in CVD and T2DM risk prediction in the group of patients with MetS
3. Assess the applicability of epigenetic biomarkers for CVD risk prediction, as an early atherosclerosis indicator

Objectives:

1. Compare the real CVD outcomes with the predicted ones for patients with MetS
2. Investigate the association between CVD outcomes and aortic stiffness, based on AoPWV
3. Identify the most significant biomarkers indicative of the onset of T2DM in patients with MetS
4. Investigate the association of miRs with biomarkers of early atherosclerosis (AoPWV, carotid intima-media thickness [CIMT], flow-mediated dilatation [FMD]).

2. CARDIOVASCULAR DISEASE RISK MODELS IN PATIENTS WITH METABOLIC SYNDROME

The concept of risk assessment and reduction forms the cornerstone of preventive cardiology. Risk factor assessment determines the therapeutic strategy, because the intensity of preventive intervention is tailored to the patient's risk of coronary heart disease [11]. Risk estimates can theoretically be used to raise population awareness of diseases such as CVD that cause a significant burden of morbidity and mortality, and to communicate knowledge about that risk to individuals and subgroups, also to motivate adherence to recommended lifestyle changes or therapies. In clinical practice, risk prediction algorithms are mostly used to identify individuals with high risk for developing CVD in the short term and to select those individuals for more intensive preventive interventions [12,13]. Therefore, risk evaluation and prediction of possible events initiate life prolonging concept and disability free aging.

Aim

To investigate whether commonly used and internationally verified risk assessment tools are accurate for MetS patients.

Literature review

Some pre-existing conditions that patients present with can interfere with risk prediction. Several studies have shown that patients with diabetes had significantly elevated risk for CV outcomes, in spite of CVD risk prediction models prognosis [14]. At the same time, there is concern over others diseases and syndromes. Evidence and clinical practice suggest that current widely used risk assessment tools are not adjusted to increasing proportion of population, such as MetS, and therefore might be a misguiding risk assessment tool.

MetS is a multiplex risk factor that is associated with increased risk for CVD, T2DM, and their associated complications. MetS consists of five risk factors, including elevated BP, hyperglycemia, dyslipidemia, a prothrombotic state, and a proinflammatory state. In most countries, MetS is common, affecting at least one-quarter

of adults [15,16]. This prevalence appears to be increasing because of a parallel rise in the incidence of obesity and physical inactivity [16]. MetS is not an absolute risk indicator; nonetheless, persons with MetS are at twice the risk for CVD years as individuals without MetS [17]. It is not surprising that almost all models had good risk prediction in the cohort that they were developed in. However, it does raise doubt about limitations of relying on such models that have not been externally validated. Cohorts in North America and Europe show the strongest evidence of external validation. These cohorts had the same outcome measure used in both development and validation studies.

However, there is still an uncertainty as to whether this exponentially increasing part of the population with comorbidities, such as MetS, estimate their risk correctly, using common and widespread risk prediction algorithms, which were frequently developed on the basis of isolated populations. Moreover, the most widely used ones, such as the Framingham, were developed over 20 years ago when obesity and other components of MetS were significantly less common than they are today, and newer, more novel CV risk factors haven't been identified.

Using European cohorts with matched outcomes to externally validate American risk models provided mixed results. Several studies having matched outcomes announced reasonable risk model performance. However, source population, including all-diabetic and elderly cohorts, in most cases was at lower risk than European cohorts [18,19]. Risk models under predicted the outcomes in some of the studies, but these under predictions occurred almost only in high-risk patient cohorts. Such cohorts included patients with poor access to health care, poorly controlled hypertension, advanced age, organ transplants, or diabetes [19,20–23]. Nonetheless, most external validations of American risk models across European cohorts identified that models over predicted the risk [16,18,20,24–28]. This over prediction was mainly because underlying outcome event rates between evaluation and model cohorts were not the same.

Methodology

Population. A cross-sectional study was performed among 3194 subjects with MetS. Mean age of the population was 54.1 (SD 6.2) years, including 2042 females and 3089 individuals with hypertension. All patients were recruited fro mthe Lithuanian High Cardiovascular Risk (LitHiR) primary prevention programme between

2006 and 2014. This long-term programme focused on women (aged 50–65) and men (aged 40–55) without overt CVD, as described previously [29,30].

Patients were diagnosed with MetS if they met three or more criteria of the revised National Cholesterol Education Program Adult Treatment Panel III (NCEP ATPIII). Hypertension was defined as an elevation of BP (>140 mmHg systolic and/or >90 mmHg diastolic) obtained on at least three separate measurements performed on different days. Dyslipidemia was defined as TC >5 mmol/L, or LDL-C >3 mmol/L, or HDL-C <1.0 mmol/L in men, or <1.2 mmol/L in women, or TG >1.7 mmol/L. Diabetes was defined according to past medical history, if the patient received pharmacologic treatment, or if the FPG level was ≥7 mmol/L. Smoking was classified as never, former, or current smoking.

All patients underwent a physical examination, risk profile (smoking, dietary patterns) analysis, anthropometry [height, weight, waist circumference (WC) and body mass index (BMI), defined as weight in kilograms divided by height (in meters squared)], BP, and pulse determination. Twelve-lead electrocardiogram (ECG) was registered. After a 12-hour fast, serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose (FPG), and high sensitivity C-reactive protein (hs-CRP) levels were assessed.

Each patient also underwent thorough investigation protocol and was followed up for up to 7 years, with mean follow-up 3.9 years.

Risk-assessment tools. A literature review was performed to identify the risk-assessment tools for inclusion. Studies that were not in English, were published over 10 years ago, did not provide information on the key question, had less than 200 participants, or were not original studies, were excluded.

Search components were developed using input from previous systematic studies. An iterative refinement method was employed using approximately 50 relevant researches, which were previously identified as a quasi-validation set, to estimate the research iterations if the search picked or missed known items of interest [31–33]. The risk prediction models that were included for further analysis were based on external validity (at least 10 times) and a primary patient group:

1. 1991 Framingham risk score(FRS) model(FRS1) for CVD (26 evaluations)
2. 1998 FRS model(FRS2) for total coronary heart disease (CHD) (24 evaluations)

3. FRS Adult Treatment Panel III (ATPIII) model(FRS3) for hard CHD (16 evaluations)
4. Prospective Cardiovascular Münster (PROCAM) model for hard CHD (11 evaluations)
5. Systematic Coronary Risk Evaluation (SCORE) model for CVD mortality (11 evaluations).

However, it is important to note that the FRS ATPIII model excludes patients with diabetes, and the PROCAM model excludes women.

Additionally, two risk-prediction models that are widely used but are without external validation were included. The first one is the Swiss coronary risk calculator [IAS-AGLA; Working Group on Lipids and Atherosclerosis (AGLA) of the Swiss Society of Cardiology (SGK) published guidelines for the prevention of atherosclerosis]. It is a recalibrated PROCAM version for Switzerland and calculates the 10-year risk of myocardial infarction (MI). According to the Swiss guidelines (2005), this score is recommended for lipid lowering guidelines and risk stratification. Moreover, it has high specificity (around 90%), but low sensitivity (around 30%) [34]. The second risk prediction model is the Reynolds risk score (RRS), which is a risk equation that includes traditional risk factors and two novel risk markers – hs-CRP and a family history of premature CAD. Studies have shown that, compared to the FRS model, RRS significantly improves global CV risk prediction, especially for those who have previously had a perceived moderate risk [35,36]. **Table 1** provides an overview of the CVD risk assessment models.

Table 1. Cardiovascular disease (CVD) risk assessment models

No.	Risk prediction model	Variables included	Follow-up, years	Outcomes	Outcomes as per ICD-10	Researched population	Geographic location	Recruitment	External validation
1.	1991 FRS model for CVD	Age (years), female (1, female; 0, male), SBP [average of two office measurements (mmHg)], DBP [average of two office measurements (mmHg)], TC [measured by the Abell-Kendall method (mg/dL)], HDL-C [determined after heparin-manganese precipitation (mg/dL)], smoking (1, cigarette smoking or quit within past year; 0, otherwise), diabetes [1,diabetes; 0, otherwise (conservative definition is treatment with insulin or oral agents or having a FPG of 140 mg/dL or >10)], and ECG-LVH (1, definite; 0, otherwise).	12	CHD, stroke, CHF, or PVD	I20-I25,I60-I63,I50,I70-I79	5,573 persons (2,983 women; 2,590 men). White men and women, aged 30–74 years, free from CVD (stroke, transient ischemia, CHD [includes AP, coronary insufficiency (UA), MI, and sudden death], CHF, and intermittent claudication) until time of risk factor measurement.	USA	1968–1975	Yes
2.	1998 FRS model for total CHD	Age, gender, TC, HDL-C, SBP, smoking, DM	12	All CHD: includes CHD death, MI, UA, and AP	I20-I22, death secondary to I20-I25 and I46	2489 men and 2856 women aged 30–74 years	USA	1971–1974	Yes

No.	Risk prediction model	Variables included	Follow-up, years	Outcomes	Outcomes as per ICD-10	Researched population	Geographic location	Recruitment	External validation
3.	FRS ATP III model for hard CHD (i.e. sudden CHD death or MI with or without cardiac procedures)	Age, gender, TC (mg/dL), HDL-C (mg/dL), SBP (mmHg), BP treatment (yes or no), current smoking (yes or no)	12	Hard CHD: CHD death and nonfatal MI	I21-I22, death secondary to I20-I25	2489 men and 2856 women aged 30–74 years	USA	1971–1974	Yes
4.	PROCAM model for hard CHD	Age, LDL-C, smoking, HDL-C, SBP, family history of premature MI, DM, and TG	10	Hard CHD: CHD death and nonfatal MI	I21-I22, death secondary to I20-I25	5389 men aged 35–65 years	Germany	1985	Yes
5.	SCORE model for CVD mortality	Age, gender, TC (mg/dL), HDL-C (mg/dL), SBP (mmHg), current smoking (yes or no), region of Europe (high risk or low risk region)	Variable	CDV death (including CHD, arrhythmia, HF, stroke, aortic aneurysm, and PVD)	Death secondary to: I20-I25,I47-I49,I50,I60-63,I70-I79	205,178 persons (88,080 women and 117,098 men), ages 45–64 years	Europe	Variable; 1972–1991	Yes
6.	IAS-AGLA	Age, LDL-C, smoking, HDL-C, SBP, family history of premature MI, DM, and TG		10 years risk for MI	I21-I22		Switzerland		No
7.	RRS								

No.	Risk prediction model	Variables included	Follow-up, years	Outcomes	Outcomes as per ICD-10	Researched population	Geographic location	Recruitment	External validation
	RRS (women)	Age, TC (mg/dL), HDL-C (mg/dL), SBP (mmHg), DM assessed by HbA_{1c} (%), current smoking (yes or no), parental history of MI before age 60 years (yes or no), serum hs-CRP (mg/L)	10.2	Global CVD: CV death, nonfatal MI, nonfatal stroke, coronary revascularization	I21-I22,I60-I63, death secondary to I20-I25	24 558 initially healthy US women 45 years or older	USA	2004	No
	RRS (men)	Age, TC (mg/dL), HDL-C (mg/dL), SBP (mmHg), current smoking (yes or no), parental history of MI before age 60 years (yes or no), serum hs-CRP (mg/L)	10.8	Global CVD: CV death, nonfatal MI, nonfatal stroke, coronary revascularization	I21-I22,I60-I63, death secondary to I20-I25	Men aged ≥50 years and free of CVD, DM, and cancer	USA	2008	No

AP, angina pectoris; ATPIII, Adult Treatment Panel III of the National Cholesterol Education Program; CHD, coronary heart disease; CV, cardiovascular; CVD, cardiovascular disease; DM, diabetes mellitus; ECGLVH, electrocardiogram for left ventricular hypertrophy; FPG, fasting plasma glucose; FRS, Framingham risk score; HbA_{1c}, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity serum C-reactive protein; IAS-AGLA, Working Group on Lipids and Atherosclerosis (AGLA) of the Swiss Society of Cardiology (SGK) published guidelines for the prevention of atherosclerosis; ICD-10, The World Health Organization's international statistical classification of diseases and related health problems, 10th revision; LDL-C, low-density lipoprotein cholesterol; MI, myocardial infarction; PROCAM, prospective cardiovascular Münster; PVD, peripheral vascular disease; RRS, Reynolds risk score; SBP, systolic blood pressure; SCORE, systemic coronary risk evaluation; TC, total cholesterol; TG, triglyceride UA, unstable angina.

Statistical analyses

The 10-year risks were calculated according to risk score models. For each patient with fewer than 10 years of follow-up, risk score was lowered proportionally. The c-statistic was calculated to examine the discriminative ability of each risk score model. The Hosmer-Lemeshow (H-L) test was used to compare agreement between the observed and predicted numbers of CV events in groups of patients stratified in deciles of predicted risk. Statistical analyses were performed using STATISTICA 10 and SPSS v17.0.

Results

Model described outcomes

Discrimination. All seven CV risk models had comparable discriminatory abilities. In general, discriminative ability was poor with c-statistic scores of 0.684 (95% CI 0.627 to 0.740), 0.650 (95% CI 0.624 to 0.676), 0.623 (95% CI 0.593 to 0.652), 0.680 (95% CI 0.595 to 0.764), 0.661 (95% CI 0.572 to 0.750), 0.643 (95% CI 0.561 to 0.725), 0.707 (95% CI 0.613 to 0.801) for the FRS1, FRS2, FRS3, PROCAM, SCORE, IAS-AGLA and RRS respectively. Related receiver operating characteristic (ROC) curves are presented in **Figure 1.**

Calibration. The observed and predicted numbers of CV events for all seven algorithms had many inconsistencies across deciles of predicted CV risk (**Figure 2**).

Observed CV risk appeared to diverge from the CV risk predictions calculated using the RRS in almost all deciles. The H-L test indicated low model fit with a P-value of 0.072. The number of CV events predicted by the FRS2 varied less from the observed number of CV events, but showed differences in all deciles. The H-L test also indicated no model fit with a P-value of 0.011. CV risk as predicted by IAS-AGLA showed deviation in all deciles, especially top ones; however, a P-value of 0.265 of the H-L test indicating moderate model fit. The observed numbers of CV events and predicted by PROCAM and FRS III showed different disparities in different deciles; however, the H-L test result indicated a good model fit with P-values of 0.570 and 0.487. The expected number of CV events using FRS1 was much higher in bottom deciles and lower in top deciles in comparison to observed number of CV

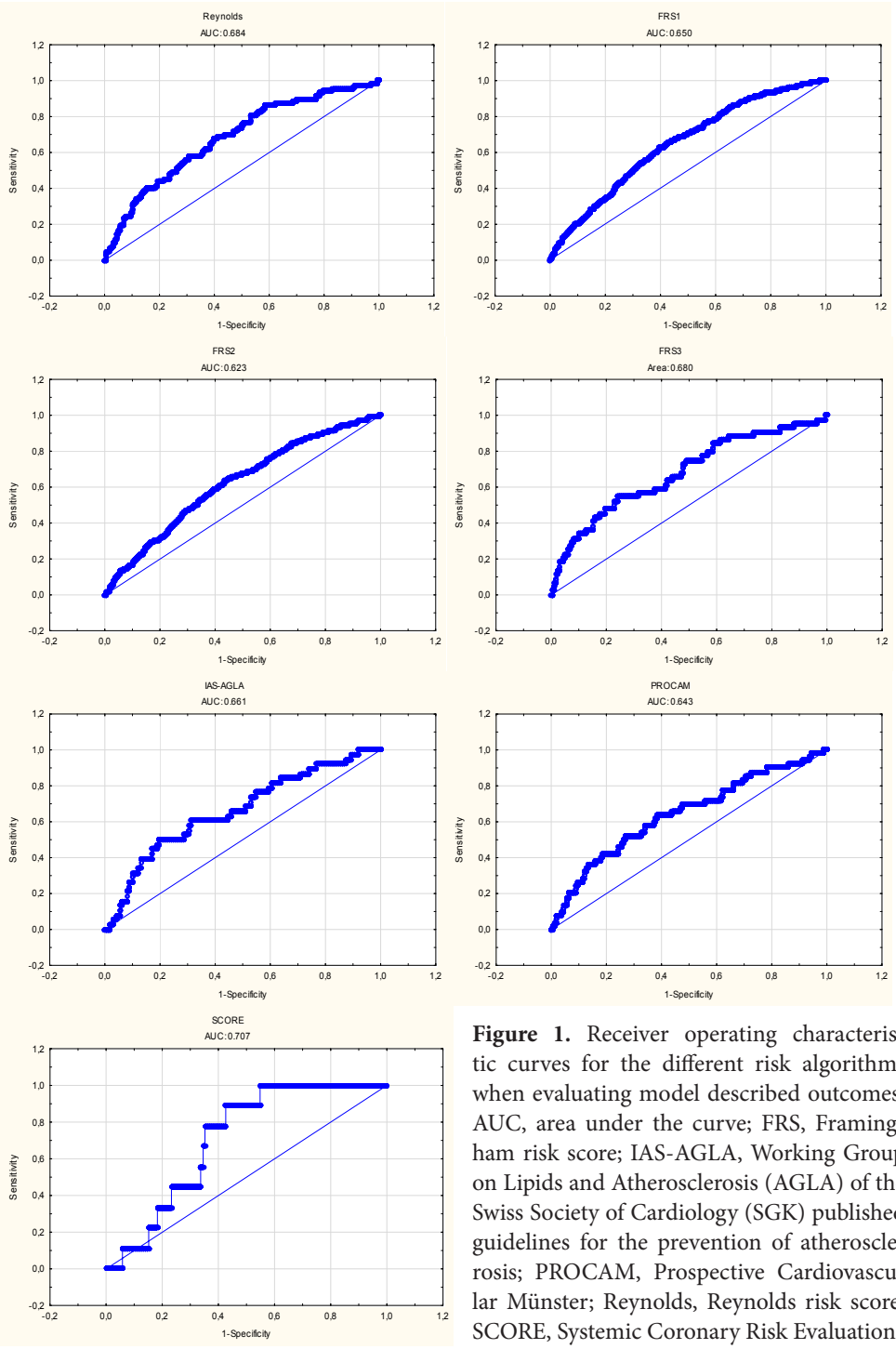

Figure 1. Receiver operating characteristic curves for the different risk algorithms when evaluating model described outcomes. AUC, area under the curve; FRS, Framingham risk score; IAS-AGLA, Working Group on Lipids and Atherosclerosis (AGLA) of the Swiss Society of Cardiology (SGK) published guidelines for the prevention of atherosclerosis; PROCAM, Prospective Cardiovascular Münster; Reynolds, Reynolds risk score; SCORE, Systemic Coronary Risk Evaluation.

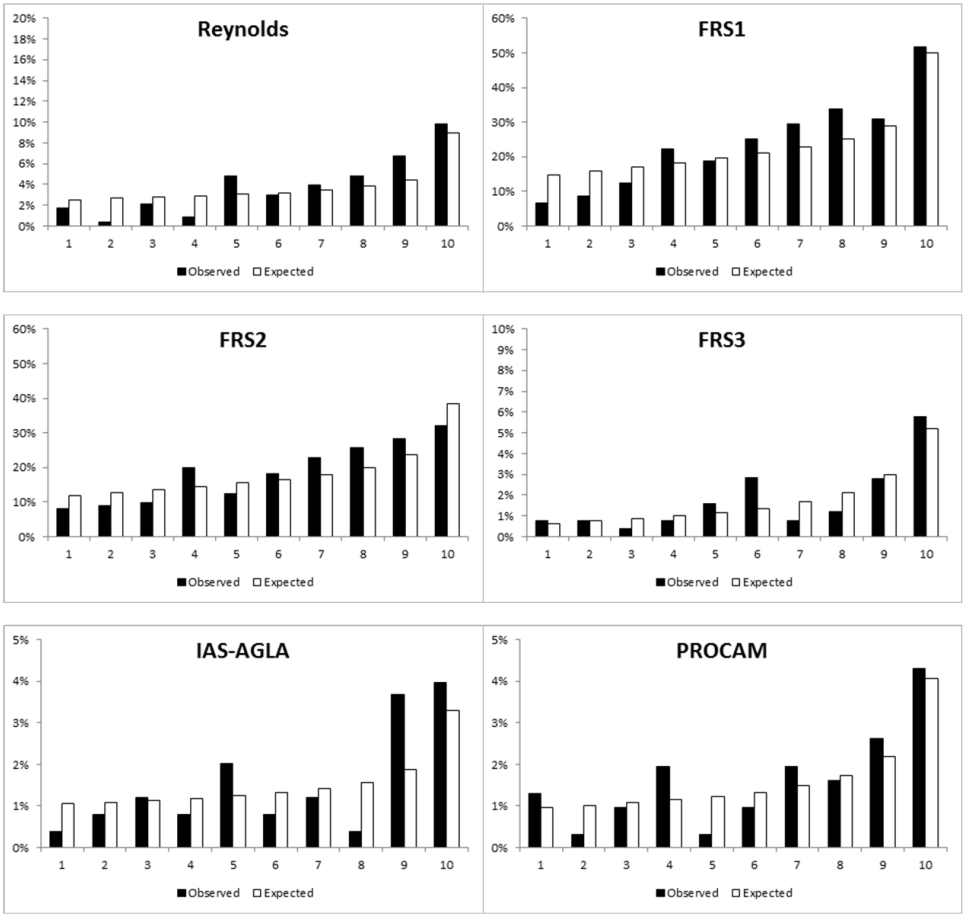

Figure 2. Observed versus predicted cardiovascular events (%) in deciles of predicted risk for the different risk algorithms when evaluating model described outcomes. FRS, Framingham risk score; IAS-AGLA, Working Group on Lipids and Atherosclerosis (AGLA) of the Swiss Society of Cardiology (SGK) published guidelines for the prevention of atherosclerosis; PROCAM, Prospective Cardiovascular Münster; Reynolds, Reynolds risk score; SCORE, Systemic Coronary Risk Evaluation.

events. The H-L test indicated moderate model fit for SCORE with a p value of 0.301. Overall, the expected CV risk calculated by these algorithms appeared to inaccurately predict an estimate of observed CV events in patients with MetS.

FRS2 described outcomes

Discrimination. All seven CV risk models had average discriminative ability with c-statistic scores of 0.629 (95% CI 0.599 to 0.659), 0.645 (95% CI 0.616 to 0.675), 0.616 (95% CI 0.586 to 0.646), 0.623 (95% CI 0.592 to 0.655), 0.625 (95% CI 0.595 to 0.656), 0.627 (95% CI 0.596 to 0.658), and 0.608 (95% CI 0.578 to 0.639) for the RRS, FRS1, FRS2, FRS3, IAS-AGLA, PROCAM and SCORE, respectively (**Figure 3**).

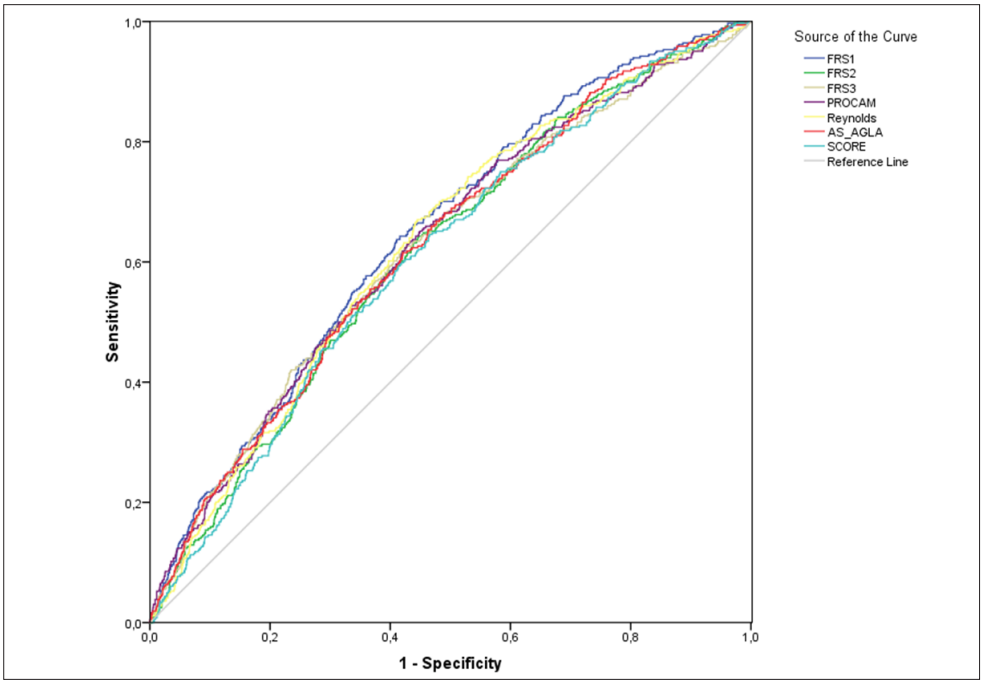

Figure 3. Receiver operating characteristic curves for the different risk algorithms when evaluating the Framingham risk score model 2(FRS2) described outcomes. IAS-AGLA, Working Group on Lipids and Atherosclerosis (AGLA) of the Swiss Society of Cardiology (SGK) published guidelines for the prevention of atherosclerosis; PROCAM, Prospective Cardiovascular Münster; Reynolds, Reynolds risk score; SCORE, Systemic Coronary Risk Evaluation.

Calibration. There were many mismatches between the observed and predicted numbers of CV events for all seven algorithms across deciles of predicted CV risk (**Figure 4**).

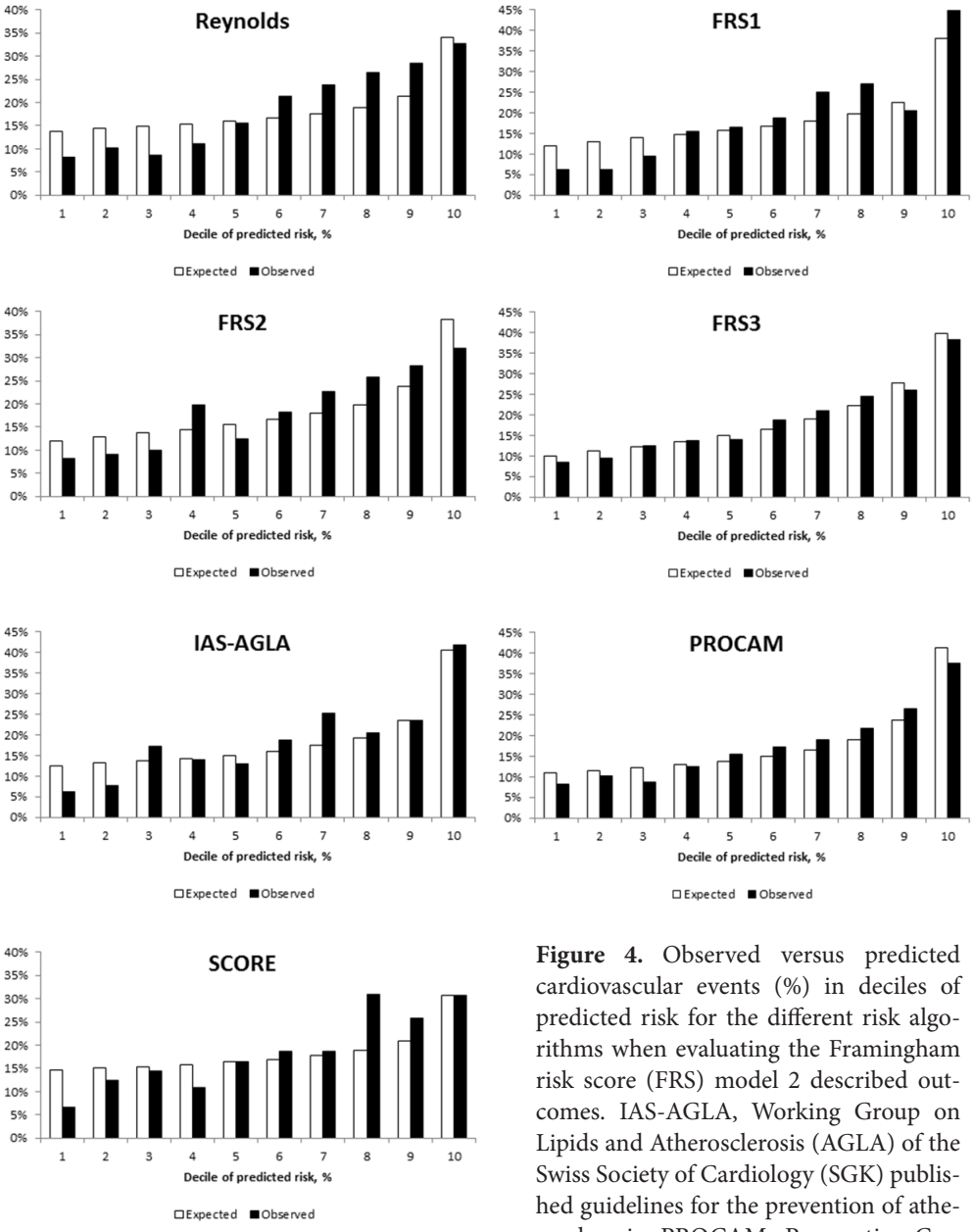

Figure 4. Observed versus predicted cardiovascular events (%) in deciles of predicted risk for the different risk algorithms when evaluating the Framingham risk score (FRS) model 2 described outcomes. IAS-AGLA, Working Group on Lipids and Atherosclerosis (AGLA) of the Swiss Society of Cardiology (SGK) published guidelines for the prevention of atherosclerosis; PROCAM, Prospective Cardiovascular Münster; Reynolds, Reynolds risk score; SCORE, Systemic Coronary Risk Evaluation.

The observed and predicted number of CV events using RRS differ significantly in all deciles both under- and overestimating CV risk. The H-L test yielded a P-value of 0.004 indicating no model fit. The number of CV events predicted by the FRS2 diverged from the observed number of CV events, but the difference did not show any strict tendency across deciles. The H-L test indicated a no model fit with a P-value of 0.011. CV risk as predicted by IAS-AGLA showed discrepancies in all deciles and had best predictions in top ones. A P-value of 0.007 of the H-L test indicated no model fit. The numbers of CV events predicted by PROCAM and the observed numbers were also inconsistent overestimating the risk in lower and underestimating in middle deciles. The result of H-L test indicated a moderate model fit with a P-value of 0.346. The observed numbers of CV events and numbers predicted by FRS1 and SCORE showed lack of similarity in lower and top deciles over- and underestimating the CV risk and looked most promising in middle deciles, however H-L test result showed a P-value of <0,001 indicating no model fit. FRS III showed best calibration with P-value of 0.961. In general, almost any of these algorithms did not manage to accurately predict an estimate of observed CV events in patients with MetS.

Discussion

Some of the risk models analyzed here proved to be better at predicting outcomes than the models that were designed to assess CV risk and predict outcome. Importantly, none of the models were adequate in predicting risk in the MetS group, even though they were developed, and considered appropriate, as a risk assessment tool.

Unstable way of life, variable diet and stress leads to changes in anthropometric parameters. This further leads to an increase of sickness rate of various diseases such as diabetes mellitus (DM), MetS, etc. These conditions have a negative effect on risk prediction. For example, DM has a significantly increased risk of CV outcomes compared to those predicted by various risk models. It is considered that other diseases also affect risk prediction therefore such patients are not suited for risk prediction models.

Due to lifestyle peculiarities, more and more people have an increased count of risk factors at a younger age. The prevalence of MetS is also increasing constantly. MetS is a multi-componential risk factor associated with an increased risk of CVD. Subjects with T2DM and MetS have twice as high risk than subjects without MetS. However, it is still not clear if such part of population causes correct prediction of

risk, using widely known risk prediction algorithms designed on the basis of separate populations. What is more, majority of these algorithms were developed more than 20 years ago when obesity and other components of MetS were not as widely spread, large part of other risk factors were not identified yet.

Nowadays data of general examination and anthropometric parameters are used in risk prediction, which are easy to measure, but can personalize an individual only on a very small basis. Such data does not represent person's genotype, way of life, etc. and this causes a lack of reliability in risk assessment.

Conclusion

- None of the risk prediction models evaluated were adequate in predicting future risk among the vulnerable population of patients with MetS
- Widely used CVD risk assessment tools either underestimate or overestimate risk in patients with MetS
- There are risk prediction models that can predict FRS2 outcomes for MetS patients better than the FRS2 model itself.

3. PERSONALISING CARDIOVASCULAR AND TYPE 2 DIABETES MELLITUS RISK ASSESSMENT WITH ADDITIONAL BIOMARKERS

Several risk scoring systems for CHD and CVD have been designed specifically for use in clinical practice, the majority of which are based on the American Framingham study [37,38]. The FRS equations are the most widely accepted method for projecting CVD/CHD risks, and are used in the British, European, and New Zealand guidelines.

A large systematic review of CV risk assessment in primary prevention has shown that the performance of FRS models varies considerably between populations and that accuracy relates to the background risk of the population to which it has been applied [39]. Framingham-based scoring systems tend to overestimate risk in low- and medium-risk groups, and underestimate risk for certain subgroups including British Asians and those with type 1 DM, T2DM and nephropathy, familial hypercholesterolemia, a strong family history of premature CHD, left ventricular hypertrophy on electrocardiography, and chronic renal disease [40]. Moreover, the work conducted in the current study confirms that this variable risk estimation also applies to patients with MetS.

For many health professionals, the calculation of absolute CV risk is the starting point for the development of CVD prevention strategies; however, there is an obvious and growing need for further personalization of CVD risk assessment. At present, patients at high risk of CVD are detected on the basis of the risk caused by the combination of their risk factors. This is calculated using risk estimation systems (SCORE, FRS). Another approach to detecting those at high risk is to look for the presence of subclinical atherosclerosis. Examples include ultrasound scanning of the carotid vessels (CIMT), aortic stiffness (AoPWV), and FMD for the presence of atherosclerosis. Advantages of AoPWV, CIMT and FMD are that they are non-invasive, and involve no exposure to radiation. AoPWV was selected for investigation as a potential biomarker for further personalising CVD risk and as a predictor of outcome in patients with MetS. We also aimed to investigate which of the more common potential biochemical biomarkers had the greatest predictive role in T2DM risk within the same sample.

3A. AORTIC PULSE WAVE VELOCITY IS AN INDEPENDENT CARDIOVASCULAR EVENT PREDICTOR IN A HIGH CARDIOMETABOLIC RISK GROUP

Literature review

MetS is a complex of atherosclerotic risk factors such as IGT, high BP, dyslipidemia and abdominal obesity [41,42]. Almost one-quarter of the world's adult population already has MetS [43] and many more remain, as yet, undiagnosed [44]. Patients with these components also frequently manifest a prothrombotic state and a proinflammatory state [45]; thus, MetS is considered to be a chronic inflammatory condition [46]. Moreover, MetS is assumed to be an indicator of long-term CV risk [47] and it is strongly associated with CVD development [48]. It is estimated that MetS provides a 2-fold increase in the risk of developing CVD [47]. Delineating those patients with MetS who are most vulnerable to the development of CVD would greatly aid disease prevention strategies.

Some risk prediction tools are applicable to patients with MetS, such as the FRS or the SCORE [49]; however, these are limited. The FRS algorithm evaluates the short-term (typically 5–10 years) risk of CVD [47] and, despite attempts to improve this risk-scoring system by adding components of MetS, such as central obesity, triglycerides, or IFG, no significant increase in predictive power for CVD has been found [45]. MetS score, defined as the number of MetS components, may also be inaccurate in predicting CVD because each component acts synergistically in CVD development [50]. Valuable efforts to improve risk assessment for CVD by adding new risk factors with an independent predictive value are ongoing.

AoPWV, a measure of arterial stiffness, represents a novel risk factor pathway under investigation. Arterial stiffness is measured either locally e.g. carotid, radial, brachial or femoral using ultrasound images of arterial changes in diameter or area and local distending pressure or regionally by measuring PWV along an arterial segment [51]. Impaired arterial stiffness, and precisely aortic PWV, is considered to be a well-recognized risk factor for CVD [52–54]. AoPWV, a direct measure of arterial stiffness, may enable the recognition of patients at low versus high risk of CV events [55]. In addition, aortic PWV may enhance risk prediction when added to classical risk factors [52] and, consequently, may provide better identification of high-risk groups [53]. Moreover, measurement of arterial stiffness may be useful in

clinical practice as it is a simple, noninvasive and reproducible method in CVD risk evaluation [56]. To date, any association of impaired arterial stiffness and CV risk in patients with MetS remains to be determined.

Aim

To assess whether arterial stiffness, as measured by AoPWV, is a viable predictor of CVD risk in patients with MetS.

Methodology

Study population

All 4259 patients included in the study were recruited from a single speciality cardiology centre participating in the Lithuanian High Cardiovascular Risk (LitHiR) primary prevention programme, which involves employable aged men (aged 40-55) and women (aged 50-65). Outcome follow-up data were derived from the national death registry and national healthcare fund disease and services databases. The cohort was divided into two groups: event group (EG) and event-free group (EFG), depending on whether they had an event recoded during the follow-up time.

High CV risk was defined as having one or more of the following conditions: 1) a SCORE risk assessment of >11; 2) diabetes; 3) MetS; 4) positive family history of CVD; and/or 4) severe dyslipidemia.

Informed consent

The local ethics committee approved the study without written informed patient consent due to retrospective design and large number of patients.

MetS

MetS was defined according to the revised NCEP ATPIII criteria, meeting three or more of the following:

- Waist circumference ≥102 cm in men, ≥88 cm in women
- TG ≥1.7 mmol/L
- HDL-C <1.03 mmol/L in men, <1.29 mmol/L in women
- BP ≥130/85 mmHg
- Fasting plasma glucose (FPG) ≥5.6 mmol/L

Arterial stiffness

Parameters of arterial stiffness and wave reflection were assessed by applanation tonometry (SphygmoCor, AtCor Medical, v. 8.0, Sydney, Australia). Radial, carotid and femoral artery pressure waveforms were recorded for 20 seconds each with single transducer synchronized with electrocardiogram (ECG) R wave, after obtaining high quality waveform. Distance between carotid and femoral arterial sites was determined using a tape measure. Carotid–femoral PWV, the 'gold standard' parameter of aortic stiffness, was calculated using subtraction method. Augmentation index (AIx), calculated from aortic pressure waveform, and adjusted for the heart rate, was automatically derived from the radial pressure waveform using previously validated transfer function.

Baseline measurements

All patients underwent a baseline examination including medical history, physical examination risk profile and lifestyle assessment, evaluation of CV family history, 12-lead ECG, laboratory blood tests, and non-invasive assessment of arterial markers of subclinical atherosclerosis. Weight, height, and waist circumference were measured with the subject wearing light clothing and without shoes. BMI was calculated as weight in kilograms divided by the square of height in meters. BP was measured after the patient rested at least five minutes, using an oscillometric semiautomatic device (Schiller Argus VCM) with a standard bladder (12–13 cm long and 35 cm wide), validated according to standardized mercury sphygmomanometer - at least one measurement per arm with additional measurements if the first two differed significantly. The reference value was the highest value or the average of the two highest values, if measured more than twice. Assessment of arterial stiffness was carried out by applanation tonometry (Sphygmocor v.7.01, AtCor Medical). Information about smoking and drug use was collected by a questionnaire. Current smoking was recorded if the subject smoked at least one cigarette per day. Positive CV family history was recorded if first-degree relatives of the patient had any CV events at a young age (men ≤45 years, women ≤55 years old).

Statistical analyses

Statistical analysis was performed using the SAS package for Windows (9.1 Version Cary, NC, USA). Means were compared using ANOVA followed by Bonferroni test. ANCOVA analysis was used to calculate least square means (±standard error of the mean, SEM) for comparison of PWV, after controlling for covariates (age, sex, non-HDL-C levels, current smoking, presence of DM, and study site for PWV). Multivariable logistic regression analysis was used to explore whether AoPWV independently predicted CV events. A two-sided P-value <0.05 indicated statistical significance.

Results

In total, 4259 high-risk subjects with MetS were followed up for a mean of 1389.3±625.73 days. On average, participants had 3.49±1.05 components of MetS as per the NCEP ATPIII criteria, and 18% of the group had five risk factors.

Baseline characteristics

The mean age in the EFG was 54 years versus 55 in the EG. There were 34 diabetic patients in the EG; however, there was no significant difference compared with the EFG (P<0.05). Baseline BMI (EFG 31.7; EG 31.6) and waist circumference (EFG 105 cm; EG 106 cm) were similar between the groups. Detailed baseline and clinical characteristics of the studied population, according to the presence or absence of the CVD events, are summarized in the **Table 2.**

CVD events

CVD events during the follow-up included fatal or non-fatal MI or stroke. No significant difference between the EFG and the EG with at least one CVD event during follow up. In the follow-up period, there were a total of 129 CVD events registered within the EG.

Association between arterial properties and CVD events

Comparing the two groups, aortic PWV was significantly lower in the EFG group 8.8±1.6 (EFG) vs. 9.41±2 (EG), P<0.001. Mean aortic pulse pressure (Ao_PP) was

Table 2. Clinical characteristics of the study population according to the absence or presence of the cardiovascular disease events.

	Total	No Event	Event			P
			Total	MI	Stroke	
n	4259	4130	129	78	50	
Age, years	54.13±6.23	54.1±6.24	55.17±5.81	55.82±5.67	54.15±6	0.055
Male gender, n (%)	1542 (36.2)	14 89 (36.1)	53 (41.1)	24 (30.8)	28 (56.0)	0.242
Hypertension, n (%)	3961 (93.0)	3835 (92.9)	126 (97.7)	78 (100.0)	47 (94.0)	0.053
DM, n (%)	751 (17.6)	717 (17.4)	34 (26.4)	20 (25.6)	14 (28.0)	0.008
Smoking, n (%)	972 (22.8)	939 (22.7)	33 (25.6)	14 (17.95)	18 (36.0)	0.448
Height, m	1.68±0.1	1.68±0.1	1.67±0.1	1.66±0.09	1.7±0.1	0.671
Weight, kg	89.36±16.83	89.38±16.85	88.93±16.15	87.63±16.94	90.78±14.93	0.767
BMI, kg/m^2	31.69±4.92	31.69±4.93	31.64±4.56	31.73±4.3	31.53±5.01	0.906
Waist circumference/height, m/m	0.63±0.07	0.63±0.07	0.63±0.07	0.63±0.06	0.63±0.07	0.642
Waist circumference, cm	105.42±11.05	105.41±11.04	105.65±11.5	104.8±11.27	106.96±11.95	0.810
TC, mmol/L	6.87±1.47	6.87±1.47	6.92±1.46	6.76±1.47	7.22±1.39	0.671
LDL-C, mmol/L	4.49±1.27	4.49±1.27	4.56±1.27	4.5±1.29	4.67±1.23	0.538
HDL-C, mmol/L	1.29±0.35	1.29±0.35	1.24±0.29	1.27±0.3	1.2±0.27	0.091
TG, mmol/L	2.38±2.33	2.38±2.35	2.38±1.52	2.1±1.09	2.82±1.96	0.989
FPG, mmol/L	6.16±1.31	6.15±1.29	6.5±1.78	6.23±0.93	6.89±2.55	0.004
hs-CRP, mg/dL	3.4±5.44	3.37±5.41	4.26±6.15	4.58±7.38	3.5±2.91	0.070
HbA_{1c}	5.92±0.66	5.92±0.66	5.98±0.72	5.86±0.51	6.16±0.94	0.381
Urea	5.36±1.39	5.35±1.38	5.55±1.55	5.5±1.71	5.57±1.25	0.135
Creatinine	71.51±16.86	71.41±16.97	74.79±12.75	73.9±13.48	75.72±11.23	0.025
Urine albumin_mg_l	15.15±47.42	15.14±47.66	15.71±25.54	18.33±30.25	9.4±4.67	0.961
Brachial PWV, cm/s	9.12±1.27	9.12±1.26	9.13±1.33	9.16±1.47	9.06±1.06	0.898
Femoral PWV, cm/s	8.82±1.61	8.8±1.6	9.41±2.00	9.48±2.04	9.3±2.0	<0.001
Aix@75	28.4±11.19	28.35±11.19	30.29±11.07	30.17±11.39	30.19±10.62	0.057
	24.39±10.44	24.33±10.43	26.31±10.53	26.55±10.02	25.60±11.22	0.038
MeanBP_Ap, mmHg	106.82±12.61	106.69±12.45	111.07±16.60	113.05±17.46	108.25±15.03	<0.001
Aortic pulse pressure, mmHg	43.37±11.07	43.28±11.02	46.25±12.32	46.82±12.84	45.56±11.64	0.003
Mean CAVI	7.89±1.61	7.89±1.61	8.14±1.62	8.16±1.63	8.08±1.63	0.086
Heart rate, beats/min	65.83±9.32	65.8±9.32	66.85±9.18	67.33±9.69	66±8.43	0.447
Follow-up, days	1389.30±625.73	1406.98±615.03	823.41±700.02	793.40±691.67	885.86±714.48	0.030

P, comparing event free vs. event (total) group. (t-test; chi-square test)

Table 3. Univariate logistic regression models (per 1 standard deviation change).

Risk factor		Event								
		Total (CV)			MI			Stroke		
	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	
AoPWV	Model 1	1.387	1.182; 1.627	<0.001	1.434	1.173; 1.753	<0.001	1.317	1.017; 1.706	0.037
	Model 2	1.332	1.129; 1.571	<0.001	1.369	1.11; 1.689	0.003	1.302	0.997; 1.7	0.052
AIx@75	Model 1	1.206	1.012; 1.438	0.036	1.232	0.987; 1.536	0.065	1.129	0.852; 1.495	0.399
	Model 2	1.343	1.1; 1.639	0.004	1.193	0.916; 1.555	0.191	1.478	1.118; 1.955	0.006
MeanBP_Ao	Model 1	1.378	1.168; 1.625	<0.001	1.559	1.275; 1.906	<0.001	1.130	0.856; 1.491	0.389
	Model 2	1.360	1.152; 1.607	<0.001	1.569	1.28; 1.922	<0.001	1.094	0.827; 1.448	0.528
Ao_PP	Model 1	1.270	1.082; 1.49	0.003	1.319	1.081; 1.61	0.006	1.207	0.933; 1.561	0.153
	Model 2	1.294	1.092; 1.533	0.003	1.265	1.019; 1.571	0.033	1.349	1.039; 1.753	0.025

Model 1: unadjusted; Model 2: adjusted for age and gender.

Aix@75, aortic augmentation index adjusted for a heart rate of 75 beats per minute; Ao_PP, aortic pulse pressure; AoPWV, aortic pulse wave velocity; CI, confidence interval; CV, cardiovascular; MeanBP_Ao, mean aortic blood pressure; MI, myocardial infarction; OR, odds ratio.

significantly lower in the EFG group, measured 43.28±11.02 (EFG) vs. 46.25±12.32 (EG), P=0.003, mean aortic BP (meanBP_Ao) 106.69±12.45 (EFG) vs. 111.07±16.6 (EG), P<0.001.

In the unadjusted logistic regression model, aorticPWV remained a strong CVD event predictor (**Table 2**). Odds ratios (OR) for a CV event, as shown in the **Table 3** illustrate that CV risk increases with the change of various arterial and hemodynamic parameters by one standard deviation (SD).

Four selected variables were divided into 3 tertiles, comparing cumulative proportion survival rate, aortic PWV remained a strong survival predictor, P=0.001 (**Figure 5**), comparing 3rd tertile vs. 1st tertile for CVD event, aortic PWV OR was 1.748 (95% CI 1.135; 2.691, P=0.011).

	1st tertile	2nd tertile	3rd tertile
AoPWV	<8	8-9.3	≥9.3
AIx@75	**<21**	**21–29**	≥29
MeanBP_Ao	**<101**	**101–111**	**≥111**
Ao_PP	<38	38-47	≥47

AIx@75, aortic augmentation index adjusted for a heart rate of 75 beats per minute; Ao_PP, aortic pulse pressure; AoPWV, aortic pulse wave velocity; MeanBP_Ao, mean aortic blood pressure; PWV, pulse wave velocity.

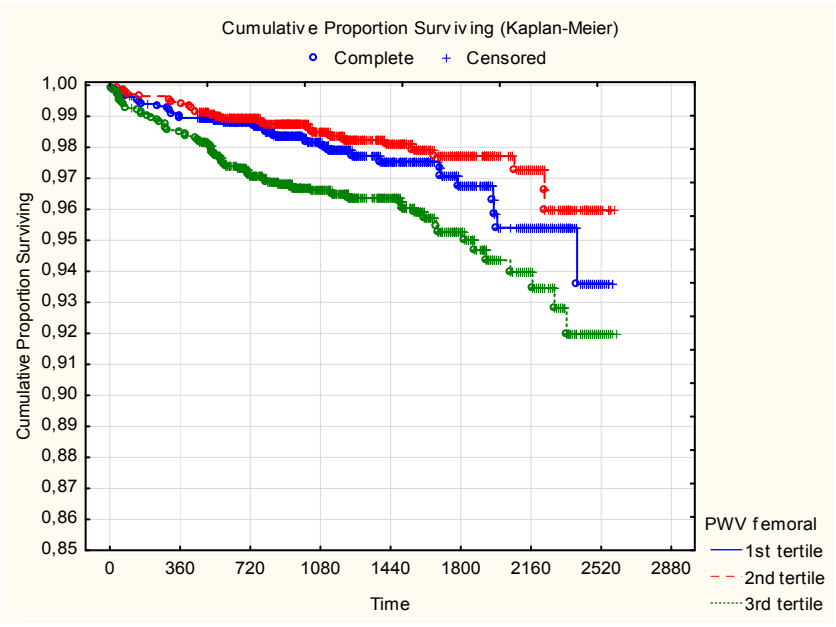

Figure 5. Cumulative proportion surviving curve (AoPWV).

Discussion

The main finding of this study was that, using a logistic regression model, AoPWV remained a strong independent CVD event predictor, indicating higher CVD risk with increasing AoPWV. In addition, survival analysis confirmed it is a viable CVD prediction indicator. These findings highlight the importance of including AoPWV into widely used CVD risk assessment tools, especially for high CVD risk groups.

The role of aortic PWV in CVD risk evaluation has been widely investigated. AoPWV has been assumed as the 'gold standard' for measuring aortic stiffness [51] and may represent a predictor of CV events because of its involvement in pathophysiological CV outcomes [57]. For example, arterial stiffness is linked to left ventricular hypertrophy, which is known as a risk factor for CV complications in hypertensive and normotensive patients [58]. As PWV reflects the long-term effect of CVD risk factors on the arterial wall, it may have a better predictive value than traditional risk factors [56]. Furthermore, a significant proportion of patients at intermediate risk after arterial stiffness measurement could be reclassified into a higher or lower CVD risk [59]. It is recognized that aortic stiffness has an independent predictive value for all-cause mortality [56]. In addition, an independent influence of arterial stiffness on survival has been demonstrated in patients with end-stage renal disease who are at high risk of mortality [60].

When assessing arterial stiffness, it is important to take into account that this parameter increases with aging [59,61]. This is induced by an increase in arterial wall thickness because of hyperplasia of the intima and also by reduction of elastin and its replacement with collagen in the media [62]. Therefore, it is not always clear whether this parameter must be determined as a function of age [63].

The impact of individual components of MetS on arterial stiffness should be discussed. Firstly, arterial stiffness strongly depends on high BP [64] and has an independent predictive value for fatal and nonfatal CVD outcomes in patients with hypertension [59]. This is because of elevated SBP, which increases left ventricular afterload, and because of lower DBP, which changes coronary perfusion [58]. Moreover, essential hypertension is often associated with various metabolic abnormalities such as dyslipidemia, elevated glucose levels, insulin resistance and abdominal obesity [65].

Secondly, arterial stiffness is also increased in people with IGT and DM[24]. Diabetes may increase arterial stiffness through pathological mechanisms, including low nitric oxide bioavailability, enhanced oxidative stress, chronic inflammation, activat-

ed sympathetic nervous system and changes in the arterial wall [66]. Furthermore, hyperglycemia might cause the glycation of matrix proteins and the accumulation of advanced glycation end products on collagen and elastin [41].

Another important cardiometabolic risk is abdominal adiposity. Central adiposity may induce the secretion of several peptides such as leptin, which may change arterial wall motion [67]. Obesity, and especially elevated leptin, has been associated with lower arterial distensibility [65]. Consequently, weight loss has been demonstrated to improve arterial stiffness [68]. On the contrary, most of the studies related to AoPWV and dyslipidemia found minimal or incompatible correlations among these factors [65]. In addition, people with obesity and DM are more likely to show increased AoPWV than people with hyperlipidemia [69].

It is important to measure arterial stiffness at different sites, as stiffness is not equal along the arterial system [70]; more elastic arteries are closer to the heart and therefore stiffness increases gradually from the ascending aorta to the peripheral arteries [71]. The estimation of local arterial wall stiffness is mostly performed using superficial arteries such as the carotid where atherosclerosis is often located [57]. Although PWV can be measured on any artery, only carotid–femoral PWV has been recognized to have predictive value for morbidity and mortality [71]. Stiffness at other arterial sites has lower or no ability to predict CV events [72].

There are some controversies surrounding the role of arterial stiffness in CV risk assessment. It has been demonstrated that carotid–femoral PWV reflects both elastic and muscular elements of the arterial system and that these may be differentially associated with CVD events and mortality [51]. In contrast to the study, the Atherosclerosis Risk In Communities (ARIC) study has reported that carotid stiffness is more strongly associated with cerebrovascular disease than with CVD, because stiffening of this artery may lead to a pressure overload on the brain [73]. Moreover, another study has demonstrated that increased carotid stiffness is more strongly associated with all-cause mortality than with CV outcomes [70].

Nevertheless, the use of arterial stiffness is advantageous in terms of CVD prognosis, especially in patients at high risk of cardiometabolic disease [74]. Additionally, this parameter may enhance the evaluation of the individual CVD risk in hypertensive patients who regularly attend the outpatient clinic [58]. Furthermore, arterial stiffness is a potential goal of treatment[34] and BP lowering drugs can promote regression of intima-media thickness [75]. It is of interest that angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARBs) independently

reduce arterial stiffness without any association of BP reduction by blocking the renin–angiotensin system (RAS) which may impair arterial stiffness through the strong vasoconstrictor, angiotensin II [76].

Finally, it is important to prevent and reduce the presence of MetS in order to reduce CVD and to prolong life in adults [48]. These data support the concept of using arterial stiffness as a predictor of future CV health among the population with MetS and to aid primary prevention.

Conclusion

- AoPWV remained a strong independent CVD event predictor, indicating higher CVD risk with increasing AoPWV
- Inclusion of AoPWV into CVD risk assessment tools is warranted, and may be particularly useful in the assessment of high CVD risk groups.

3B. PREDICTIVE MODELS TO IDENTIFY BIOMARKERS FOR PROGRESSION FROM METABOLIC SYNDROME TO TYPE 2 DIABETES MELLITUS

Literature review

MetS is a cluster of serious CV risk factors including abdominal obesity, high cholesterol and high BP that is prevalent in a large proportion of the world's population. Individuals presenting with this cluster of risk factors are three times as likely to have, and twice as likely to die from, a heart attack or stroke, compared to people without the syndrome [77]. Several population-based studies have shown that MetS is a risk factor for the future development of T2DM [78–82]. Importantly, a meta-analysis of prospective studies showed that people with MetS have a five-fold greater risk of developing T2DM than individuals without the syndrome [83]. The cluster of risk factors that typify MetS is driving an epidemic of CVD and DM.

Predicting the likelihood of T2DM among patients with MetS is a clinical challenge. In this era of personalized and patient-centric care comes the desire to predict the likely progression of a pre-diabetic state and prevent the associated risk of premature illness and death. Elements of MetS such as impaired fasting glucose (IFG) and IGT are shown to be strong predictors of T2DM in many studies [84–86], while

waist circumference, BMI, and TG have shown associations with the incidence of T2DM in subjects with high post-prandial glucose [87] and in the general population [88]. When atherosclerosis is already present, the presence of a high waist circumference alone is associated with an increased risk of developing T2DM, as is the presence of ≥3 metabolic risk factors [89]. Together, the presence of ≥3 metabolic risk factors and a high waist circumference is associated with a 10-fold increased risk of T2DM in this atherosclerotic population [89]. Many of these studies are associative in nature, and analysis of the predictive and cumulative value of these factors is warranted. Furthermore, the scale of risk among patients with MetS who do not yet show overt atherosclerotic disease is, as yet, undetermined.

Aim

To determine the predictive value of clinical biomarkers for the incidence of T2DM in patients with MetS who do not yet show atherosclerotic disease.

Methodology

Study design

A prospective study of 525 non-diabetic, middle-aged Lithuanian men (n=187, 36%) and women (n=338, 64%) with MetS but without overt atherosclerotic disease from the Lithuanian High Cardiovascular Risk (LitHiR) primary prevention programme was conducted between 2007 and 2011. This programme includes patients from primary and secondary care settings and they were followed up for a median period of 3.3 years, for identifying the new T2DM cases.

Definition of MetS

MetS was diagnosed if a patient presented with three or more of the revised NCEP ATPIII criteria [90, 91]:

- Waist circumference ≥102 cm in men, ≥88 cm in women
- TG ≥1.7 mmol/L
- HDL-C <1.03 mmol/L in men, <1.29 mmol/L in women
- BP ≥130/85 mmHg
- FPG ≥5.6 mmol/L

The metabolic syndrome score was calculated as the sum of MetS components present.

Assessments

Baseline assessments were recorded for medical history, physical examination, risk profile, and lifestyle parameters, evaluation of CV family history, 12-lead electrocardiogram, laboratory blood tests, and non-invasive assessment of arterial markers of subclinical atherosclerosis, weight, height, waist circumference, body mass index (BMI), BP, and arterial stiffness.

Fasting venous blood samples were collected for estimation of serum TC, TG, glucose, HDL-C, LDL-C, hs-CRP, HbA_{1c} and insulin. A standard 75-g oral glucose tolerance test (OGTT) was carried out after patients completed a 12-hour overnight fast. Plasma glucose and insulin concentrations were measured at 0 and 120 minutes.

Subjects were classified into various categories of glucose tolerance using the WHO criteria [92]. Normal glucose tolerance was defined by fasting glucose <6.1 mmol/L and 2-h OGTT glucose <7.8 mmol/L. IFG was defined by fasting glucose ≥6.1 mmol/L and <7.0 mmol/L and 2-h OGTT glucose <7.8 mmol/L. IGT was defined by fasting glucose <7.0 mmol/L and 2-h OGTT glucose between 7.8 and 11.0 mmol/L. Diabetes was defined by fasting glucose ≥7.0 mmol/L and/or 2-h OGTT glucose ≥11.1 mmol/L.

Four surrogate indices of insulin resistance or insulin sensitivity were calculated: the homeostasis model assessment insulin resistance (HOMA-IR) index [93] was calculated as fasting insulin [μU/mL] × FPG [mmol/l] / 22.5; the quantitative insulin-sensitivity check index (QUICKI) index [94] was calculated as 1/[log(fasting insulin [μU/mL]) + log(FPG [mg/dL])]; the Cederholm insulin sensitivity index (ISI) [95], representing peripheral insulin sensitivity, was calculated as $ISI_{Cederholm} = 75000 + (G_0\text{-}G_{120}) \times 1.15 \times 180 \times 0.19 \times \text{weight}/120 \times G_{mean} \times \log (I_{mean})$, where G_0 and G_{120} are plasma glucose (mmol/L) concentrations at 0 and 120 minutes. The Matsuda insulin sensitivity index [96,97], reflecting a composite estimate of hepatic and muscle insulin sensitivity, was calculated as $ISI_{Matsuda} = 10{,}000 / \sqrt{(G_0 \times I_0 \times G_{120} \times I_{120})}$, where G_0, G_{120}, and I_0, I_{120} are the plasma glucose (mg/dL) and the plasma insulin (μU/mL) concentrations respectively at time 0 and 120 minutes.

Statistical analyses

Descriptive analyses were applied to the baseline measures: mean and SD for the continuous variables and the frequency, and proportion for the categorical variables.

Logistic regression was used to develop predictive models for incident cases and to investigate the association between various markers and the onset of T2DM. Gender-adjusted odds ratios (ORs) were calculated and p values (Bonferroni corrected) were based on two-sided tests with a cutoff for statistical significance of 0.05. Little's method was applied to control for bias due to missing data [98–100].

A stepwise, multivariate logistic regression model was developed to determine which factors predicted progression to T2DM most strongly. Bayesian Information Criterion (BIC) was used to assess model fit based on a log likelihood function [101]. The model with the lowest BIC value was preferred. A "forward" approach was taken, starting with the gender variable, then adding one variable at each step that maximally reduced the BIC statistic and terminated when the BIC statistic stopped decreasing. Model accuracy was estimated by using each subject as a validation set, and the remaining subjects to generate the model. ROC curves with area under the curve (AUC) values were calculated for predicting T2DM. Statistical and modeling analyses employed MATLAB 7.13 (R2011b) and SPSS Statistics 19.0.0.

Results

Baseline characteristics of the cohort who progressed to diabetes and those who did not are shown in **Table 4**. As would be expected, many of the baseline parameters were higher among the group of later progressors.

Table 5 presents the odds-ratio results for all variables after they were adjusted for gender. In the first iteration, 12 significant predictors (presented in decreasing order of their association) were identified: FPG, BMI, waist circumference, OGTT glucose, HbA_{1c}, QUICKI, metabolic syndrome score, weight, ISIMatsuda, OGTT insulin, HOMA-IR, and fasting insulin. In the second iteration, after adjusting for FPG and gender, the BMI showed the most significant association. After the selection of BMI (third iteration), only HbA_{1c} remained a significant predictor. The final set included: gender, FPG, BMI, and HbA_{1c}. The selected variables: FPG, BMI, and HbA_{1c} each showed a significant cumulative effect in the final model (FPG: P=0.000001; BMI: 0.00001; HbA_{1c}: P=0.0004).

Figure 6 shows the predictive power of FPG, BMI, and HbA_{1c}, in the stepwise multivariate logistic regression model. It also shows the improvement in the prediction for the combined, gender-adjusted score of these three variables, by plotting the ROC curves of the corresponding models. The model showed a high level of accuracy (AUC=0.91).

Table 4. Baseline characteristics of the cohort.

Variable	Complete case	Men		Women	
		Non-progressors	Progressors	Non-progressors	Progressors
	n (%)	value (SD)	value (SD)	value (SD)	value (SD)
FPG, mmol/L	521 (99%)	5.9 (0.8)	7.1 (1.2)	5.7 (0.6)	6.7 (0.6)
BMI, kg/m^2	524 (100%)	30.3 (3.8)	35.6 (5.4)	30.7 (4.6)	34.6 (5.4)
Waist circumference, cm	520 (99%)	106.6 (9.4)	116.5 (9.7)	100.8 (9.6)	108.9 (7.3)
OGTT glucose, mmol/L	425 (81%)	5.4 (1.6)	6.9 (1.8)	6.3 (1.7)	7.8 (1.8)
HbA_{1c}, %	405 (77%)	5.6 (0.2)	6.0 (0.5)	5.7 (0.3)	5.9 (0.2)
QUICKI	326 (62%)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)
Metabolic syndrome score, 0–5	525 (100%)	3.3 (1.0)	3.8 (0.8)	3.4 (1.0)	4.3 (0.7)
Weight, kg	524 (100%)	95.4 (13.9)	107.6 (16.2)	80.0 (12.9)	87.6 (12.9)
ISIMatsuda	299 (57%)	7.8 (5.4)	3.8 (2.4)	6.6 (4.8)	3.3 (2.2)
OGTT insulin, pmol/L	301 (57%)	254.5 (199.3)	473.7 (209.2)	415.5 (345.8)	694.7 (492.8)
HOMA-IR	326 (62%)	3.2 (1.8)	5.7 (2.3)	3.4 (2.6)	4.9 (2.0)
Fasting insulin, pmol/L	326 (62%)	84.3 (43.0)	131.9 (49.3)	88.0 (61.6)	115.5 (49.0)
HDL-C, mmol/L	523 (100%)	1.2 (0.3)	1.1 (0.2)	1.4 (0.3)	1.2 (0.2)
LDL-C, mmol/L	524 (100%)	4.3 (1.2)	3.7 (1.1)	4.8 (1.3)	4.4 (0.9)
TC, mmol/L	525 (100%)	6.7 (1.4)	6.1 (1.3)	7.1 (1.4)	6.7 (1.2)
hs-CRP, mg/L	502 (96%)	4.0 (6.8)	3.9 (4.3)	2.9 (3.2)	7.2 (13.7)
ISICederholm	299 (57%)	75088.2 (1048.6)	75363.3 (1453.0)	74441.7 (1223.9)	73737.3 (1848.3)
Age, years	525 (100%)	48.0 (4.0)	49.1 (4.4)	56.9 (4.1)	56.9 (3.6)
Smoking status	522 (99%)				
Never		82 (49%)	8 (50%)	267 (83%)	15 (94%)
Former		13 (8%)	0 (0%)	7 (2%)	0 (0%)
Current		73 (43%)	8 (50%)	48 (15%)	1 (6%)
TG, mmol/L	525 (100%)	2.7 (2.4)	3.0 (1.6)	2.1 (2.2)	2.2 (0.9)
Statin treatment	501 (95%)	163 (98%) 4 (2%)	13 (100%) 0 (0%)	303 (99%) 3 (1%)	15 (100%) 0 (0%)
AoPWV, m/s	480 (91%)	8.6 (1.4)	9.3 (1.8)	8.8 (1.4)	9.1 (1.6)
rPWV	496 (94%)	9.1 (1.2)	9.0 (1.5)	8.9 (1.3)	8.5 (1.3)
MAP, mmHg	493 (94%)	107.5 (13.2)	103.9 (9.9)	106.7 (14.6)	106.4 (16.3)
AIx@75 (%)	499 (95%)	18.1 (8.8)	16.3 (9.1)	30.3 (7.8)	28.1 (14.1)

Table 5. Odds ratios of the various investigated markers, adjusted for gender

Variables		OR (95% CI) Bonferroni corrected P-value	
	All	Men	Women
FPG	4.3 (2.6-7.2) P<0.0001	3.9 (1.9-8.1) P=0.01	4.8 (2.4-9.5) P=0.0002
BMI	1.2 (1.1–1.3) P<0.0001	1.3 (1.2–1.5) P=0.0005	1.2 (1.1–1.3) P=0.04
Waist circumference	1.09 (1.05–1.13) P<0.0001	1.1 (1.0–1.2) P=0.01	1.1 (1.0–1.1) P=0.1
OGTT glucose	1.6 (1.3–2.0) P=0.0002	1.6 (1.2–2.2) P=0.05	1.7 (1.2–2.3) P=0.04
HbA_{1c}	13.0 (4.1-41.7) P=0.0003	33.1 (4.5–240.9) P=0.01	6.5 (1.5–27.6) NS
QUICKI	0.00 (0.00–0.00) P=0.001	0.00 (0.00–0.00) P=0.1	0.00 (0.00–0.00) NS
Metabolic syndrome score	2.5 (1.6–3.8) P=0.001	1.8 (1.0–3.3) NS	3.4 (1.8-6.7) P=0.01
Weight	1.0 (1.0–1.1) P=0.003	1.1 (1.0–1.1) P=0.1	1.0 (1.0–1.1) NS
ISIMatsuda	0.6 (0.5–0.8) P=0.01	0.6 (0.4–0.9) NS	0.6 (0.4–0.9) NS
OGTT insulin	1.002 (1.001–1.003) P=0.02	1.00 (1.00–1.01)NS	1.002 (1.000–1.003) NS
HOMA-IR	1.3 (1.1–1.5) P=0.02	1.5 (1.1–2.0) NS	1.2 (1.0–1.3) NS
Fasting insulin	1.01 (1.00–1.01) NS	1.02 (1.00–1.03) NS	1.00 (1.00–1.01) NS
HDL cholesterol	0.1 (0.0–0.6) NS	0.2 (0.0–2.5) NS	0.1 (0.0–0.7) NS
LDL cholesterol	0.7 (0.5–1.0) NS	0.6 (0.4–1.0) NS	0.8 (0.5–1.2) NS
Total cholesterol	0.7 (0.6–1.0) NS	0.7 (0.5–1.1) NS	0.8 (0.5–1.1) NS
hs-CRP	1.0 (1.0–1.1) NS	1.0 (0.9–1.1) NS	1.1 (1.0–1.2) NS
ISICederholm	0.99 (0.99–1.00) NS	1.00 (1.00–1.00) NS	1.00 (0.99–1.00) NS
Age	1.0 (0.9–1.1) NS	1.1 (0.9–1.2) NS	1.0 (0.9–1.1) NS
Smoking (never, former, current)	0.9 (0.6–1.4) NS	1.1 (0.6–1.8) NS	0.6 (0.2–1.7) NS
Triglycerides	1.0 (0.9–1.2) NS	1.0 (0.9–1.3) NS	1.0 (0.8–1.2) NS
Statin treatment (no, yes)	0.0 –	0.0 –	0.0 –

P-values are Bonferroni corrected. P>0.1 is marked not significant (NS). For the binary statin treatment variable, all incident cases had a negative value. Hence odds ratio for statin treatment is 0, and the confidence interval and P-value are not defined.

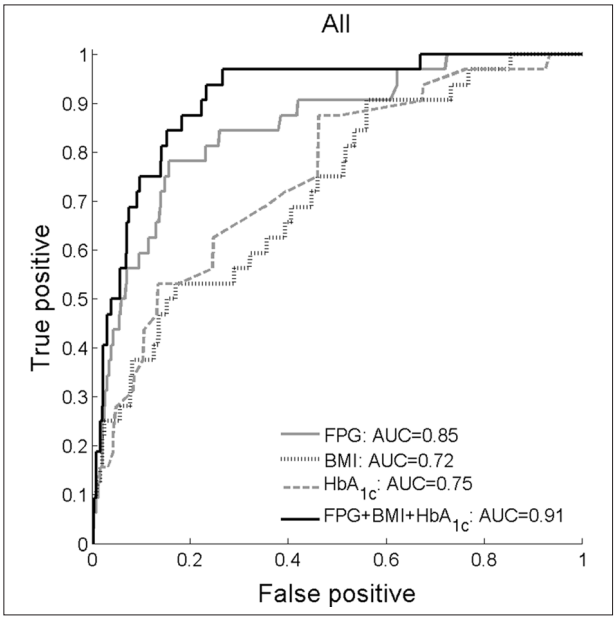

Figure 6. Comparison of prediction models. Receiver operating characteristic curves of four diabetes onset prediction models: Fasting plasma glucose (FPG)-model, body mass index (BMI)-model, glycosylated haemoglobin (HbA_{1c})-model, and a FPG-BMI-HbA_{1c}-model. All models were adjusted for gender.

Using the model to test the cumulative value of the obesity measures, BMI had the most significant cumulative effect (P=0.003, odds ratio test), compared to weight (P=0.03, odds ratio test), or waist circumference (P>0.1, odds ratio test).

FPG was superior to OGTT glucose in predicting T2DM, showing a very significant cumulative effect (P<0.00001, odds-ratio test), while OGTT glucose showed a milder cumulative effect in the gender-adjusted model (P=0.007, odds-ratio test), and no significant effect when BMI and HbA_{1c} were added to the model (P>0.1, odds-ratio test).

Measures of arterial stiffness - aortic augmentation index adjusted for a heart rate of 75 beats per minute (AIx@75) and radial PWV - showed no significant association with either progression to diabetes or IGT/IFG pre-diabetic conditions. Interestingly, another measure, aortic PWV, was significantly associated with progression to diabetes (P=0.04; odds ratio test).

Discussion

In this study, the combination of FPG, BMI, and HbA_{1c} was shown to be a powerful predictor for the development of T2DM in subjects with MetS. FPG was shown to be superior to OGTT glucose in predicting T2DM, with OGTT glucose showing no cumulative value to FPG. These findings are consistent with general population

studies showing that IGT and IFG are similarly associated with an increased risk of diabetes, and that risks are higher when IGT and IFG coexist [102]. The OGTT findings, however, differ from previous studies [103,104]. The higher predictive value of FPG presented herein may be explained by the high prevalence of elevated FPG observed. However, performing a 2-h OGTT should be considered superfluous to the prediction of candidates for future diabetes, as supported by previous authors [105–107].

BMI and HbA_{1c} were also evaluated as predictors of diabetes in previous studies [88,104]. The risk of T2DM increased exponentially with HbA_{1c} in both genders [108]. In another large study, a model including both FPG and HbA_{1c} was more effective for T2DM prediction than models including FPG alone or HbA_{1c} alone [109]. Recently, a study confirmed that HbA_{1c} of ≥5.6% had an increased risk for progression to T2DM, independent of other confounding factors [110]. These prior studies support the current finding on the cumulative effect of HbA_{1c} with respect to FPG and HbA_{1c}. Four common insulin resistance/sensitivity indices were less predictive for T2DM than FPG and OGTT glucose, which is consistent with previous findings [105,111].

The applanation tonometry results correspond with previous studies, suggesting an association between aortic PWV and diabetes, and a lack of association between elevated augmentation index and the presence of diabetes [112]. Similar to previous reports [113], this study demonstrated that the association between increased aortic stiffness and glucose metabolism abnormalities (IGT) is already found in pre-diabetic stages, and that IGT is more strongly associated with CV risk than IFG. The increased aortic PWV in the current study cannot be explained by the elevation of CRP, and is predominantly associated with elevated 2h-OGTT glucose measurements.

To my knowledge, no previous study has established a predictive model for new onset diabetes in subjects with MetS. This study focused on middle-aged metabolic-syndrome subjects in Lithuania; thus, the findings are limited in their generalizability to subjects without MetS or to race/ethnicity populations that are differentially affected by diabetes. Additional limitations are the small dataset (525 subjects) and short duration of follow-up (2-4 years), resulting in only 32 participants who developed diabetes during the study. Subsequently, there is uncertainty in assessing the level of the risk estimate. Another drawback of the study design is the lack of information on diabetes familial history, which has been shown to be a strong predictor for T2DM in the general population as well as in subjects with MetS [88].

T2DM onset in middle-aged subjects with MetS can be predicted with remarkable accuracy using the combination of FPG, BMI, and HbA_{1c}, and is related to elevated aortic PWV measurements. As previous authors have noted, complicated predictive modeling and clinical biomarker measurements may not enhance clinical practice to the same degree as a few simple routine clinical measures to identify diabetes risk [114]. This study shows that simple measures, such as BMI, FPG, and HbA_{1c}, can accurately predict the development of T2DM in subjects with MetS. Application of these predictors in real-life practice may provide great value in delineating those patients at greatest risk of progression to T2DM. Meta-analysis of data from many population-based studies has shown that MetS, regardless of how it is defined, is a significant predictor of incident diabetes in many different populations [83]. The current study adds to the current knowledge that for subjects who already have MetS: fasting plasma glucose is the strongest predictor, with BMI and glycosylated hemoglobin having cumulative value.

Conclusion

- T2DM onset in middle-aged subjects with MetS can be predicted with remarkable accuracy using a combination of FPG, BMI, and HbA_{1c}, and is related to elevated AoPWV measurements
- FPG was shown to be superior to OGTT glucose in predicting T2DM among MetS subjects.

4. CIRCULATING MICRORNAS AS BIOMARKERS OF EARLY ATHEROSCLEROSIS

MicroRNAs (miRs) are endogenous, non-coding, small (18–22 nucleotides) RNA molecules. They negatively regulate gene expression at the posttranscriptional level by imperfect base pairing with the 3′-untranslated region of target mRNA, leading to miR degradation or translation repression [115]. This mechanism of controlling gene expression by miRs plays a crucial role in normal physiology and development, regulating several cellular processes such as differentiation, growth, proliferation and apoptosis. miRs are present in cells, such as endothelial cells, monocytes and macrophages, vascular smooth cells, and in platelets and plasma. miRs in plasma avoid degradation by being packaged in microparticles (exosomes, microvesicles and apoptotic bodies) or bound with proteins or high-density lipoproteins (HDL) [116–118].

In addition to their physiological functions, miRs are indicated in the pathogenesis of metabolic diseases. They participate in the control of cholesterol, lipoprotein, glucose and hormone (e.g. estrogen) metabolism, as well as affecting the production of adipokines; thus, miRs represent critical regulators of metabolic homeostasis. When these pathways are disrupted, miRs have a potential pathological role in obesity, insulin resistance, MetS and T2DM (which contributes to the development of vessel wall inflammation and atherosclerosis) and cardiac injury/remodeling after MI [119]. Certain miRs in plasma or serum are already elevated or decreased in association with metabolic diseases prior to CAD, PAD, HF, ACS, and stroke; others appear to be related to CV complications [120–132]. The enormous burden of CVDs – contributing to 48% of global deaths due to non-communicable diseases [133] – has prompted the search for prognostic markers that may serve to guide intervention to prevent illness. Detection of circulating miRs – these guides of metabolic homeostasis – generated much interest in their potential as biomarkers or predictors of disease that would be accessible by routine diagnostic methods. Owing to the limited follow-up times of the investigated subjects, correlations between the miRs and the wide research on early atherosclerosis markers were therefore relied on. Those include arterial stiffness (AoPWV), as previously described, but also extending to endothelial function tests (FMD) and CIMT measurements.

FMD is an endothelium-dependent, NO-mediated process that uses high-resolution ultrasound in the brachial artery. The method is completely non-invasive and safe, easy to use, reliable and repeatable in expert laboratories. FMD is limited by technical difficulties and methodological shortcomings (for example, FMD measurements require significant technical expertise; guidelines propose a minimum number of 100 supervised scans prior to scanning independently and at least 100 scans/year to maintain competency) [134]. Extensive training of the operator is needed resulting in a long learning curve, and image analysis may be labor-intensive. Potential environmental/physiological influences (e.g. food, caffeine, temperature, stress) need to be controlled for. Methodological standardization is needed (cuff positioning, timing of response, edge detection, software analysis, stereotactic probe-holding devices) to reduce operator-dependence, improve reproducibility and allow comparison among laboratories [135].

AoPWV, CIMT and FMD are already used in daily clinical practice. They are useful in determining the degree of subclinical carotid atherosclerosis or endothelial dysfunction, and have the advantage of being noninvasive and safe. However, their applicability in routine screening for risk determination is limited because they are operator dependent, time-consuming and relatively expensive (requirement of specific equipment, well trained staff and specially prepared patient). Moreover, it is difficult to reproduce. Because of this there is a need of simpler, less time consuming biomarkers, which would correlate with AoPWV, CIMT and FMD.

Literature review

Narrowing the search for miRs responsible for CVDs presents many challenges. miRs are known to regulate multiple target genes and in turn, one gene can be regulated by several miRs, making an assessment of the specific effects of any miRs cumbersome. Moreover, deriving the pathophysiological program that is changed by a set of differentially expressed miRs and their target genes requires a reductionist approach that extracts the essential features of the miRs regulation pattern in a structured and scientifically logical manner. A systems biology approach may assist in the identification of patterns of miR effects relevant to CV and metabolic diseases. These patterns can then be used as a basis for further functional investigation. A systematic literature review was conducted with the aim of reviewing the existing literature on miRs as biomarkers of CVDs – atherosclerosis, CAD, and ACS – with

the aim of selecting the 10 most promising miRs for further research in the current study's patient group.

Information resources, search and study selection

The PubMed database for English-language articles related to miRs as biomarkers of CV and metabolic diseases were searched. The key search terms in relation to CVDs were (miR) AND (atherosclerosis OR cardiovascular OR cerebrovascular OR acute coronary syndrome OR heart failure OR stroke). The key search terms in relation to metabolic diseases were (miR) AND (biomarker) AND (overweight OR obesity OR type 2 diabetes OR insulin resistance OR hypertension OR dyslipidemia OR metabolic syndrome). Based on the abstracts, experimental studies in preclinical models were excluded. Only original research papers were included for the final assessment. No lower date limit was used.

The initial literature search identified 164 articles. A total of 101 reviews, which cited other research papers, were excluded. Overall, 63 original, full-text research papers were included for analysis. The summary of the literature search and selection is depicted in **Figure 7**. The different miR groups and the key features of the included studies are summarized in **Table 6**.

Cardiovascular diseases

Atherosclerosis. Six experimental studies related to miRs in atherosclerosis were identified. Three of them concentrated on CAD. Fichtlscherer et al. [120] examined plasma miR profiles of 67 stable CAD patients and 31 healthy subjects. The two groups differed significantly in hypertension and diabetes status, as well as levels of total and low-density lipoprotein (LDL) cholesterol, but no adjustments were made for these differences. Caenorhabditis elegans miR-39 was used for normalization of the RNA preparation. Circulating levels of miR-126, miR-17, miR-92a, smooth muscle-enriched miR-145 and the inflammation-associated miR-155 were significantly reduced in patients with CAD, whereas cardiac muscle-enriched miRs (miR-133a, miR-208a) tended to be higher in patients with CAD [120]. Weber et al. [122] identified 11 miRs (miR-150, miR-584, miR-19a, miR-145, miR-155, miR-222, miR-378, miR-29a, miR-30e-5p, miR-342, miR-181d) that were significantly downregulated in whole blood of CAD patients (n=10) compared to age-matched healthy

controls (n=15). Both studies suggested that significantly dysregulated miRs could serve as diagnostic markers for CAD. However, the metabolic profiles of CAD patients and healthy controls were found to be very different [122].

Li et al. [121] focused on atherosclerosis in peripheral arteries. This group examined 104 patients with atherosclerosis obliterans or PAD and 105 age-matched healthy controls. They identified three miRs (miR-130a, miR-27b, miR-210) that were significantly upregulated in the serum of patients with atherosclerosis obliterans or PAD. U6 snRNA was used as the housekeeping gene. They also found a significant difference in the levels of miR-130a between patients with atherosclerosis obliterans stage Fontaine I and stage Fontaine III, suggesting that miR-130 might reflect the progression of atherosclerosis obliterans [121]. Stather et al. [136] examined 15 PAD patients and compared their miR expression profiles to those of 16 healthy controls. Twelve miRs (let-7e, miR-15b, miR-16, miR-20b, miR-25, miR-26b, miR-27b, miR-28-5p, miR-126, miR-195, miR-335, and miR-363) were significantly different between the two groups. The geometric mean of mammU6 and RNU48 expression levels was used as normalization factor in all miR qRT-PCR data. Based on the receiver operating characteristic (ROC) curves, miR-16, miR-363, and miR-15b had the best predictive value with an area under the curve >0.92 ($P<0.001$) [136]. However, no adjustments were made for eventual differences in metabolic factors.

Fan et al. [137] investigated 63 patients with non-calcified plaques, 62 patients with calcified plaques and 61 healthy controls. Cel-miR-39, cel-miR-54 and cel-miR-238 were used as housekeeping miR. The level of serum miR-21 was significantly lower and MMP-9 was significantly higher in patients with non-calcified coronary artery lesions, compared with the control and calcified plaque groups. This finding suggests that miR-21 could be a biomarker for plaque instability by suppressing target gene RECK to promote the expression and secretion of MMP-9 in macrophages, which lead to fibrous cap thinning and plaque rupture [137].

In summary, miR-1, miR-126, miR-485-3p, and miR133a were confirmed to be associated with CAD. In addition, miR-122, miR-133b, miR-145 and miR-155 were found to be dysregulated in atherosclerosis and might therefore be used as biomarkers. However, in most studies, no adjustments were made for differences in metabolic factors.

Acute coronary syndrome. D'Alessandra et al. [138] examined the plasma of 19 troponin-negative patients with unstable angina (UA), 34 patients with stable angina (SA) and 20 healthy control subjects (n=20), matched for sex, age, smoking

habit, hypertension, and diabetes, and without a history of CAD or inflammatory disorders. Two miRs (miR-16 and miR-17-5p) were used as internal controls. The levels of 10 miRs were measured: miR-337-5p, miR-433, miR-485-3p, miR-1, miR-122, miR-126, miR-133a, miR-133b, miR-199a, and miR-145. Only three of these showed a statistically significant ($P<0.05$) up-regulation, when compared to controls: miR-337-5p, -433, and -485-3p. In addition, the investigators measured the expression of literature-selected miRs (miR-17-5p, -92a, -126, -133a, -145, -155, -199a, and -208a, and STEMI-related miR-1, -122, -133a, -133b, -375, and -499-5p). The expression of six of these miRs was increased in both SA and UA patients: miR-1, miR-122, miR-126, miR-133a, miR-133b, and miR-199a. The authors concluded that particular miRs could detect, with high efficacy, stable (miR-1, miR-126, miR-485-3p) or unstable (miR-1, miR-126, miR133a) CAD when compared with matched controls. However, the ability of plasmatic miRs to discriminate between SA and UA was found to be low [138]. Nevertheless, there were significant differences in the metabolic profiles of patients and controls. Wang et al. [139] measured muscle-enriched miRs (miR-1, miR-133a, and miR-499) and cardiac-specific miR-208 in 33 patients with AMI, 33 non-AMI patients, and 30 healthy controls. All of the tested miRs were elevated in the plasma of AMI patients compared with the non-AMI groups. Notably, miR-208a was able to differentiate AMI from non-AMI patients with a high sensitivity and specificity. The authors concluded that miR-208a is elevated in a cardiac-specific fashion, suggesting that the circulating level of miR-208a is minimally affected by non-cardiac tissue injury [139].

Wang et al [140] measured miR-133a in the plasma of 13 AMI patients, 176 stable CAD patients, and 127 healthy controls. U6 was measured as an endogenous control for normalizing the data of experimental qRT-PCR. miR-133a was significantly upregulated in the plasma of AMI patients compared with controls. Moreover, miR-133a positively correlated with the severity of coronary artery stenosis in CHD patients with single left anterior descending coronary artery atherosclerosis, and was also more informative in CHD diagnosis than troponin I in non-AMI CHD patients with single stenotic LAD lesion [140].

Adachi et al. [141] measured plasma concentrations of miR-499 in 14 ACS patients, 15 patients with CHF, and 10 healthy controls. miR expression was normalized to that of the small RNA 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ACT CA-3'. miR-499 was significantly upregulated in all patients with AMI, but failed to detect possible myocardial micronecrosis caused by CHF [141].

Vogel et al. [142] performed a whole-genome miR kinetic study in 18 STEMI patients and 21 healthy controls. miR expression levels were measured at multiple time points (0, 2, 4, 12, 24 h after initial presentation) in STEMI patients. The small RNA RNU-6b served as a reference. Seventeen miR markers, of which two were upregulated and 15 were downregulated in AMI patients, showed a high consistency between the different time points. Seven miRs (miR-636, miR-7-1, miR-380, miR- 1254, miR-455-3p, miR-566, and miR-1291) were downregulated at all time points. These seven miRs were investigated in additional cohorts, and only miR-1291 did not reach statistical significance. In addition to 6 significantly downregulated miRminas, miR-1915 (downregulated) and miR-181c (upregulated) were also significantly deregulated in early AMI [142].

Wang et al. [143] recruited 51 patients with AMI and 28 healthy controls, which differed significantly when comparing their total and LDL cholesterol. The plasma levels of miR-133 and miR-328 were measured. U6 was used as an internal control. They were both found to be upregulated in AMI patients when compared to the healthy cohort. Moreover, elevated levels of both miR-133 and miR-328 correlated with cardiac trooping I levels and presented no significant differences between patients with or without arrhythmias [143]. Meder et al. [144] enrolled 20 AMI patients and 20 healthy controls. Out of 121 significantly deregulated mIRminas in AMI patients, miR-1291 and miR-636 were the most predictive ones with AUC values of up to 0.94. MiR-145 and miR-30c levels significantly correlated with Trooping T levels. The small nuclear RNA RNU6B-2 served as reference. Furthermore, the study concluded a unique mIRmina signature of 19 mIRminas (miR-142-5p, miR-498, miR-492, miR-1281, miR-497, miR-151-5p, miR-802, miR-23b, miR-455-3p, miR-1250, miR-380, miR-135b, miR-345, miR-566, miR-631, miR-1254, miR-139-5p, miR-892b, and miR-146b-3p) with AUC of 0.99, which proved to effectively differentiate AMI patients from the healthy ones [144]. Hsu et al. [145] examined 39 AMI patients and 39 age- and gender-matched normal controls. They found 25 significantly deregulated miRs (miR-193a-5p, miR-147b, miR-497-5p, miR-542-5p, miR-885-3p, miR-150-3p, miR-877-5p, miR-31-5p, miR-760, miR-17-3p, miR-486-3p, miR-124-3p, miR-20a-5p, miR-18a-5p, miR-26a-5p, miR-17-5p, miR-106a-5p, let-7d-5p, miR-191-5p, miR-26b-3p, miR-126-3p, miR-487b, miR-127-3p, miR-199a-3p, and miR-29c-5p) in AMI patients. A subsequent validation study (synthetic spiked-in miR was used for normalization) confirmed that serum miR-486-3p and miR-150-3p were upregulated while miR-126-3p, miR-26a-5p, and miR-191-5p

were significantly downregulated in the sera of AMI patients compared to healthy controls [145]. Peng et al. [146] included 76 AMI patients and 110 healthy controls. Expression of the target mIRminas in plasma was normalized to endogenous control miR-16. The study demonstrated that miR-133, miR-1291 and miR-663b were significantly upregulated in AMI patients. The three mIRminas confirmed to be possible diagnostic biomarkers for AMI [146]. Olivier et al. [147] focused on geriatric patients and recruited 92 NSTEMI patients, 81 acute CHF patients without AMI, and 99 age-matched healthy controls. All mIRminas were normalized to miR-17. Out of 5 mIRminas (miR-1, miR-21, miR-133a, miR-208a, miR-423-5p, and miR-499-5p), only miR-499-5p and miR-21 were elevated in NSTEMI patients compared to controls and could distinguish NSTEMI patients from patients with acute heart failure. MiR-499-5p showed the highest increase in NSTEMI patients and significantly correlated with the levels of cardiac Trooping T in NSTEMI patients, CHF patients and controls [147]. Li et al. [148] collected plasma samples of 67 patients with AMI and 32 age- and sex-matched healthy volunteers. Plasma levels of miR-1, miR-133a, miR-208b, and miR-499 were significantly higher in AMI patients than in healthy controls. But they were not superior to cardiac trooping T for the diagnosis of AMI [148]. Long et al. [149] analyzed plasma samples of 18 AMI patients with AMI and 30 healthy controls. MiR-30a was higher at 4 h, 8 h and 12 h after onset of AMI, and miR-195 was higher at 8 h and 12 h, while let-7b was lower in AMI patients than in controls. MIRs were normalized to U6. Interestingly, in plasma of AMI patients, miR-30a, miR-195 and let-7b all reached their peak levels at 8 h, which is similar to the peak time of cTnI [149]. Long et al. [150] investigated the plasma of 17 AMI patients and 25 healthy adult volunteers. miR-1 was increased and miR-126 was decreased in AMI patients. miRMyrna expression was normalized to U6 [150]. Devaux et al. [151] examined 510 AMI and 87 healthy controls. They found that miR-208b and miR-499 were highly increased in plasma of AMI patients, but they were nearly undetectable in healthy controls, when normalizing to 3 synthetic C. elegans miRs. Patients with STEMI had higher miR concentrations than patients with NSTEMI. Both miRs correlated with peak concentrations of creatine kinase and Troponin T [151]. Ai et al. [152] examined 93 AMI patients and 66 healthy subjects for quantification of miR-1 level in plasma, using U6 as reference. miR-1 was significantly higher in plasma of AMI patients compared with non-AMI subjects and the level dropped to normal on discharge following medication. Their study results revealed that circulating miR-1 may be a novel, independent biomarker for diagnosis of AMI

[152]. Nabiałek et al. [153] recruited 17 AMI, 4 stable CAD, 5 healthy controls and measured plasma levels miR-423-5p, miR-1 and miR-208, normalizing to miR-39. miR-423-5p was significantly increased early in AMI with subsequent normalization within 6 hours [153]. Li et al. [154] recruited 117 AMI patients, 182 angina pectoris (AP) patients, and 100 age-and gender-matched controls. The results showed that six miRs (miR-1, miR-134, miR-186, miR-208, miR-223, and miR-499) were significantly elevated in the sera of AMI patients and presented significant differences between the AMI and AP patients. Two miRs (miR-208 and miR-499) were lower in AMI than in AP patients, suggesting that they are more sensitive in diagnosing AP. The authors commented that since U6 and 5S rRNA are degraded in serum samples and there is no current consensus on housekeeping miRs for qRT-PCR analysis of serum miRs, the expression levels of miRs were directly normalized to serum volume in this study [154].

Oerlemans et al. [155] measured serum levels of circulating miR-1, miR-208a, miR-499, miR-21, and miR-146a in 332 patients with chest pain. Expressions were also normalized all RT-PCR data to U6. Levels of all miRs were increased in 106 patients with ACS. The most important three miRs (miR-1, miR-499, and miR-21) significantly increased the prognostic value when added to hs-troponin T (AUC 0.90) [155]. Eitel et al. [156] measured the level of serum miR-133 in 216 STEMI patients. miR expressions were normalized to that of cel-miR-39. miR-133a levels higher than the median value were associated with decreased myocardial salvage, larger infarcts, more pronounced reperfusion injury, and left ventricular dysfunction as assessed by cardiovascular magnetic resonance (CMR) [156]. Widera et al. [157] selected 6 cardiomyocyte-enriched miRs and measured their levels in 444 patients with acute coronary syndrome. Patients with NSTEMI or STEMI had higher levels of miR-1, miR-133a, and miR-208b than UA patients. miR-133a and miR-208b were also associated with all cause mortality at 6 months [157]. Goretti et al. [158] followed a cohort of 510 AMI patients for 6 years. miR-208b and miR-499 were significantly upregulated in AMI patients. Furthermore, significant associations between miR-208b, age and BMI were observed [158]. Corsten et al. [159] included 32 AMI and 36 normal coronary angiography patients. Plasma levels of miR-1, miR-133a, miR-208b, miR-499, miR-21, miR-29b, miR-146, miR-155, and miR-223 were subsequently assessed. miR levels were normalized to a mix of 3 spiked-in synthetic Caenorhabditis elegans miRs. miR-208b and miR-499 were highly elevated in AMI patients and showed significant association with plasma troponin T [159]. Gidlöf

et al. [160] examined the plasma of 424 patients with suspected AMI. They found that circulating miR-208b and miR-499-5p specifically reflected myocardial damage. Moreover, upregulated miR-208b and miR-499-5p strongly correlated with troponin levels. MIRs were normalized to miR-17. This established an association of increased miR-208b and miR-499-5p levels with reduced systolic function after MI and risk of death or heart failure [160]. Zampetaki et al. [161] performed a prospective study including 820 participants. miR-126 was positively whereas miR-223 and miR-197 were inversely associated with risk of incident AMI. miRs were normalized to U6 [161].

Two studies focusing on left ventricular remodeling after MI were identified. Devaux et al. [162] measured plasma levels of miR-150 in 90 patients after AMI. miR-150 was downregulated in patients with left ventricle remodeling after AMI, normalizing for spiked-in synthetic C. elegans miRs. miR-150 may also have additive predictive value for left ventricle (LV) remodeling to either N-terminal prohormone of brain natriuretic peptide (NT-proBNP) alone or a multi-parameter model, including serum markers of troponin I, creatine kinase and NT-proBNP at admission, age and sex [162]. Lv et al. [163] recruited 359 post-AMI patients and divided them into LV-remodeling (n=116) and non-LV-remodeling (n=243) based on the echocardiographic parameters of left ventricle. miR-208b and miR-34a, normalized to spiked-in SV40, were higher in plasma of patients with left ventricle remodeling. miR-208b and miR-34a were also associated with increased risk of mortality or heart failure within six months. miR levels were normalized using spiked-in SV40. The two miRs may be potential diagnostic markers of left ventricle remodeling in post-MI patients [163].

The study by D'Alessandra et al. [138] appears to give the most reliable data on diagnosis unstable angina, as the study used miR references and included controls, matched for sex, age, smoking habit, hypertension, and diabetes, and without a history of CAD or inflammatory disorders. Of 26 studies that investigated ACS and miR association, 4 studies identified miR-499, miR-1 and miR-208b as potential diagnostic markers of ACS. Furhtermore, miR-133a and miR-133b may also be potential biomarkers as they were found to be dysregulated in ACS by Wang et al. [139,140], Eitel et al. [156], Widera et al. [157], and Peng et al. [146].

Heart failure. Bauters et al. [164] measured miR-133a and miR-423-5p in plasma of 246 patients with a first anterior Q-wave MI during a 1-year follow-up. Cel-miR-39 was used as normalization control. miR-133a and miR-423-5p, nor-

malized to Cel-miR-39, were not associated with indices of left ventricle function and remodeling or with B-type natriuretic peptide. They concluded that circulating levels of miR-133a and miR-423-5p are not useful as biomarkers of left ventricular remodeling after MI [164].

Zhao et al. [165] examined serum of 22 heart failure (HF) patients, 18 healthy controls, and the umbilical venous blood of 9 fetuses from 9 independent parturitions. Synthetic Arabidopsis thaliana miR solution was used for normalization. Nine miRs (miR-210, miR-27a, miR-30a, miR-21, miR-29a, miR-19a, miR-27b, miR-24, and miR-19b), normalized to Synthetic Arabidopsis thaliana miRs, were markedly upregulated in the HF and fetus groups, compared to controls. miR-210 and miR-30a were significantly upregulated both in HF patients and in umbilical cord blood. The latter also showed high diagnostic accuracy and positive correlation with NT-proBNP levels, suggesting that they may be potential biomarkers for HF [165].

Matsumoto et al. [166] investigated 21 AMI patients who developed HF within one year after AMI and 65 matched controls without subsequent CV events (score-based matching of age, sex, diabetes, hypertension, dyslipidemia, smoking, previous MI, Killip class >II at AMI onset, infarction size, reperfusion therapy rates, and medication at the time of serum collection to adjust for potential baseline differences between the two groups). The expression level of each miR was normalized to that of U6 snRNA, miR-766 or let-7d using the calculated mean control of each sample. The p53-responsive miR-192 was significantly upregulated in HF group. miR-194 and miR-34a also positively correlated with left ventricular diastolic dimension and left ventricle ejection fraction [166].

Qiang et al. [167] investigated 106 patients with chronic HF (55 with ischemic cardiomyopathy and 51 with non-ischemic cardiomyopathy) and 30 age-matched controls. Sixteen miRs (miR-126, miR-508-5p, miR-34a, miR-210, miR-490-3p, miR-513-5p, miR-517c, miR-518e, miR-589, miR-220c, miR-200a, miR-186, miR-7i, miR-200b, miR-595, and miR-662) were differentially expressed between ICM and NICM patients, normalizing their expression to that of U6. The study concluded that decreased miR-126 and elevated miR-508-5p levels in endothelial progenitor cells were independent prognostic factors for the outcome of ICM or NICM patients with chronic heart failure [167].

A study by Fan et al. [168] examined the plasma miR in 45 dilated cardiomyopathy patients and 39 age- and sex- matched healthy controls. miR-423-5p, normalized to cel-miR-39, was significantly upregulated in patients with DCM. Furthermore,

the plasma levels of miR-423-5p positively correlated with NT-proBNP and had a high diagnostic accuracy [168].

Goren et al. [169] found elevated levels of circulating miR-423-5p, miR-320a, miR-22, miR-92b in sera from patients with stable chronic heart failure (n=30) compared to age-, gender- and ethnically matched healthy controls (n=30). Data were normalized by scaling with the mean control of the samples. Combination of the four miRs resulted in a threshold score of 1.98 with a high sensitivity and specificity for the significant detection of HF patients. A high score correlated with serum BNP levels, increased left ventricular end-diastolic dimension and increased left atrial dimension [169].

Fukushima et al. [170] investigated 33 patients with ischemic heart disease and 17 healthy controls. They found that plasma concentrations of miR-126, normalized to the small RNA, negatively correlated with brain natriuretic peptide, with age and New York Heart Association (NYHA) class, suggesting that miR-126 may be able to be a useful biomarker for heart failure [170].

Tijsen et al. [171] selected 12 patients with heart failure and 12 healthy controls. They identified six miRs (miR-18b, miR-129-5p, miR-1254, miR-675, HS_202.1, and miR-622) that are elevated in patients with heart failure, normalizing for miR-1249, a miR that was found to be unchanged in the arrays were used for normalization. MiR-423-5p was most strongly related to the clinical diagnosis of HF as well as related to NT-proBNP levels and NYHA class [171].

Vogel et al. [172] examined whole blood of 53 patients with non-ischemic HF with reduced ejection fraction and 39 healthy subjects. The study found over 20 miRs that were deregulated in HF patients compared to controls. However, there was no stable house-keeping miR to normalize miR expressions. The AUC of the combination of 8 miRs (miR-558, miR-122, miR-520d-5p, miR-200b, miR-622, miR-519e, miR-1231, and miR-1228) was 0.81, representing a significant improvement in diagnosing HF compared to each single miR marker (miR-520-d-5p AC=0.71, miR-558 AUC=0.7, miR-122 AUC=0.7). In addition, miR-622, miR-520d-5p, miR-519e, miR-200b, miR-122, and miR-558 also showed positive correlation with left ventricular ejection fraction [172].

Endo et al. [173] selected 39 patients with HF. miR-210 expression levels in mononuclear cells were significantly higher in patients with NYHA III and IV HF than those with NYHA II or healthy controls. No significant correlation between plasma BNP and miR-210 levels in patients with NYHA II heart failure was observed [173].

Ellis et al. [174] collected plasma samples of 135 dyspnea patients and 15 healthy volunteers. They identified 17 significantly dysregulated miRs, normalizing for four reference miRs (miR-20a, miR-106b, miR-363, and miR-140-3p). Seven miRs (miR-103, miR-142-3p, miR-199a-3p, miR-23a, miR-27b, miR-324-5p, and miR-342-3p) were associated with HF in multivariate regression and receiver operating characteristic (ROC) analyses. Another four miRs (miR-103, miR-142-3p, miR-30b, and miR-342-3p) were all significantly downregulated in HF patients compared with non-HF dyspnea and healthy control groups [174].

Zhang et al. [175] examined miR-1 levels in the plasma of 49 patients with AMI. They first compared the relationship between miR-1 level and ejection fraction and found that miR-1 levels negatively correlated with EF. The author states that circulating miR-1 may be a novel biomarker for predicting the onset of heart failure in AMI patients with high level of miR-1 [175].

Several miRs were found to be abundantly expressed in HF patients. Among them, miR-423-5p was one of the most studied ones (showed significant dysregulation in heart failure in studies by Fan et al. [168], Goren et al. [169], and Tijsen et al. [171]). Matsumoto et al. [166] and Fan et al. [168] examined adjusted control groups and used miR reference, which makes their results more reliable. They found that miR-192, miR-194, miR-34a, as well as miR-423-5p were significantly dysregulated in heart failure. Qiang et al. [167] investigated the largest number of patients and identified miR-126 and miR-508-5p dysregulation in HF.

Stroke. Long et al. [176] recruited 197 patients with ischemic stroke and collected their blood samples at 24 h, 1 week, 4 weeks, 24 weeks and 48 weeks after symptoms onset. The results were compared with 50 healthy volunteers. The researchers found that circulating miR-30a and miR-126, normalized to snU6, were downregulated in ischemic stroke patients at 24 h, 1 w, 4 w and 24 weeks. Let-7b was lower than in controls in large-vessel atherosclerosis, while in other subtypes of ischemic stroke (small artery stroke, cardio-embolic stroke and stroke due to undetermined causes) let-7b was upregulated [176]. Gan et al. [130] found miR-145 to be significantly upregulated in ischemic stroke patients (n=32), compared 14 healthy control subjects, normalizing miR expressions to Ribosomal 18s rRNA [130]. Tan et al. [131] investigated a cohort of 19 young ischemic stroke patients aged between 18–49 years and compared their miR profiling with 5 healthy controls. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter. The study found that 8 miRs (hsa-let-7f, miR-126, miR-1259, miR-

142-3p, miR-15b, miR-186, miR-519e, and miR-768-5p) were downregulated across the three subtypes of stroke (large artery stroke, small artery stroke, and cardioembolic stroke); in contrast, 17 miRs (hsa-let-7e, miR-1184, miR-1246, miR-1261, miR-1275, miR-1285, miR-1290, miR-181a, miR-25, miR-513a-5p, miR-550, miR-602, miR-665, miR-891a, miR-933, miR-939, miR-923) were upregulated in the subtypes. 79 miRs were also able to distinguish SA and LA subtypes of ischemic stroke [131]. Tsai et al. [177] enrolled 167 subjects with ischemic stroke, 66 atherosclerosis subjects with any carotid plaque score and 157 healthy controls. miR-21 was found to be significantly higher and miR-221 significantly lower in serum of ischemic stroke and atherosclerosis patients than in healthy controls, normalizing to miR-16 [177]. Zeng et al. [178] measured miR-210 at 3, 7 and 14 days after stroke in 60 patients. miR-210, normalized to sn U6, was significantly decreased in stroke patients compared to 112 healthy controls, especially at 7 days and 14 days of stroke onset [178].

The most reliable of the miRs in diagnosing acute cerebral ischemia were considered to be miR-126, let-7b, miR-145, miR-21, and miR-210. Interestingly, miR-126 and miR-145 were also found to be significantly associated with coronary atherosclerosis by Fichtlscherer et al. [120] and D'Alessandra et al. [138] miR-21 was also found to be downregulated in unstable coronary plaques in a study by Fan et al. [137].

Metabolic diseases

Obesity. Ortega et al. [179] compared miR profiles in plasma of 49 non-obese patients, 19 obese patients and 12 morbidly obese patients. Three miRs (miR-15a, miR-520c-3p and miR-423-5p, normalizing to miR-106a, miR-146a, miR-19b, miR-223, miR-186, and miR-199a-3p) were significantly downregulated in morbidly obese patients. The discriminant function of these three miRs was specific for morbid obesity with an accuracy of 93.5% [179]. Wang et al. [180] found elevated levels of circulating miR-130b in sera from obese patients (n=23) compared to healthy controls (n=21), normalizing to miR-223. Moreover, the circulating level of miR-130b positively correlated with body mass index and could be a diagnostic biomarker reflecting the degree of obesity [180]. Wang R et al. [181] observed 34 circulating miRs that were expressed differently in obese patients compared with controls. In particular, miR-122 was significantly upregulated in obese patients (n=123) compared with controls (n=107) and was found to be an independent risk factor of insulin resistance after controlling for confounding factors [181]. Pescador et al.

[182] selected thirteen patients with T2DM, 20 obese patients, 16 obese patients with T2DM, and 20 healthy controls. miR-138 and miR-376a, normalized to miR-30c, miR-103, miR-191 and miR-423-3p, were significantly downregulated in serum of obese subjects. In contrast, serum miR-15b level was significantly higher when compared to controls, diabetic and obese diabetic patients [182]. Heneghan et al. [183] examined whole blood of 30 obese and 20 non-obese patients. The controls consisted of age-matched healthy volunteers with BMI less than 25kg/m^2 as well as the non-obese surgical patients who donated a whole-blood sample in addition to omental and subcutaneous fat samples at the time of elective abdominal surgery. miR-16 was used as an endogenous control, given its stable expression in all samples in the profiling experiment. Two miRs (miR-17–5p and miR-132) were found to be significantly downregulated in blood of obese patients compared to non-obese subjects [183].

Among four studies focusing on obesity, Haneghan et al. [183] were the only researchers who used adjustment of controls and miR reference, which makes these results the most reliable. The study identified miR-17-5p and miR-132 as being significantly downregulated in obesity. The remainder of the reviewed studies found miR-15a, miR-15b, miR-520c-3p, miR-423-5p, miR-130b, miR-138, and miR-376a to be significantly deregulated in obese patients compared with controls.

T2DM. T2DM is another important risk factor for CVD. Although the literature search identified a wide spectrum of studies investigating miR pattern changes in T2DM, only five significant experimental studies were selected. Karolina et al. [126] examined the whole blood of 6 patients with IFG, 8 patients with T2DM and 7 healthy controls. Eight important miRs (upregulated miR-144, miR-150, miR-182, miR-192, miR-29a, miR-320, and downregulated miR-146a, miR-30d in diabetes patients) were found to be related to different stages of diabetes progression [126]. miR expressions were normalized to U6 snRNA. Another study by Zampetaki et al. [127] included 80 patients with T2DM and 80 healthy controls. The study found reduced miR-15a, miR-29b, miR-126, miR-223, and elevated miR-28-3p levels in plasma of diabetic patients compared to healthy subjects, normalizing to miR-454 and RNU6b. These five miRs are necessary and sufficient for a non-redundant classification of DM and might antedate the manifestation of disease [127]. The third study by Kong et al. [128] recruited 18 newly diagnosed T2DM patients, 19 pre-diabetes patients and 19 T2DM-susceptible individuals with normal glucose tolerance. Seven miRs (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a and miR-375, normal-

ized to RUN6B) showed significant up-regulation in sera from newly diagnosed T2DM group compared with the T2DM susceptible individuals with normal glucose tolerance group. However, T2DM-susceptible individuals with normal glucose tolerance and pre-diabetes patients showed no significant difference in levels of these 7 miRs, suggesting that expression patterns of diabetes-related miRs had not changed dramatically in pre-diabetic stage [128]. Párrizas et al. [184] screened sera from 17 healthy patients, 10 IFG, 9 IGT, and 10 T2D patients. Three miR (miR-150, miR-192, and miR-193b), normalizing for let-7b, let-7g and let-7i, were significantly increased in IFG and IGT groups, but remained unchanged in T2D subjects. This suggests, that circulating miR-150, miR-192 and miR-193b may act as a common signature for pre-diabetes [184]. Finally, Liu et al. [185] examined 82 IGT patients, 75 IFG patients, 160 n-T2D patients, and 138 healthy controls. Serum miR-126 concentration was significantly lower in IGT/IFG and T2DM patients than in healthy controls, normalizing to cel-miR-39. Moreover, significantly lower serum miR-126 concentration was detected in T2DM patients than in the IGT/IFG subjects. Further ROC analysis showed that serum miR-126 may be able to distinguish IFG/IGT and T2DM patients from healthy individuals [185].

In conclusion, three miRs (miR-29a, miR-30d, and miR-146a) were found to be significantly deregulated in T2DM. The studies of Liu et al. [185] and Zampetaki et al. [127] highlighted miR-126 as a potential biomarker of T2DM, which was already regulated years before manifestation of the disease and therefore could be useful for risk prediction.

Hypertension. Li et al. [125] studied the miR profile changes in essential hypertension. They examined the plasma of 13 hypertensive patients and 5 healthy controls. The relative expression of 20 miRs was normalized to expression of the internal control (small nuclear U6). Three miRs were significantly deregulated in hypertensive patients compared to healthy controls – human cytomegalovirus (HCMV)-encoded miR, hcmv-miR-UL112, and let-7e were upregulated, whereas miR-296-5p was downregulated. The authors state they are first to report a link between HCMV replication and essential hypertension [125].

Hyperlipidemia. Gao et al. [124] compared hyperlipidemia patients (n=255) with controls (n=100), using synthetic C. elegans for normalization. Plasma levels of miR-122 and miR-370 were significantly increased in hyperlipidemia patients. Therefore, it was concluded that miR-122 and miR-370 may be associated with hyperlipidemia and severity of CAD [124].

MetS. Karolina et al. [132] examined whole blood of 46 healthy controls, 50 MetS, 50 T2DM, 89 hypercholesterolemia, and 30 hypertension patients. Three clusters of miRs could characterize different metabolic disorders that potentially contribute to the pathogenesis of MetS. Expression of miR-197, miR-23a, and miR-509-5p were identified as potential contributors of dyslipidemia in MetS and miR-130a and miR-195 as contributors of hypertension. A plausible association of miR-27a and miR-320a with MetS and T2DM patients has also been found because these miRs remained dysregulated in both cases [132]. Raitoharju et al. [186] examined 72 patients from the Young Finns Study. The expression data was processed using non-parametric background correction, followed by quintile normalization with control and expression probes. As a result they found nine miRs that correlated with MetS components. miR-144-5p correlated with glucose levels, hsa-1207-5p with glycosylated hemoglobin and hsa-miR-484 with metabolites related to insulin resistance. Hsa-miR-625-3p correlated with cholesterol levels, hsa-miR-1237-3p and hsa-miR-331-3p expression with certain fatty acids levels and hsa-miR-129-1-3p, -129-2-3p, and -1288-3p with glycerol levels [186].

Both studies investigated only correlations between miRs and specific components of MetS. Interestingly, miR-197, associated with dyslipidemia in this group, in coronary heart disease group was also significantly dysregulated. miR-130a and miR-195 in MetS group associated with hypertension, was also significantly deregulated in atherosclerotic patients in other previously described studies, miR-195, also highlighted in obesity, smoking and acute coronary syndrome groups. miR-27a, associated between MetS and DM Type 2, has been highlighted in smoking, atherosclerosis and heart failure patient groups. It is difficult to highlight particular miRs that could potentially be used for diagnosis of MetS, because of its association with so many different factors.

Literature review conclusion

Published studies have reported the promising role of miRs as early diagnostic biomarkers in CVD. Indeed, the results of this systematic revi ew demonstrate that many miRs are significantly up- or downregulated in numerous metabolic and CV diseases, including obesity, MetS, T2DM, hypertension, dyslipidemia, CAD, PAD, HF, ACS, and stroke. With appropriate biomarkers, the latter diseases could be diagnosed before the clinical symptoms arise and measures to prevent disease progression could be initiated.

Since this work was completed, another review has corroborated some of these findings, supporting the role of miRs as biomarkers of metabolic and CV diseases [187] miR-122 has received particular focus in studies to date, due to its abundance in the liver and its tissue specificity. This miR has been suggested to play a central role in the maintenance of lipid and glucose homeostasis and may represent a novel biomarker for CV and metabolic diseases [188]. Features of miRs that predispose them to be potentially useful biomarkers include their tissue- and cell-type specific expression, the essential roles they play in many biological and pathological processes and their disease-specific release patterns into peripheral blood [189,190].

The current systematic literature review has some limitations. Due to the novelty of this topic in cardiology, most of the selected studies enrolled small numbers of subjects and included no follow up of the patients. Another limitation was the inability to conduct any meta-analysis on the datasets due to their heterogeneity. There is also inherent bias in the selection of the miRs examined in the original studies because only a few of the known miRs have been studied to date in this emerging field. It should also be noted that the clinical applicability of the miRs as early diagnostic biomarkers is, as yet, unclear. Most of the studies selected in the review state the potential prognostic function of the miRs based on the miRs detected in the disease state (e.g. coronary artery disease, hypertension or T2DM). Therefore, it is not possible to confirm whether particular miRs are significantly deregulated prior to the onset of the disease. However, the source studies do show strong connections between deregulated miR expression and risk factors of CVD. Thus, miRs could potentially lead to earlier identification of at-risk patients and better control of their metabolic and CV risk profile. This aspect does warrant further research in order to determine whether miRs posited as biomarkers for CVD have already been deregulated prior to disease onset. In order to study this effectively, large-scale, high-quality studies with longer patient follow-up periods are required. Biobanks may provide a useful resource of blood samples representative of both healthy and diseased populations.

In conclusion, miRs measured in plasma or bodily fluids may represent potentially useful disease biomarkers and predictors of metabolic disease and associated CV complications. An urgent need exists for novel biomarkers that can distinguish patients with obesity or MetS who are most likely to develop future metabolic and CV complications. Such markers could greatly assist in reducing the burden of diabetes to healthcare systems and society. Further elucidation of miR-mediated pathways in cardiometabolic disease shows promise to provide such biomarkers and this review provides guidance on potentially valuable areas of research.

Aim

To identify miR that may be used as a biomarker for the early atherosclerosis diagnosis.

Methodology

Based on the literature review, described above, the following miRs were selected for further investigations: miR-1, miR-122, miR-126, miR-132, miR-133a, miR-133b, miR-370, miR-145, miR-155, miR-195.

Patients included in this part of the study were recruited between 2007 and 2011 from the Lithuanian High Cardiovascular Risk (LitHiR) primary prevention program. This long-term program has focused on employable-aged women (aged 50–65) and men (aged 40–55) without overt CVD. CVD was defined as stable AP, CAD, AMI, coronary artery bypass grafting, percutaneous coronary intervention, transient ischemic attack or stroke, and PAD. As part of the program, a two-level approach involving primary healthcare institutions (PHCI) and specialized CV prevention units (CVPU) was applied. Participants of the first level of the program were recruited in three ways. The first group consisted of people registered in PHCI and invited by general practitioners to participate in the program. The second group consisted of people who visited PHCIs for reasons other than CV problems. The third group included people who found out about the program via local mass media. Only participants matching the program criteria were recruited. After CV risk evaluation at the PHCI level, subjects with high CV risk were directed to the CVPUs (secondary level) for further examination and treatment. High CV risk was defined as having one or more of the following conditions: 1) SCORE risk score of ≥11; 2) diabetes; 3) MetS; 4) positive family history of CVD; and/or 4) severe dyslipidemia.

Selecting patients with MetS

From 2006 to 2010, 266,391 patients were examined at the PHCIs. Of these patients, the current study included 2891 [1072 (37%) men and 1819 (63%) women] patients who were diagnosed with MetS and referred to the CVPU at the Vilnius University Hospital Santariškių Klinikos for additional assessment, risk stratification, and creating an individual prevention plan.

MetS was diagnosed if three or more criteria of the revised NCEP ATPIII were met:

- Waist circumference ≥102 cm in men, ≥88 cm in women
- TG ≥1.7 mmol/L
- HDL-C <1.03 mmol/L in men, <1.29 mmol/L in women
- BP ≥130/85 mmHg
- FPG ≥5.6 mmol/L

Sample collection and miR detection

Venous blood samples (three ethylenediaminetetraacetic acid tubes, two tubes containing clot activator and gel for serum separation, 1 tube containing sodium citrate and liquid density medium) were collected after patients completed a 12-hour fast. All blood samples were transported with 30 minutes, under room temperature.

If the sample does not meet the requirements, the sample is not registered and investigated. All samples are noticed in a register paper. Each sample and each sample position in the freezer was identified by a code. Registered and coded samples were delivered to the sample preparation room.

Cell sample preparation

EDTA blood was diluted in EL buffer with ratio 1:5, incubated on ice for 10-15 minutes and centrifuged (400 × g for 10 min, 4°C). After withdrawing supernatant pellet was resuspended in EL buffer (1:2) and centrifuged (400 × g for 10 min, 4°C). After withdrawing supernatant pellet was stored under -80°C.

Serum sample preparation

Blood with clot activators war placed in vertical position for 30 minutes, centrifuged (1100–1300 × g, 10–15 min). Supernatant containing serum was stored under -80°C. Of 2891 initially screened patients, after a 3-year follow-up period, randomly selected 182 returning patients were prospectively selected for this study.

Statistical analyses

Correlation coefficient. Pearson correlation coefficient was used to measure the linear dependence between micro RNA and medical tests. Correlation coefficient between two variables $x = \{x_1, x_2, \ldots, x_n\}$ and $y = \{y_1, y_2, \ldots, y_n\}$ was calculated using formula:

$$r_{xy} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})\sum_{i=1}^{n}(y_i - \bar{y})}{n\sigma_x\sigma_y}, \tag{1}$$

where $\bar{x}, \bar{y}$ are averages of x and y, $\sigma_x\sigma_y$ are SDs of x and y and n denotes the sample size. Coefficient values are between -1 and 1. The closer the absolute value of r_{xy} gets to 1, the stronger linear relationship between the variables is. The null hypothesis is tested to determine whether this relationship is significant:

$$\begin{aligned} &H_0: r_{xy} = 0, \\ &H_1: r_{xy} \neq 0. \end{aligned} \tag{2}$$

It was done by comparing the P-value to the selected significance level (in this case $\alpha = 0{,}05$).

To examine whether the linear relationship exists between continuous and categorical variables, a simple linear regression and test on the slope of the regression line were performed.

Linear regression. Linear regression assumes that explanatory variables and a response variable are systematically linked by a linear relationship:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n + \varepsilon, \tag{3}$$

where $x_1, \ldots, x_n$ are the input variables, y is the response (predicted) variable, $\beta_0, \ldots, \beta_n$ are model parameters, and ε is a random error. Usually least squares method [191] is used for estimating the unknown model parameters $\beta_0, \ldots, \beta_n$ given a set of observations $x_1, \ldots, x_n, y$. The t tests [191] are used to conduct hypothesis tests on the regression coefficients obtained in simple linear regression. The statements for the hypothesis test are expressed as:

$$\begin{aligned} &H_0: \beta_i = 0, \\ &H_1: \beta_i \neq 0. \end{aligned} \tag{4}$$

The null hypothesis is rejected when the P-value is less than the level of significance ($\alpha = 0{,}05$) and when no linear relationship exists between x_i and y. In order to choose the most appropriate regression model, a stepwise selection procedure based on test criteria was used.

Binary logistic regression. Binary logistic regression [192] is a special type of regression where the dependent variable is a categorical variable having two categories. This model is used to estimate the probability of a binary response based on one or more predictor (or independent) variables. Formally, the model can be written as

$$P(y = 1|x_1, \cdots, x_n) = \frac{e^{\beta_0+\beta_1 x_1+\beta_2 x_2+\cdots+\beta_n x_n}}{1 + e^{\beta_0+\beta_1 x_1+\beta_2 x_2+\cdots+\beta_n x_n}}, \tag{5}$$

where y is a binary response variable, x_1, ..., x_n a set of explanatory variable, β_0, ..., β_n are model parameters and $P(y = 1|x_1, ..., x_n)$ is interpreted as the probability of an event $y = 1$. The regression coefficients are estimated using maximum likelihood estimation [192]. The Wald statistic [192], analogous to the t-test in linear regression, is used to assess the significance of coefficients. Model selection can be performed using stepwise selection procedure.

Evaluation of performance. Two different measures were used to evaluate the performance of the linear regression models:

1. Mean absolute error (MAE) [193], i.e. the average difference between predicted value $\hat{y}_i$ and real observation y_i:

$$\text{MAE} = \frac{1}{n}\sum_{i=1}^{n} |\hat{y}_i - y_i|. \tag{6}$$

   This measure was selected due to its simplicity – easy to understand and calculate.

2. Mean absolute percentage error (MAPE) [193], i.e. the average value of the unsigned percentage error:

$$\text{MAE} = \frac{1}{n}\sum_{i=1}^{n} \frac{100|\hat{y}_i - y_i|}{y_i}. \tag{7}$$

MAPE was selected because this measure provides the answer in percentage terms, making it easy to interpret, especially, when the item's demand volume is unknown.

Binary logistic regression model is evaluated using a confusion matrix, i.e. a table with two rows and two columns that reports the frequency of each possible case:

	$\hat{y}_i = 1$	$\hat{y}_i = 0$
$y_i = 1$	**TP** (true positive)	**FN** (false negative)
$y_i = 0$	**FP** (false positive)	**TN** (true negative)

These values can be used to calculate statistical measures of the performance of a binary classification [191]:

$$precision = \frac{TP}{TP + FP} \tag{8}$$

$$recall = \frac{TP}{TP + FN} \tag{9}$$

$$F = 2\frac{precision \cdot recall}{precision + recall} \tag{10}$$

Results

Correlation analysis

A Pearson correlation coefficient (if both variables are continuous) and a linear regression -test (in case one variable is continuous and other categorical variable) were used to determine whether a significant linear relationship exists between miR and selected arterial markers. Analysis results showed that there is some weak linear relationship (7). Cardio-ankle vascular index (CAVI) was associated with miR-1, miR-122, miR-133a, miR-133b results, and augmentation index (AIx) normalized to a heart rate of 75 beats per minute (AIx@75) was associated with miR-1, miR-145, miR-133a, miR-133b. AoPWV had a linear relationship with single miR-122. Carotid artery stiffness, measured on the right and left carotid arteries, was only associated with miR-1, and only limited to the left side measurements.

Results of a linear regression -test for categorical values are provided in Klaida! Nerastas nuorodos šaltinis.8, highlighting significant correlation between miR-370 and plaques in the right carotid artery as well as miR-132 and plaques in the left carotid artery. On both occasions, the relationship existed only on one side of the carotid artery, but not the other.

There was statistical significance miR and AoPWV, as categorical value (cut off 9.6 m/s); however, logistic regression models for AoPWV suggested that the cut-off value for the selected intermediate CV risk patient group of 9.6 m/s was inaccurate, and might need to be reconsidered for future research (**Table 9**).

Linear regression models demonstrated an association between the investigated arterial markers and miRs with promising forecasting results (**Table 10**).

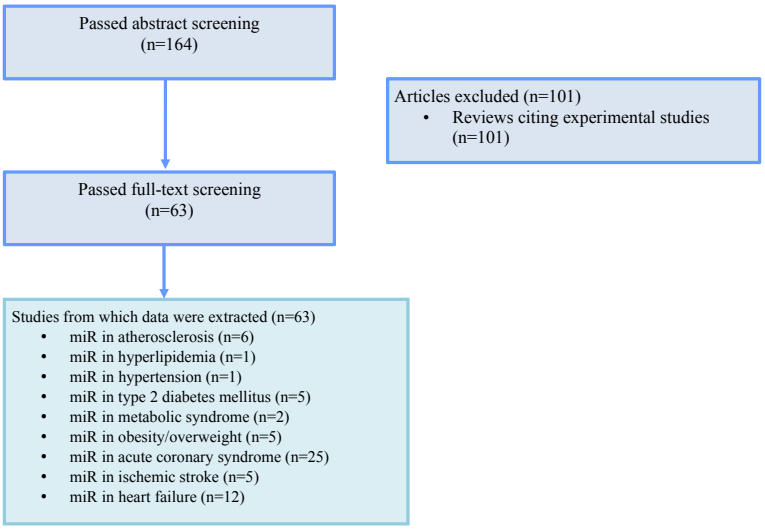

Figure 7. Summary of literature search and selection.

Table 6. MicroRNA (miR) groups and key features and findings of included studies.

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
Atherosclerosis	miR-1	(+)	Plasma	Array	67 stable CAD, 31 healthy	Diagnostic or disease monitoring	Fichtlscherer et al., 2010 [120]
	miR-133a (P>0.05)	(+)					
	miR-133b	(+)					
	miR-208a (P>0.05)	(+)					
	miR-208b	(+)					
	miR-499-3p	(+)					
	miR-499-5p	(+)					
	miR-126	(-)					
	miR-17	(-)					
	miR-20a	(-)					
	miR-92a	(-)					
	miR-221	(-)					
	miR-199a-5p	(-)					
	miR-27a	(-)					
	miR-130a	(-)					
	let-7d	(-)					
	miR-21	(-)					
	miR-155	(-)					
	miR-143	(-)					
	miR-145	(-)					
	miR - 21	(+)	Serum (stored at -80°C)	Array	104 ASO/ PAD, 105 healthy	Risk or diagnostic markers for ASO	Li et al., 2010 [121]
	miR-130a	(+)					
	miR-27b	(+)					
	let-7f	(+)					
	miR-210	(+)					
	miR-221	(-)					
	miR-222	(-)					
	miR-150	(-)	Whole blood	Array	10 CAD, 15 healthy	Diagnostic or disease monitoring	Weber et al., 2011 [122]
	miR-584	(-)					
	miR-21	(-)					
	miR-24	(-)					
	miR-126	(-)					
	miR-92a	(-)					
	miR-34a	(-)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-19a	(-)					
	miR-145	(-)					
	miR-155	(-)					
	miR-222	(-)					
	miR-378	(-)					
	miR-29a	(-)					
	miR-30e-5p	(-)					
	miR-342	(-)					
	miR-181d	(-)					
	let 7e	(-)	Whole blood	qRT-PCR	15 PAD, 16 healthy	Potential biomarkers for PAD	Stather et al., 2013 [136]
	miR-15b	(-)					
	miR-16	(-)					
	miR-20b	(-)					
	miR-25	(-)					
	miR-26b	(-)					
	miR-27b	(-)					
	miR-28-5p	(-)					
	miR-126	(-)					
	miR-195	(-)					
	miR-335	(-)					
	miR-363	(-)					
	miR-720	(+)					
	miR-1274A	(+)					
	miR-337-5p	(+)	Plasma (stored at -80°C)	qRT-PCR	19 UA, 34 SA, 20 healthy	Potential biomarkers for CAD	D'Alessandra et al., 2013 [138]
	miR-433	(+)					
	miR-485-3p	(+)					
	miR-1	(+)					
	miR-122	(+)					
	miR-126	(+)					
	miR-133a	(+)					
	miR-133b	(+)					
	miR-199a	(+)					
	miR-145	(+)					
	miR-17-5p	(+)					
	miR-92a	(+)					
	miR-155	(+)					
	miR-208a	(+)					
	miR-375	(+)					
	miR-499-5p	(+)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-21	(-) non-calcified plaque	Serum	Array	63 non-calcified plaque, 62 calcified plaque, 61 controls	Diagnostic marker for coronary atherosclerotic plaque instability	Fan et al., 2014 [137]
Hyperlipidemia	miR-122	(+)	Plasma (stored at -80°C)	qRT-PCR	255 hyperlipidemia, 100 healthy	Diagnostic or disease monitoring	Gao et al., 2012 [124]
	miR-370	(+)					
	miR-33a	undetectable					
	miR-33b	undetectable					
Hypertension	miR-605	(+)	Plasma	Array	13 hypertensive, 5 healthy	Disease monitoring	Li et al., 2011 [125]
	miR-1252	(+)					
	miR-600	(+)					
	miR-516b	(+)					
	miR-623	(+)					
	miR-602	(+)					
	kshv-miR-K12-6-3p	(+)					
	HCMV-miR-UL112	(+)					
	let-7e	(+)					
	miR-625	(-)					
	miR-486--5p	(-)					
	ebv-miR-BART17-3p	(-)					
	kshv-miR-K12-10b	(-)					
	miR-664	(-)					
	ebv-miR-BART19-5p	(-)					
	miR-615-5p	(-)					
	miR-18b	(-)					
	miR-1249	(-)					
	miR-30d	(-)					
	miR-324-3p	(-)					
	kshv-miR-K12-10a	(-)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-296-5p	(-)					
	miR-518b	(-)					
	miR-1236	(-)					
	miR-133b	(-)					
	miR-634	(-)					
	miR-1227	(-)					
T2DM	miR-144	(+)	Whole blood	qRT-PCR	6 IFG, 8 T2D, 7 healthy	Disease monitoring	Karolina DS et al., 2011 [126]
	miR-146a	(-)					
	miR-150	(+)					
	miR-182	(+)					
	miR-192	(+)					
	miR-29a	(+)					
	miR-30d	(-)					
	miR-320a	(+)					
	miR-24	(-)	Plasma	Array	80 DM, 80 healthy	Disease monitoring	Zampetaki et al., 2010 [127]
	miR-21	(-)					
	miR-20b	(-)					
	miR-15a	(-)					
	miR-126	(-)					
	miR-191	(-)					
	miR-197	(-)					
	miR-223	(-)					
	miR-320	(-)					
	miR-28-3p	(+)					
	miR-486	(-)					
	miR-150	(-)					
	miR-29b	(-)					
	miR-9	(+)	Serum (stored at -80°C)	qRT-PCR	18 n-T2D, 19 pre-T2D, 19 s-NGT	Disease monitoring	Kong et al., 2011 [128]
	miR-29a	(+)					
	miR-30d	(+)					
	miR-34a	(+)					
	miR-124a	(+)					
	miR-146a	(+)					
	miR-375	(+)					
	miR-191	(+)	Serum	qRT-PCR	17 healthy, 10 IFG, 9 IGT, 10 T2D	New biomarkers for pre-diabetes	Párrizas et al., 2014 [184]
	miR-193b	(+)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-126	(-)	Serum	qRT-PCR	82 IGT, 75 IFG, 160 n-T2DM, 138 healthy	Potential biomarker for pre-diabetes and DM	Liu et al., 2014 [185]
MetS	miR-197		Whole blood	qRT-PCR	46 healthy, 50 MetS, 50 T2DM, 89 hypercholesterolemia, 30 hypertension	Correlate with dyslipidemia in MetS	Karolina DS et al., 2012 [132]
	miR-23a						
	miR-509-5p						
	miR-130a					Correlate with hypertension	
	miR-195						
	miR-27a					Correlate with MetS and T2DM	
	miR-320a						
	miR-103					No correlation	
	miR-17						
	miR-183						
	miR-584						
	miR-652						
	miR-92a						
	miR-150						
	miR-192						
	hsa-miR-129-1-3p		Whole blood (stored at -80°C)	qRT-PCR	72 patients from Young Finns Study	Correlates with glycerol levels	Raitoharju et al., 2014 [186]
	miR-129-2-3p						
	miR-1288-3p						
	miR-144-5p					Correlates with glucose levels	
	miR-484					Correlates with metabolites related to insulin resistance	

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-625-3p					Correlates with cholesterol levels	
	miR-1207-5p					Correlates with HbA_{1c}	
	miR-1237-3p					Correlates with certain fatty acids levels	
	miR-331-3p						
Obesity	miR-142-3p	(+)	Plasma	qRT-PCR	49 non-obese, 19 obese, 12 morbidly obese	Potential biomarkers for risk estimation and classification of morbidly obese patients	Ortega et al., 2013 [179]
	miR-140-5p	(+)					
	miR-221	(-)					
	miR-15a	(-)					
	miR-520c-3p	(-)					
	miR-130b	(-)					
	miR-532-5p	(-)					
	miR-125b	(-)					
	miR-423-5p	(-)					
	miR-130b	(+)	Serum	qRT-PCR	23 overweight/obese, 21 healthy	Potential biomarker for overweight	Wang et al., 2013 [180]
	miR-138	(-)	Serum (stored at -80°C)	qRT-PCR	13 T2D, 20 obese, 16 obese with T2D, 20 healthy	Potential biomarkers for obesity prediction	Pescador et al., 2013 [182]
	miR15b	(+)					
	miR-376a	(-)					
	miR-122	(~)/(+)	Whole blood	qRT-PCR	30 obese, 20 non-obese	Potential biomarkers for obesity	Heneghan et al., 2011 [183]

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-17-5p	(-)					
	miR-132	(-)					
	miR-143	(+)					
	miR-145	(-)					
	miR-34a	(-)					
	miR-99a	(-)					
	miR-195	(-)					
	miR-122	(+)	Serum (stored at -80°C)	qRT-PCR	56 obese, 56 healthy	Potential biomarker of obesity and insulin resistance	Wang et al., 2015 [181]
ACS	miR-499	(+)	Plasma (stored at -80°C)	qRT-PCR	33 AMI, 33 non-AMI, 30 healthy	Potential biomarker for early detection of myocardial injury	Wang et al., 2010 [139]
	miR-208a	(+)					
	miR-1	(+)					
	miR-133a	(+)					
	miR-133a	(+)	Plasma (stored at -80°C)	qRT-PCR	13 AMI patients, 176 CHD and 127 healthy	New potential biomarker for AMI	Wang et al., 2013 [140]
	miR-1	(+)	Serum (stored at -80°C)	qRT-PCR	332 patients with chest pain	Potential biomarkers for ACS	Oerlemans et al., 2012 [155]
	miR-208a	(+)					
	miR-499	(+)					
	miR-21	(+)					
	miR-133a	(+)	Serum (stored at -80°C)	qRT-PCR	216 STEMI	Potential prognostic biomarker in STEMI patients	Eitel et al., 2012 [156]
	miR-1	(+)	Serum	qRT-PCR	117 UAP, 131 NSTEMI, 196 STEMI	Diagnostic and prognostic markers in ACS	Widera et al., 2011 [157]
	miR-133a	(+)					
	miR-133b	(+)					
	miR-208a	(+)					
	miR-208b	(+)					
	miR-499	(+)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-499	(+)	Plasma	qRT-PCR	14 ACS, 15 CHF, 10 healthy	Biomarker of MI	Adachi et al., 2010 [141]
	miR-150	(-)	Plasma	qRT-PCR	90 AMI	Biomarker of LV remodeling post-AMI	Devaux et al., 2013 [162]
	miR-1254	(-)	Whole blood	qRT-PCR	18 STEMI, 21 healthy	Potential diagnostic biomarkers for AMI	Vogel et al., 2013 [142]
	miR-380	(-)					
	miR-455-3p	(-)					
	miR-566	(-)					
	miR-636	(-)					
	miR-7-1	(-)					
	miR-1291	(-)					
	miR-1915	(-)					
	miR-181c	(+)					
	miR-133	(+)	Plasma (stored at -80°C)	qRT-PCR	51 AMI, 28 healthy	Potential diagnostic biomarkers for AMI	Wang R et al., 2011 [143]
	miR-328	(+)					
	miR-1291	(-)	Whole blood	qRT-PCR	20 STEMI, 20 healthy	Potential diagnostic biomarkers for AMI	Meder et al., 2010 [144]
	miR-663b	(-)					
	miR-193a-5p	(+)	Serum (stored at -80°C)	qRT-PCR	39 STEMI, 39 healthy	Potential diagnostic biomarkers for STEMI	Hsu et al., 2014 [145]
	miR-147b	(+)					
	miR-497-5p	(+)					
	miR-542-5p	(+)					
	miR-885-3p	(+)					
	miR-150-3p	(+)					
	miR-877-5p	(+)					
	miR-31-5p	(+)					
	miR-760	(+)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-17-3p	(+)					
	miR-486-3p	(+)					
	miR-124-3p	(+)					
	miR-20a-5p	(-)					
	miR-18a-5p	(-)					
	miR-26a-5p	(-)					
	miR-17-5p	(-)					
	miR-106a-5p	(-)					
	let-7d-5p	(-)					
	miR-191-5p	(-)					
	miR-26b-3p	(-)					
	miR-126-3p	(-)					
	miR-487b	(-)					
	miR-127-3p	(-)					
	miR-199a-3p	(-)					
	miR-29c-5p	(-)					
	miR-133	(+)	Plasma	qRT-PCR	76 AMI, 110 healthy	Potential biomarkers for AMI	Peng et al., 2014 [146]
	miR-1291	(+)					
	miR-663b	(+)					
	miR-208b	(+)	Plasma (stored at -80°C)	qRT-PCR	116 remodeling, 243 non-remodeling	Biomarker of LV remodeling after AMI	Lv et al., 2014 [163]
	miR-34a	(+)					
	miR-208b	(+)	Plasma	qRT-PCR	510 AMI	Potential biomarkers for AMI	Goretti et al., 2013 [158]
	miR-499	(+)					
	miR-1	(+)	Plasma	qRT-PCR	92 NSTEMI, 81 acute CHF without AMI, 99 healthy	Potential biomarker for NSTEMI	Olivieri et al., 2013 [147]
	miR-21	(+)					
	miR-133a	(+)					
	miR-208a	(+)					
	miR-423-5p	(+)					
	miR-499-5p	(+)					
	miR-1	(+)	Serum (stored at -80°C)	qRT-PCR	117 AMI, 182 AP, 100 healthy	Potential biomarkers for AMI	Li et al., 2013 [148]

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-134	(+)					
	miR-186	(+)					
	miR-208	(+)					
	miR-223	(+)					
	miR-499	(+)					
	miR-1	(+)	Plasma (stored at -80°C)	q-RT-PCR	32 AMI, 36 normal coronary angiography	Possible biomarkers for cardiac damage	Corsten et al., 2010 [159]
	miR-133a	(+)					
	miR-208b	(+)					
	miR-499	(+)					
	miR-223	(-)					
	miR-1	(+)	Plasma (stored at -80°C)	qRT-PCR	424 patients with suspected ACS	Potential diagnostic biomarkers for AMI	Gidlöf et al., 2013 [160]
	miR-208b	(+)					
	miR-499-5p	(+)					
	miR-1	(+)	Plasma	qRT-PCR	67 AMI, 32 healthy	Potential diagnostic biomarkers for AMI	Li et al., 2013 [154]
	miR-133a	(+)					
	miR-208b	(+)					
	miR-499	(+)					
	miR-30a	(+)	Plasma (stored at -80°C)	qRT-PCR	18 AMI, 30 healthy	Potential diagnostic biomarkers for AMI	Long et al., 2012 [149]
	miR-195	(+)					
	let-7b	(-)					
	miR-126	(+)	Plasma	qRT-PCR	820 from Bruneck Study	Potential diagnostic biomarkers for AMI	Zampetaki et al., 2012 [161]
	miR-223	(+)					
	miR-197	(+)					
	miR-1	(+)	Plasma	qRT-PCR	17 AMI, 25 healthy	Potential diagnostic biomarkers for AMI	Long et al., 2012 [150]
	miR-126	(-)					
	miR-208b	(+)	Plasma (stored at -80°C)	qRT-PCR	510 AMI, 87 healthy	Potential diagnostic biomarkers for AMI	Devaux et al., 2013 [151]
	miR-499	(+)					
	miR-1	(+)	Plasma	qRT-PCR	93 AMI, 66 healthy	Potential diagnostic biomarkers for AMI	Ai et al., 2010 [152]

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-423-5p	(+)	Plasma (stored at -80°C)	qRT-PCR	17 AMI, 4 stable CAD, 5 healthy	Potential early marker of myocardial necrosis	Nabiałek et al., 2013 [153]
	miR-208a	(+)					
	miR-1	(+)					
HF	miR-125a-5p	(-)	Serum	Array	21 HF, 65 controls	Potential predictors of ischemic HF after AMI	Matsumoto et al., 2013 [166]
	miR-15b	(+)					
	miR-20a	(+)					
	miR-30b	(+)					
	miR-146a	(-)					
	miR-192	(+)					
	miR-19a	(+)					
	miR-200c	(-)					
	miR-345	(-)					
	miR-374b	(-)					
	miR-485-3p	(-)					
	miR-518d-3p	(-)					
	miR-125a-5p	(-)					
	miR-215	(+)					
	miR-532-5p	(+)					
	miR-194	(+)					
	miR-34a	(+)					
	miR-210	(+)	Serum	qRT-PCR	22 HF, 18 healthy controls, 9 fetuses	Possible biomarkers of HF	Zhao et al., 2013 [165]
	miR-27a	(+)					
	miR-30a	(+)					
	miR-21	(+)					
	miR-29a	(+)					
	miR-19a	(+)					
	miR-27b	(+)					
	miR-24	(+)					
	miR-19b	(+)					
	hsa-let-7i	(-)	Whole blood	Array	CHF (55 ICM + 51 NICM), 30 healthy	Novel targets for diagnosis, prevention and treatment of CHF	Qiang et al., 2013 [167]
	hsa-miR-508-5p	(+)					
	hsa-miR-589	(-)					
	hsa-miR-518e	(-)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	hsa-miR-662	(-)					
	hsa-miR-200b	(+)					
	hsa-miR-517c	(+)					
	hsa-miR-126	(-)					
	hsa-miR-210	(-)					
	hsa-miR-513-5p	(+)					
	hsa-miR-34a	(+)					
	hsa-miR-490-3p	(+)					
	hsa-miR-186	(+)					
	hsa-miR-220c	(-)					
	hsa-miR-595	(+)					
	miR-200a	(+)					
	miR-423-5p	(+)	Plasma	qRT-PCR	45 DCM, 39 healthy	Diagnostic biomarker for HF caused by DCM	Fan et al., 2013 [168]
	miR-126	(+)					
	miR-361-5p	(+)					
	miR-155	(-)					
	miR-146a	(+)					
	miR-423-5p	(+)	Serum (stored at -80°C)	qRT-PCR	30 CHF, 30 healthy	Diagnostic and prognostic markers for HF	Goren et al., 2012 [169]
	miR-320a	(+)					
	miR-22	(+)					
	miR-92b	(+)					
	miR-17	(+)					
	miR-532-3p	(+)					
	miR-92a	(+)					
	miR-30a	(+)					
	miR-21	(+)					
	miR-101	(+)					
	miR-126	(+)	Plasma	qRT-PCR	33 ischemic heart disease, 17 healthy	New biomarker for CHF	Fukushima et al., 2011 [170]

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-423-5p	(+)	Plasma	Array	30 HF, 20 non-HF, 39 healthy	Potential biomarkers for HF	Tijsen et al., 2010 [171]
	miR-129-5p	(+)					
	miR-675	(+)					
	miR-18b	(+)					
	HS_202.1	(+)					
	miR-1254	(+)					
	miR-622	(+)					
	miR-302d	(+)					
	miR-654-3p	(+)					
	miR-346	(+)					
	miR-1301	(+)					
	miR-24-2	(-)					
	miR-15b	(+)					
	miR-142-3p	(+)					
	HS_239	(-)					
	so-lexa3927-221	(-)					
	miR-520d-5p	(+)	Serum (stored at -80°C)	qRT-PCR	53 HF-REF, 39 healthy	Potential biomarkers for systolic HF	Vogel et al., 2013 [172]
	miR-122	(+)					
	miR-643	(+)					
	miR-548i	(+)					
	miR-718	(+)					
	miR-935	(+)					
	let-7e	(+)					
	miR-376a	(+)					
	miR-1225-5p	(+)					
	miR-675	(+)					
	miR-622	(+)					
	miR-582-3p	(+)					
	miR-551b	(+)					
	miR-224	(+)					
	miR-670	(+)					
	miR-331-5p	(+)					
	miR-369-3p	(+)					
	miR-944	(+)					
	miR-200b	(+)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-519e	(+)					
	miR-558	(-)					
	miR-1302	(-)					
	miR-146b-3p	(-)					
	miR-345	(-)					
	miR-760	(-)					
	miR-218	(-)					
	miR-1301	(-)					
	miR-604	(-)					
	miR-370	(-)					
	miR-144	(-)					
	miR-574-5p	(-)					
	miR-566	(-)					
	miR-1321	(-)					
	miR-143	(-)					
	miR-551b	(-)					
	miR-20b	(-)					
	miR-1914	(-)					
	miR-597	(-)					
	miR-623	(-)					
	miR-421	(-)					
	miR-1231	(+)					
	miR-1228	(+)					
	let-7b	(+)	Plasma	qRT-PCR	135 dyspnea, 15 healthy	Potential biomarkers for HF	Ellis et al., 2013 [174]
	miR-103	(-)					
	miR-142-3p	(-)					
	miR-150	(-)					
	miR-185	(+)					
	miR-1909	(-)					
	miR-199a-3p	(-)					
	miR-2110	(+)					
	miR-23a	(-)					
	miR-27b	(-)					
	miR-29a	(-)					
	miR-30b	(-)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-324-5p	(-)					
	miR-342-3p	(-)					
	miR-423-5p	(+)					
	miR-598	(-)					
	miR-940	(-)					
	miR-210	(+)	Plasma + mononuclear cells	qRT-PCR	39 CHF [plasma] / 13 CHF, 6 healthy [mononuclear cells]	Potential biomarker for CHF	Endo et al., 2013 [173]
	miR-1	(+)	Plasma	qRT-PCR	49 AMI	Potential diagnostic biomarkers for AMI	Zhang et al., 2013 [175]
Stroke	miR-30	(-)	Plasma (stored at -80°C)	qRT-PCR	197 ischemic stroke, 50 healthy	Potential biomarkers for ischemic stroke	Long et al., 2013 [176]
	miR-126	(+)					
	let-7b	(+/-)					
	miR-145	(+)	Whole blood	qRT-PCR	32 ischemic stroke, 14 healthy	Potential biomarkers for ischemic stroke	Gan et al., 2012 [130]
	miR-21	(+)	Serum (stored at -80°C)	qRT-PCR	167 ischemic stroke, 66 atherosclerosis, 157 healthy	Potential biomarkers for ischemic stroke	Tsai et al., 2013 [177]
	miR-221	(-)					
	miR-210	(-)	Whole blood	qRT-PCR	112 ischemic stroke, 60 healthy	Potential biomarkers for ischemic stroke	Zeng et al., 2011 [178]
	hsa-let-7f	(-)	Whole blood	qRT-PCR	19 ischemic stroke	Potential biomarkers for ischemic stroke	Tan et al., 2009 [131]
	miR-126	(-)					
	miR-1259	(-)					
	miR-142-3p	(-)					
	miR-15b	(-)					

Disease	miR	Regulation	Source	Method	Number of samples	Potential clinical applicability	Reference
	miR-186	(-)					
	miR-519e	(-)					
	miR-768-5p	(-)					
	hsa-let-7e	(+)					
	miR-1184	(+)					
	miR-1246	(+)					
	miR-1261	(+)					
	miR-1275	(+)					
	miR-1285	(+)					
	miR-1290	(+)					
	miR-181a	(+)					
	miR-25	(+)					
	miR-513a-5p	(+)					
	miR-550	(+)					
	miR-602	(+)					
	miR-665	(+)					
	miR-891a	(+)					
	miR-933	(+)					
	miR-939	(+)					
	miR-923	(+)					

miRs shaded with green were statistically significantly upregulated (+) or downregulated (-) and might be potentially applicable.

ACS, acute coronary syndrome; ASO, arteriosclerosis obliterans; CAD, coronary artery disease; DM, diabetes mellitus; HF, heart failure; IFG, impaired fasting glucose; n-T2DM, newly diagnosed type 2 diabetes mellitus; PAD, peripheral artery disease; qRT-PCR, quantitative real-time polymerase chain reaction; s-NGT, susceptible individuals with normal glucose tolerance; T2DM, type 2 diabetes mellitus; pre-T2DM, pre type 2 diabetes mellitus.

Table 7. Pearson correlation analysis between microRNAs (miRs) and selected arterial markers.

	miR-1		miR-126		miR-145		miR-155		miR-122		miR-370		miR-133a		miR-133b		miR-195		miR-132	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*		
FMD, %	-0.086	0.264	-0.03	0.695	-0.066	0.392	-0.074	0.34	0.002	0.98	-0.004	0.964	-0.067	0.389	-0.073	0.347	-0.068	0.382	0.091	0.24
AoPWV	$3.7 \cdot 10^{-5}$	0.99	-0.003	0.972	0.005	0.944	-0.064	0.393	-0.15	0.049	0.094	0.213	-0.003	0.964	-0.09	0.233	0.075	0.317	0.057	0.453
AIx@75	-0.201	0.005	-0.041	0.585	-0.151	0.044	-0.126	0.095	0.032	0.671	$9 \cdot 10^{-4}$	0.99	-0.235	0.002	-0.242	0.001	-0.012	0.873	0.008	0.92
MAP	-0.076	0.315	-0.009	0.907	-0.039	0.603	0.041	0.589	-0.1	0.188	0.068	0.369	-0.071	0.345	-0.066	0.385	-0.124	0.101	0.011	0.889
CAVImean	-0.218	0.004	-0.054	0.479	0.037	0.629	-0.136	0.073	0.152	0.046	-0.058	0.449	-0.177	0.02	-0.221	0.003	0.065	0.398	0.025	0.747
MMI	0.08	0.294	0.036	0.643	0.125	0.1	-0.011	0.882	0.088	0.251	-0.005	0.946	0.056	0.465	0.095	0.215	-0.048	0.53	0.01	0.898
Stiffness right carotid artery	-0.024	0.754	0.119	0.116	0.038	0.615	-0.032	0.679	0.102	0.181	0.095	0.209	0.014	0.858	0.024	0.757	0.103	0.173	0.165	0.029
Stiffness left carotid artery	0.154	0.042	-0.002	0.985	0.001	0.986	-0.035	0.642	0.036	0.639	0.026	0.729	0.053	0.482	0.109	0.151	0.047	0.536	0.018	0.818
Stiffness carotid artery	0.075	0.324	0.067	0.382	0.022	0.769	-0.038	0.616	0.078	0.306	0.069	0.364	0.039	0.613	0.076	0.318	0.085	0.261	0.103	0.174

AIx@75; augmentation index normalized to a heart rate of 75 beats per minute; AoPWV, aortic pulse wave velocity; CAVImean, mean cardio-ankle vascular index; FMD, flow-mediated dilatation; MAP, mean arterial pressure; MMI, myocardial mass index ; r, correlation coefficient.

Table 8. P-values from a linear regression -test for an association between microRNAs and carotid arterial plaques.

	miR-1	miR-126	miR-145	miR-155	miR-122	miR-370	miR-133a	miR-133b	miR-195	miR-132
Right carotid plague	0.629	0.382	0.811	0.953	0.244	0.005	0.712	0.855	0.104	0.257
Left carotid plaque	0.789	0.306	0.74	0.834	0.512	0.636	0.699	0.835	0.537	0.04

Table 9. Logistic regression models for aortic pulse wave velocity.

Cut off	Precision	Recall	F-score
8.6 (median)	–	–	–
8.7	0.509	0.785	0.593
8.8	0.518	0.835	0.623
8.9	0.598	0.88	0.697
9	0.615	0.908	0.719
9.1	0.633	0.926	0.74
9.2	0.656	0.935	0.76
9.3	0.662	0.936	0.763
9.4	–	–	–
9.5	0.725	0.967	0.821
9.6	0.749	0.974	0.839
9.7	0.784	0.994	0.871
9.8	0.796	1	0.881
9.9	0.812	0.986	0.886
10	0.848	1	0.914
10.1	0.864	0.982	0.915
10.2	0.864	0.982	0.915
10.3	0.87	0.988	0.922
10.4	0.87	0.988	0.922
10.5	0.88	0.988	0.929
10.6	0.887	0.988	0.932

Table 10. Linear regression models.

	Model	MAE	MAPE
FMD	–	–	–
AoPWV	8.34 +0.32 miR-1-0.37 miR-133b	1.34	15.09
AIx@75	38.55-3.14 miR-133b	9.87	145.89
MAP	98.82-1.63miR-122	10.7	34.92
CAVImean	10.51- 0.28 miR-133b + 0.27miR-122 -0.25miR-155	1.09	16.63
Stiffness right carotid artery	1.61+0.46miR-126 + 0.33miR-132	1.39	40.35
Stiffness left carotid artery	–	–	–
Stiffness carotid artery	3.07+0.27miR-132	1.16	30.69
MMI	91.8+5.15miR-145	18.67	23.97

AoPWV, aortic pulse wave velocity; CAV MAE, mean absolute error; FMD, flow-mediated dilatation; MAP, mean arterial pressure; MMI, myocardial mass index ; MAPE, mean absolute percentage error.

Discussion

Although accumulating evidence suggests that miRs may be potential biomarkers for atherosclerosis, hypertension, and CAD, little is known about the circulating levels of miRs and their association with arterial biomarkers, such as AoPWV, CAVI, carotid artery stiffness, and others. In the present study, to our knowledge, a relationship between miR-1 and AIx@75, CAVI, and carotid artery stiffness, as well as between miR-133a/b and AIx@75, and CAVI, have been demonstrated for the first time. These findings indicate a link between miRs and vascular stiffness. Additionally, miR-122 demonstrated a linear relationship with AoPWV, the most extensively investigated arterial marker. However, miR-122 was the only miR tested that demonstrated this relationship. Thus, further research is required.

Despite the statistically significant findings of linear relationships between arterial markers and miR-1, miR-122, miR-133a, and miR-133b, the relationships were weak, possibly due to insufficient numbers of patients. Moreover, no linear relationship was observed between arterial markers and miR-126, miR-155, and miR-195.

Considering that CVDs are usually multifactorial diseases, caused by various mechanisms, it is likely that a combination of miRs will have a stronger predictive or diagnostic power, as demonstrated in the present study. To further clarify these findings, the current linear regression models need to be tested on wider populations.

The miRs tested here were not associated with outcomes, as the follow-up time from collection of blood samples was too short. Therefore, the associations are limited to other test results, rather than to CV outcomes. However, this would be an invaluable aspect to study in longer-term studies.

Conclusion

- miR-122 showed a linear relationship with the most commonly investigated arterial marker, AoPWV
- Demonstrated relationships between miR-1 and arterial markers such as AIx@75/CAVI/carotid artery stiffness, as well as between miR-133a/b and AIx@75/CAVI, suggests some association between the miRs and vascular stiffness, as measured by several different methods
- miRs measured in plasma or bodily fluids may represent potentially useful disease biomarkers and predictors of metabolic disease and associated CV complications.

5. CLINICAL IMPLICATIONS

Clinical practitioners must consider the adaption of current risk prediction tools to encompass wider arterial and molecular indicators. These studies on the MetS patient group suggest that assessment of CVD risk as well as potential for progression to T2DM are inadequately assessed based solely on the traditional risk assessment tools. The current observations confirm the insufficiency of FRS assessment among the important subgroup of people with metabolic disease. This should be considered when applying FRS in clinical practice. The research described herein demonstrates that AoPWV is a viable risk stratification biomarker, directly related to CVD outcomes. It would be valuable to incorporate this or other measures of arterial stiffness in the assessment of patients with metabolic disease. Also demonstrated was an association of miR with early atherosclerosis, suggesting that it could be a biomarker of subclinical atherosclerosis in the foreseeable future. As the timing and intensity of preventive intervention are dependent on the outcomes of a patient's risk assessment, it is imperative that the most accurate and specific risk prediction tools are applied.

6. NOVELTY

This is the largest study, to my knowledge, of exploring CVD risk assessment among patients with MetS. The breadth of these studies across several potential avenues for enhancing current risk prediction makes this a valuable body of research. The quality of the investigations and their potential to improve risk prediction for large numbers of patients makes them a valuable addition to this field. Also provided is original research demonstrating an association between early atherosclerosis and multiple miRs, highlighting the potential of miRs to be used as early biomarkers.

7. SUMMARIZED DISCUSSIONS

This series of studies was conducted with the principal aim of investigating various arterial and molecular biomarkers for their usefulness as predictors of T2DM and CVD risk. T2DM and CVD are both leading causes of morbidity and mortality worldwide. Primary and secondary prevention approaches are crucial to avoid the associated burden and impact on the patient and society. It is well established that CVD is a life-long condition that begins with risk factors that predispose to the development of subclinical atherosclerosis, which in turn contributes to the occurrence of overt CVD [194,195]. MetS is a cluster of CV risk factors that can progress to diabetes without appropriate intervention and management.

As CVD remains the leading cause of death worldwide, and the associated mortality is estimated to reach more than 23.6 million deaths per year by 2030 [196], CVD risk assessment and early prevention remain important priorities in daily medical practice. In adults, it is recommended to assess the traditional atherosclerotic CVD risk factors every 4-6 years between the ages of 20-79 years. Several risk screening models have been developed for major CV events; for example, the FRS, PROCAM, QRISK, EURO-SCORE, QRISK2, the RRS, and the ASSIGN score [197].

The FRS is one of the most widely utilized methods for predicting CV risk. It can be used to predict 10- and 30-year mortality based on age, diabetes, smoking status, SBP, TC, and HDL-C (male sex and use of antihypertensive treatment included for 30-year risk prediction) [198]. However, this model is not suitable for all patient groups and may under- or overestimate overall CV risk, particularly in high-risk groups. With the advent of more personalized medicine, it is hoped that the accuracy of this, and other risk models, can be improved by adding novel risk markers and biomarkers into the risk prediction algorithm. Biomarkers may assist with both primary and secondary prevention by identifying high-risk individuals, by enabling early and accurate diagnosis, and by predicting the future evolution of the disease. The research undertaken here, therefore, focussed on biomarkers and explored potential candidate markers from several different angles.

In the first series of investigations, studies were undertaken to assess whether AoPWV, as a measure of arterial stiffness, can improve risk stratification in patients with MetS. Although it is known that arterial wall function contributes to CVD risk

[199], relatively few studies to date have investigated subjects at high risk of CVD, such as those with MetS - the population examined herein. AoPWV was significantly higher among those who later experienced a CVD event (fatal or non-fatal MI or stroke) during the follow-up period (9.41±2 vs 8.8±1.6, P<0.001). In an unadjusted logistic regression model, AoPWV remained a strong predictor of a CVD event. These findings are of interest because AoPWV appears to be a true predictor, independent of confounding factors such as age or BP. Furthermore, it a simple, accurate marker, measured using non-invasive ultrasound methods that are highly reproducible [200]. Arterial stiffness may, therefore, represent one important pathway linking MetS/diabetes to the increased CV risk observed in these patients. Indeed, increased arterial stiffness predicts the development of CV illnesses and mortality in the general population and in those with T2DM. These data support the hypothesis of increased arterial stiffness in MetS, which may explain, at least in part, the increased CV risk in these individuals, and emphasize the importance of primary prevention.

In the second series of investigations, predictive models for diabetes were developed and a range of variables was assessed for their prognostic value among middle-aged subjects with MetS. Importantly, it was found that T2DM in middle-aged MetS subjects could be predicted with a good level of accuracy. Routine measures such as FPG, BMI, and HbA_{1c} provided a solid foundation for risk assessment. FPG was the strongest predictor for the development of T2DM in subjects with MetS, with BMI and HbA_{1c} having cumulative value. Furthermore, risk was also related to elevated AoPWV measurements. These are important findings because markers that can identify those patients most at risk of progressing to T2DM might enable prevention and management programs to be targeted appropriately. A proportion of the 3.2 million deaths that result from complications associated with diabetes each year [201], could, therefore, be averted. Thus, the predictive value of clinical biomarkers for the development of T2DM in patients with MetS, who do not yet show atherosclerotic disease, may prove useful in clinical practice.

In the final series of investigations, the potential of miRs to diagnose or predict the prognosis for patients with CVD was examined. A systematic review of published literature on miRs to date was performed, allowing us to select the most promising miRs for further research. From the 19 selected studies, 52 distinct miRs were investigated and several were identified as potentially useful diagnostic and/or prognostic markers across different CVD progression stages, for example, miR-133a/b, miR-208a/b, miR-499, miR-1, and miR-145b. Functional data on these miRs

were also derived from the published literature to explore their potential mechanisms for contributing to atherosclerosis or CV risk. These miRs were implicated in diverse CV functions such angiogenesis, apoptosis, cardiac myocyte differentiation, and communication between vascular smooth muscle and endothelial cells. Thus, miR-mediated pathways may be implicated in the development of cardiometabolic disease. This literature review provides guidance on the potential direction of future investigations relating to miRs as biomarkers of CVD, which was the basis for the 10 selected miRs for the current research. The study demonstrated the association between some of the investigated miRs and early atherosclerosis, suggesting it as a possible standardized replacement for the diagnostic purposes in the foreseeable future, contributing to personalized CVD risk assessment.

Overall, these studies contribute significantly to our understanding of predictive biomarkers in CV medicine and their potential utility in enhancing the prevention, diagnosis, and management of CV risk factors, thereby improving the lives of patients. This knowledge should be incorporated into routine clinical practice in primary and secondary care to ensure efficiency and effective patient care.

8. CONCLUSIONS

1. Commonly used risk prediction tools are inaccurate for use among patients with MetS
2. Even the FRS2 model itself proved inadequate to predict FRS2 outcomes for patients with MetS
3. AoPWV is an independent CVD event predictor that may provide additive value when used concomitantly with traditional risk prediction tools
4. Progression to T2DM among middle-aged subjects with MetS can be accurately predicted using the combination of easily accessible parameters such as FPG, BMI, and HbA_{1c}, and is related to elevated AoPWV measurements
5. OGTT is less useful than FPG in predicting T2DM in patients with MetS
6. MiRs may represent potential early atherosclerosis biomarkers:
   a) miR-122 showed a linear relationship with arterial stiffness, as measured by AoPWV
   b) miR-1 showed associations with Aix@75, CAVI, and carotid artery stiffness, while miR-133a/b showed associations with Aix@75 and CAVI

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