Circulating microRNAs and other biomarkers for premature atherosclerosis

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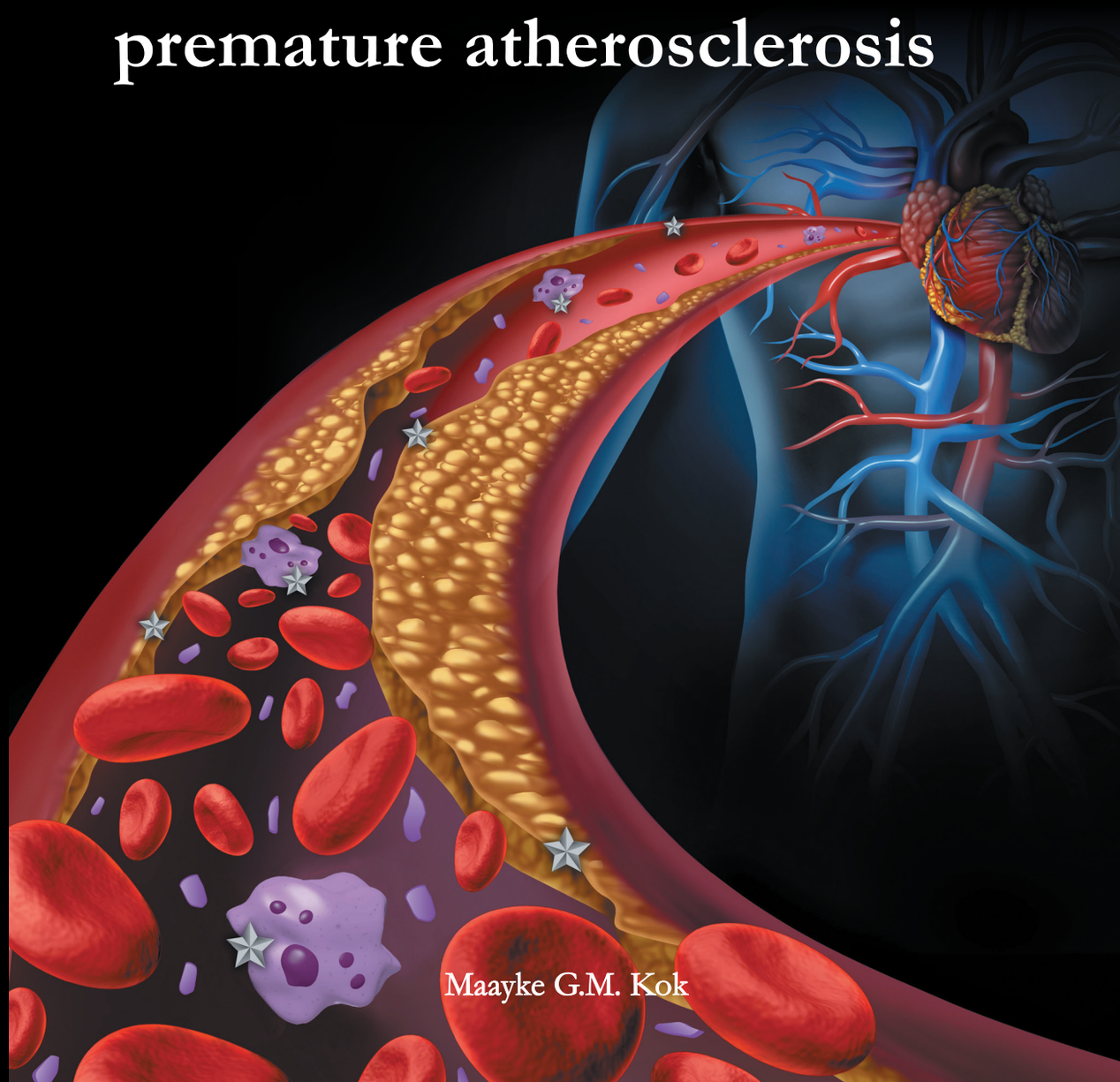

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CHAPTER 1

Introduction and outline of this thesis

Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the world. It has been estimated that 4 million people die of this disease each year in Europe and disease-related costs are estimated to reach up to €196 billion a year[1]. Introduction of primary and secondary prevention measures, such as risk reduction programs and the use of cardio-protective medication, have contributed substantially to the decrease in deaths that has been observed between 1980 and 1990[2]. However, these strategies only achieved a 20-30% reduction in event rate[3], leaving CVD a major health issue.

The understanding of the development of CVD took an important turn with the initiation of the Framingham heart study in 1949, in which they aimed to study the epidemiology of cardiovascular disease and hypertension. Until then, it was generally accepted that the development of CVD was a natural process that accompanies normal aging. As such, elevated cholesterol levels and hypertension were not considered risk factors for a serious and possibly chronic disease, but merely a natural phenomenon associated with progression of life[4,5].

To date, CVD is an extensively studied process and a lot of knowledge has been acquired regarding the development of and the risk factors for this disease. It has been shown that atherosclerosis, the primary underlying mechanism, originates from a combination of lipid accumulation, vessel wall inflammation and consequent thrombotic reactions[6]. Although this process also has a degenerative character, the severity and progression rate of the disease are mostly consequences of certain risk factors.

The risk factors for CVD arise from a combination of physiological processes, including aging, life style habits, such as smoking and obesity, and pathological mechanisms like elevated cholesterol levels, blood pressure and diabetes mellitus. Mathematical algorithms, incorporating these risk factors, have been created to calculate the individual risk of developing CVD of your patient in general practice[7,8]. Unfortunately, the process of atherosclerosis cannot be reversed yet, but based on the risk calculations, preventive measures can be taken to halt the progression of the disease and reduce the risk of myocardial infarction.

Premature cardiovascular disease

Unfortunately, the performance of the available prediction models is only poor in certain popu-lations. In the Framingham Risk Score one of the major determinants of CVD risk is the individual's age. In fact, for young individuals the influence of age garbles the calculation in such way that it underestimates the CVD risk, even when multiple risk factors are present[9].

Of all people that develop CVD, 6-10% is affected at a very young age[10]. In this perspective, patients are usually considered young if they develop CVD before the age of 50 or 60 years, depending on the definition used. Regarding these individuals, traditional cardiovascular risk factors can be identified, but are usually not the predominant cause of the disease[11]. A more pronounced risk factor in this group is the presence of CVD within the family. It is well established that a family

history of premature CVD is a robust risk factor for the development of cardiovascular events, even after adjustment for traditional risk factors[12,13]. In fact, it may increase the risk of CVD two- to three-fold[14,15].

A positive family history is often identified during the initial clinical examination of a patient with premature CVD, however a definitive genetic diagnosis is rarely made[16]. Well established genetic defects, for example in the cholesterol metabolism account for only part of the population with premature CVD. It has been postulated that rather than a monogenetic disease, premature CVD in these patients results from a combination of polygenetic predisposition and environmental risk factors.

It is important to emphasize that a positive family history for premature CVD only identifies families at risk, but holds little information on the individual risk of each family member. Calculation of the coronary calcium score can be used as a sensitive diagnostic tool for the identification of subclinical atherosclerosis in individuals at risk of premature CVD, but is only applicable once the atherosclerotic process is already visible. Ideally, subjects at risk should already be identified during adolescence, when atherosclerosis is still restricted to a minimum. Newly identified biomarkers could be a suitable tool for this purpose.

MiRNAs as biomarker of premature cardiovascular disease

MiRNAs, i.e. short RNA molecules with an average length of 22 nucleotides, are important regulators of gene expression. In normal cells, they are involved in crucial biological processes, such as cell differentiation, proliferation and apoptosis[18]. However, in pathological conditions the involvement of miRNAs has also been described and many miRNAs have been reported to be associated with specific diseases.

The first report on the involvement of miRNAs in disease originates from studies on cancer[19]. Expression levels of specific miRNAs were shown to be up regulated in leukemia cells compared to healthy cells[20]. Shortly thereafter, miRNAs were also described to be involved in angiogenesis and cardiac development, suggesting a role of miRNAs in CVD[21]. By now, multiple studies have focused on the involvement of miRNAs in the development of CVD and several miRNAs were shown to contribute to this process[22].

It was hypothesized that changes in miRNA expression during disease could be used as a marker for diagnosis. The proposed suitability of miRNAs as biomarker was based on two principal characteristics. Firstly, miRNAs can not only be detected in isolated cells, but are also present in the circulation[23]. Moreover, these circulating miRNAs are easily accessible, remarkably stable and even withstand repetitive freezing/thawing cycles and are protected against degradation[24,25]. Secondly, changes in miRNA expression profiles can be detected in a diseased state. It was hypothesized that changes in miRNA expression in diseased tissue are reflected in the circulation and that by detection of these changes in circulating miRNA expression profiles a disease could be diagnosed. Taken together, circulating miRNAs were considered an attractive tool for early identification of subjects at risk of developing CVD.

OUTLINE OF THIS THESIS

Part I

This thesis focusses on the identification of subjects at risk of developing premature CVD and on characterization of specific subpopulations of these individuals. Early identification of an increased cardiovascular risk will enable subjects to take preventive measures early in the development of the disease, thereby reducing the risk of morbidity and mortality.

In Part I, the diagnostic potential of circulating miRNAs as a biomarker for CVD is studied. Research on circulating miRNAs has emerged over the last decade, but is still in a premature stage. It was shown that differences in sample processing and data analyses can result in different outcomes, underlining the need of standardized protocols. Chapter 2 deals with one of these issues, namely normalization of miRNA real time quantitative polymerase chain reaction (RT-qPCR) data. We aimed to select normalization panels for RT-qPCR experiments in whole blood, platelets and serum samples, using a standardized method. These normalization panels could then be used in the miRNA quantification experiments in this thesis.

In chapter 3 and 4 we investigated whether changes in miRNA expression profiles could be used to identify patients at risk for cardiovascular events in different high risk populations. Chapter 3 focusses on subjects with established CVD, who are treated with aspirin to prevent further cardiovascular events. It is known that inter-individual differences exist in the response of platelets to aspirin. In some patients platelet function is almost completely inhibited, whereas in other patients inhibition of platelet function does not occur, resulting in an increased risk of re-events in these individuals. We hypothesized that we could identify these patients based on changes in platelet miRNA expression profiles. Furthermore, we hypothesized that alterations in the expression of specific platelet miRNAs could be a suitable biomarker for aspirin insensitivity.

In chapter 4 a different population with an increased risk of developing CVD is studied, namely smokers. It is known that the risk of developing CVD is twice as high amongst smoking individuals compared to non-smokers. Monocytes play a key role in the development of CVD through their involvement in atherosclerotic plaque formation. We hypothesized that smoking alters the function of circulating monocytes and that these changes in monocyte function are reflected by changes in monocyte miRNA expression profiles. Furthermore, we hypothesized that based on expression levels of specific monocyte derived miRNAs smokers with an increased risk of developing CVD can be identified.

When performing miRNA research several difficulties can be encountered. During the course of our studies we found that a small sample size is a limitation of multiple miRNA experi-ments. In chapter 5 we address this topic. We aimed to underline the importance of a sufficient sample size and do some recommendations on how to perform a suitable power calculation for future experiments.

Part II

For patients with premature CVD the multifactorial origin of the disease is even more pronounced than for patients that present with CVD at an older age. Different genetic predispositions are present in premature CVD patients, making them more sensitive to environmental risk factors. Further characterization of subpopulations is necessary to identify new factors that better predict the risk of developing premature CVD than the established mathematical risk prediction models.

The coagulation system plays an important role in the development of CVD and the presence of a hypercoagulable state has been shown to be associated with an increased risk of a cardiovascular event, especially in young individuals[26,27]. The metabolic syndrome (MS) is an important risk factor for the development of CAD, but is also known to be associated with a higher incidence of thrombo-embolic events[28]. In chapter 6 we hypothesized that individuals with premature CAD and MS have an increased prothrombotic potential, resulting in a hypercoagulable state.

Chapter 7 focusses on changes in the endothelium of subjects with premature CVD. Blood outgrowth endothelial cells (BOECs) play a crucial role in the regenerative capacity of the endothelium[29,30]. Since endothelial damage is an important component of the atherosclerotic process, reduced regenerative capacity of the endothelium will enhance the formation of atherosclerotic plaques. We hypothesized that subjects with premature CAD have lower levels of circulating BOECs and that proliferation of these cells is impaired compared with healthy controls. Additionally, we wanted to evaluate the influence of statin treatment on circulating BOEC precursors in subjects with subclinical atherosclerosis.

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PART I

MicroRNAs as biomarker for premature atherosclerosis

CHAPTER 2

Normalisation panels for the reliable quantification of circulating miRNAs by RT-qPCR

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ABSTRACT

Circulating microRNAs (miRNAs) have been reported as biomarkers for disease diagnosis. RT-qPCR is most commonly used to detect miRNAs, however no consensus on the most appropriate method for data normalisation exists. Via a standardised selection method, we aimed to determine separate miRNA normalisation panels for RT-qPCR measurements on whole blood, platelets and serum.

Candidate miRNAs were selected from studies describing circulating miRNA microarray data in the Gene Expression Omnibus or ArrayExpress. MiRNA expression data of healthy controls were retrieved from each study. For each sample type we selected those miRNAs that were least variable and sufficiently highly expressed in multiple microarray experiments, performed on at least two different platforms. Stability of the candidate miRNAs was assessed using NormFinder and geNorm algorithms in a RT-qPCR cohort of 10 patients with coronary artery disease and 10 healthy controls.

We selected miRNA normalisation panels for RT-qPCR measurements on whole blood, platelets and serum. As a validation, we assessed the precision of all 3 panels in 3 independent RT-qPCR cohorts and compared this with normalisation for miR-16 or RNU6B.

The proposed normalisation panels for whole blood, platelets and serum show better precision than normalisation for miR-16 or RNU6B.

INTRODUCTION

MiRNAs are 18 to 25 nucleotides long, noncoding RNAs that regulate gene expression post-transcriptionally by targeting the 3'-untranslated region of specific messenger RNAs (mRNA)[1]. Hereby, several biological processes, like cell differentiation and apoptosis, are affected[2]. To date, more than 2500 individual miRNAs have been identified in the human genome[3]. Since miRNAs can be detected in the circulation and are easily accessible and stable within the circulation, it has been suggested that they are very powerful biomarkers for disease. Indeed, many studies have shown the pathophysiological involvement of miRNAs, and multiple miRNAs have been reported as useful biomarkers for specific diseases[4,5].

To be able to use these miRNAs as biomarker, reliable measurement is a necessity. Because reverse transcription quantitative real-time PCR (RT-qPCR) is the highly sensitive, specific and has a high reproducibility, it is a commonly used method to quantify miRNAs[6]. To obtain reliable RT-qPCR results, possible processing variations between samples have to be corrected by normalisation[7]. In the field of messenger RNA research the optimal way of normalising RT-qPCR data has been extensively investigated and nowadays the preferred method is to normalise by a panel of different RNA molecules[7]. Many different ways of miRNA RT-qPCR data normalisation have been described[8–10], however there is no consensus on the most appropriate method yet. The three most commonly used normalisation strategies are: normalisation to the geometric mean of all detected miRNAs[11], normalisation to a single endogenous control (for example RNU6B or miR-16)[8,12], and the use of a synthetic or biological spike-in[13]. Pros and cons have been described for all these normalisation methods, but none of them seem ideal for universal use in RT-qPCR experiments[14]. We hypothesised that more reliable quantification of miRNA RT-qPCR data, and hence better precision, could be obtained by normalising miRNA RT-qPCR data using a panel of stably expressed miRNAs. The aim of this study was to establish such normalisation panels for whole blood, platelet and serum samples for studies in coronary artery disease (CAD) patients. To do so, we developed a standardised method to extract stable, highly expressed miRNAs from currently available miRNA microarray experiments. Candidate miRNAs were subsequently validated by RT-qPCR in an independent cohort using the geNorm and Normfinder algorithms.

We compared the precision of our normalisation panels with normalisation for miR-16 or RNU6B, two small RNAs that have often been used as normalisation miRNAs in literature[5]. We chose not to normalise for a spike-in, because this method only corrects for either extraction or reverse transcription efficiency and therefore it does not correct for remaining experimental variability including variability in sample collection and processing. Furthermore, normalisation for the geometrical mean of all detected miRNAs was omitted in the comparison, since too few miRNAs were detected per experiment to reliably use this normalisation method.

METHODS

To be able to construct the optimal normalisation panel for cardiovascular disease, several steps had to be taken. Firstly, in the panel discovery phase, we selected candidate miRNAs from all available miRNA microarray experiments published to date, to construct different discovery miRNA panels for either whole blood, platelets or serum. Secondly, to be able to construct the final normalisation panel, we validated the different discovery panels using RT-qPCR in a cohort of subjects with cardiovascular disease and healthy controls in the panel selection phase. Finally, in the panel validation phase, we evaluated the precision of each panel and compared this to the other normalisation methods.

Panel discovery phase

The identification of the discovery panels was done on all published miRNA micro-array experiments. The discovery panels were selected from previously performed studies that reported miRNA microarray data in the Gene Expression Omnibus (GEO) or ArrayExpress and for that matter PubMed, GEO and ArrayExpress were extensively searched for studies in which miRNA microarray experiments were performed on either whole blood, platelet or serum samples. The search terms "miRNA microarray" in combination with either the terms whole blood, platelets or serum were used. We only included the microarray studies if the normalised expression intensities were reported for at least 10 healthy control individuals (Table 1). We only used microarray data of healthy control samples to minimise influences of disease on the stability of expressed miRNAs. The purpose of the normalisation panel is to overcome technical differences within an experiment. Therefore, an ultimate 'normal' situation should be constructed as a gold standard. In the ideal situation one should have access to miRNA microarray data of all existing diseases in the discovery phase, to be able to check for universal stability. Since this was not possible, we decided to exclude all miRNAs, which were known to be differentially expressed in any disease. The stability of these discovery miRNA panels were validated in both diseased subjects and healthy controls.

All micro-array studies that reported ratio data (for example two-colour platforms), Z-transformed data, pooled sample data or data that was not normalised, were excluded.

Normalised miRNA expression data of the control subjects was extracted from GEO using the GEOquery R package. Expression intensities were log2-transformed, if the extracted data had not been transformed yet. For each sample type (whole blood, platelets and serum) the discovery panels were constructed according to stringent criteria. First, the discovery panels were selected based on two specific criteria. A. The 35% (40% for serum) most stable miRNAs were selected using the coefficient of variation (CV). To limit the influence of outlying measurements, for each miRNA the CV was calculated using robust statistics as the median absolute deviation (MAD) divided by the median; B. Sufficiently highly expressed miRNAs were selected by requiring that the expression levels of a particular miRNA had to be between the 50^{th} and 97.5^{th} percentile in at least 75% (50% for serum) of the arrays of the microarray experiment under consideration.

Second, for each sample type (whole blood, platelets and serum) we selected those candidate normalisation miRNAs for the discovery normalisation panel that met the above mentioned selection criteria in the majority of the selected microarray experiments, to make sure that the observed stability of the constructed discovery panels was not caused by one specific experiment. Third, miRNAs selected for the discovery panel had to be proven stable according to the above mentioned criteria in microarray experiments performed using at least two different platforms, to be sure that the observed stability was not due to a specific platform. This rendered three discovery panels, which had to be validated in the miRNA panel selection phase, the second step in the construction of the optimal normalisation panel. Candidate miRNAs that had been reported to be involved in any disease state in the sample type under consideration, as reported in PubMed, were excluded from this list.

Panel selection phase

Stability of RT-qPCR expression of the discovery panels was assessed in a cohort of 10 patients with CAD and 10 healthy controls. From these individuals whole blood, platelet and serum samples were collected from which RNA was isolated using the miR-Easy kit (Qiagen, Hilden, Germany) for whole blood samples and the *mir*Vana miRNA Isolation kit (Ambion, Inc, Foster City, CA, United States) for platelet and serum samples according to the manufacturer's instructions. For each miRNA specific reverse transcription was performed on 100ng of purified total RNA, including miRNAs, for whole blood and platelets and 2µl of purified total RNA for serum using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium). RT-qPCR reactions were carried out, in duplo, on a LightCycler 480 system II (Roche, Basel, Switzerland). Data were analysed using LinRegPCR quantitative PCR data analysis software, version 11[15].

Stability of RT-qPCR expression of the candidate normalisation miRNAs was analysed using the NormFinder[7] and geNorm[16] algorithms to obtain the final miRNA normalisation panels. Normfinder identifies the optimal normalisation miRNA by ranking all candidate miRNAs in terms of stability in a given sample set[7] It calculates a stability value that combines the intra- and intergroup variability within the RT-qPCR experiment and thereby adds the two sources of variation and represents a practical measure of the systematic error that will be introduced when using the investigated miRNA. Additionally, it identifies the optimal pair of miRNAs that can be used for normalisation. GeNorm uses a stability measure that quantifies to what extent the expression ratio of two candidate miRNAs is identical in all samples. For every candidate miRNA the algorithm determines the pairwise variation with all other candidates and determines the stability measure M[16]. GeNorm also determines a V-value, that is the pairwise variation between two consecutive normalisation miRNAs starting with the candidate miRNA with the lowest M value. The combination of miRNAs that resulted in the lowest V-value was selected as the optimal set of normalisation miRNAs[11]. The final normalisation panels consisted of those candidate miRNAs selected by both geNorm and Normfinder. If both algorithms selected different normalisation panels, both panels were combined in the final normalisation panel.

Panel validation phase

To be able to establish the validity of the normalisation panels we analysed the precision of the panels and compared this to the precision of other normalisation methods. Precision of the whole blood, platelet and serum normalisation panels was tested in an independent cohort for each sample type.

Whole blood

At the outpatient clinic for premature CAD (first CAD event before the age of 51 in men and 56 in women) of the Academic Medical Center in Amsterdam asymptomatic first-degree relatives (FDRs) of patients with premature CAD are screened for risk assessment. Between August 2009 and May 2011 we included 90 consecutive FDRs over the age of 30. Based on the coronary calcium (CAC) score, these subjects were divided in a group of 52 subjects with a coronary calcium score below the 80th percentile for age and gender, considered to be healthy FDRs and a second group of 38 subjects with a CAC score above the 80th percentile for age and gender, designated CAD cases.

Platelets

The platelet validation cohort consisted of 25 healthy Caucasian male volunteers. This group was part of a previously reported study[17] in which healthy controls were matched to patients with coronary artery disease (CAD). Healthy volunteers were eligible for participation if they were between the age of 35 and 65 years and did not have a personal or family history of cardiovascular disease (CVD). They did not use any medication. Inclusion took place between December 2009 and June 2010 and between November 2012 and April 2013..

Serum

The serum validation cohort consisted of 24 patients that underwent percutaneous trans-luminal coronary angioplasty because of existing coronary artery disease. Patients were between 35 and 70 years old. Samples were collected between January 2001 and December 2006. All three cohorts were included in accordance with the Declaration of Helsinki. The Medical Ethical Committee of the AMC in Amsterdam approved the study protocol and written informed consent was obtained from all participants.

For each sample type we performed RT-qPCR of the particular normalisation panel, miR-16, RNU6B and a selected miRNA that was highly expressed in that particular sample type, to function as reference miRNA. The reference miRNA was used to compare the expression after normalisation between the different normalisation methods. In a precision analysis all experiments should be performed twice to be able to assess the similarity of the results. Therefore, all RT-qPCR measurements were performed twice and per sample type. We then analysed the precision of the first run compared to the second run of our specific miRNA normalised for either the normalisation panel, miR-16 of RNU6B. The specific miRNA was normalised using the geometric mean of the miRNAs included in the normalisation panel. Precision was determined by calculating the correlation between the first and the second RT-qPCR run for each normalisation method using Spearman's rank test.

Statistical analyses

For each sample type (whole blood, platelets and serum) the value of the normalisation panel was determined by calculating the geometric mean of the expression levels of the miRNAs in the normalisation panel. The value of the miRNA of interest was subsequently divided by the value of the normalisation panel.

Spearman's rank test was used to calculate correlations in the validation studies. All analyses were performed using SPSS for Windows 19.0 and the statistical software package R.

RESULTS

Panel discovery phase

Whole blood normalisation panel

Published studies reporting miRNA microarray experiments on whole blood samples were included until April 2013. In total four microarray experiments, consisting of 183 healthy controls, met the inclusion criteria and were included in this study[18–21]. Detailed information on the included studies can be found in Table 1.

In silico analysis revealed that 5 miRNAs met the selection criteria for candidate normalisation miRNAs in all four microarray experiments. An additional 9 miRNAs met the inclusion criteria in three out of four microarray experiments, performed using at least two different platforms (Table 1). Seven out of 14 candidate normalisation miRNAs were detectable by RT-qPCR. It is commonly observed that microarray and qPCR result in disagreement, and it is well documented that both qPCR and microarray analysis have inherent pitfalls that may significantly influence the data obtained from each method[22]. In our case, we could validate the expression of 7/14 candidates for whole blood by PCR, 9/13 candidates for platelets, and 8/10 candidates for serum. Reasons for this discrepancy are most likely technical. For example, miRNA detection in microarrays may be less specific than when using Taqman PCR system, which relies on miRNA specific cDNA synthesis in combination with a miRNA specific detection system, while microarrays only rely on probe detection. This means that miRNA families with similar but not identical sequence may cross-hybridize in the array, but not in the PCR. Other causes of this discrepancy may relate to differences in sample collection and processing between the different labs. Stability of expression of the candidate normalisation miRNAs was further evaluated by Normfinder and geNorm software. Normfinder selected miR-130b as the most stable miRNA with a stability value of 0.108. MiR-130b and miR-342-3p were selected as the best combination of miRNAs with a stability value of 0.085. The geNorm analysis confirmed that miR-130b was most stable (Supplemental Figure 1A). No combination of reference miRNAs reached a V-value<0.15 (Supplemental Figure 1B). Therefore, we selected the combination of miRNAs with the lowest V-value. This panel consisted of miR-130b and miR-342-3p and had a V-value of 0.20. Since geNorm and Normfinder gave the same results the final normalisation panel consisted of miR-130b and miR-342-3p (Table 2).

Table 1 Overview of included studies

Author	GSE	Disease	Controls (n)	Platform	Probes	Selected miRNAs (n)	Selected candidates in the majority of studies
Whole blood							
Cox et al.(18)	21079	multiple sclerosis	37	Illumina Human v1 beadchip	733	56	
Keller et al.(19)	31568	miRNOme profiling	70	Febit Homo Sapiens miRBAse 13.0	863	176	
Keller et al. unpublished(21)	24709	lung disease	19	Febit Homo Sapiens miRBAse 13.0	863	187	
Schrauder et al.(20)	31309	breast cancer	57	Febit Homo Sapiens miRBAse 15.0	1100	230	
Total			183		448	14	miR-101, **miR-130b, miR-148a, miR-15a,** *miR-182*, miR-23b, miR-24, *miR-29b*, **miR-324-3p,** miR-324-5p, **miR-342-3p, miR-574-3p,** miR-590-5p, **miR-652**
Platelets							
Duttagupta(23)	32273	ulcerative colitis	22	Affymetrix	847	210	
Xu et al.(24)	39046	thrombocytemia	30	Agilent-021827 Human miRNA Microarray V3	392*	106	
Jain et al.(25)	41574	sickle cell disease	17	Agilent-021827 Human miRNA Microarray V3	939	298	

Table 1 Continued

Author	GSE	Disease	Controls (n)	Platform	Probes	Selected miRNAs (n)	Selected candidates in the majority of studies
Kok et al. unpublished(43)	59421	coronary artery disease	37	Agilent-021827 Human miRNA Microarray V3	961	25	
Total			106		377	13	*miR-106b*, **miR-140-5p, miR-148b, miR-151-3p, miR-18a,** miR-20b, *miR-25*, miR-93
Serum							
McCann et al.(26)	27474		14	Illumina Human v2 MicroRNA expression beadchip	1145	36	
Murakami et al.(27)	33857	liver disease	12	Agilent-029297 Human miRNA Microarray v14	263[#]	36	
Godfrey et al.(28)	44281	breast cancer	205	Affymetrix Multispecies miRNA-2_0 Array	2226	324	
Total			231		230	10	**miR-1228, miR-1238, miR-1260, miR-1280,** *miR-1825*, miR-193a-5p, *miR-197*, **miR-324-3p, miR-484, miR-718**

Detailed overview of the included studies. The total number of probes represents the number of probes overlap in all microarray experiments. In the last column the miRNAs indicated in bold were included in the analysis. MiRNAs indicated in italic have been reported to be involved in any disease for the specific sample type. GSE; NCBI Gene Expression Omnibus accession number.

[*] Lowly expressed miRNAs were excluded in the data available on GEO

[#] After filtering out probes that were detected on less than three arrays.

Platelet normalisation panel

We included papers reporting microarray experiments on isolated platelets until April 2013. Three published microarray experiments met our inclusion criteria[23–25]. We also included data from an in-house miRNA microarray experiment on isolated platelets of patients with premature coronary artery disease and healthy controls (GSE59421). In total we analysed data from 4 microarray experiments, consisting of 106 healthy controls. Detailed information on the included studies can be found in Table 1.

Thirteen miRNAs met the selection criteria of the in silico analysis in all 4 included experiments. Since this was a sufficient number of candidates for further analysis, we did not select any additional miRNAs that met the selection criteria in 3 microarray experiments. Nine out of 13 candidate normalisation miRNAs were detectable by RT-qPCR.

Normfinder selected miR-148b as the most stable reference miRNA with a stability value of 0.086. MiR-148b and miR-18a were selected as the best combination of miRNAs with a stability value of 0.064. GeNorm analysis revealed that miR-151-3p was the most stable reference miRNA (Supplemental Figure 2A). The lowest V value was obtained for a combination of 6 miRNAs (miR-151-3p, miR-28-5p, miR-331-3p, miR-29c, miR-148b and miR-18a; Supplemental Figure 2B), including the two miRNAs selected by Normfinder. Therefore, we chose the combination of these 6 miRNAs as normalisation panel (Table 2).

Table 2 Overview of the normalisation panels

Specimen	Whole blood	Platelets	Serum
Included studies	4	4	3
Selected miRNAs in all included studies	5	13	1
Selected from all studies minus one	9	-	9
Total number of selected miRNAs	14	13	10
Number of miRNAs in RT-qPCR experiments	7	9	8
miRNAs selected by Normfinder	miR-130b; miR-342-3p	miR-148b; miR-18a	miR-1260; miR-1280
miRNAs selected by geNorm	miR-130b; miR-342-3p	miR-148b; miR-151-3p; miR-18a; miR-28-5p; miR-29c; miR-331-3p	miR-1260; miR-1280; miR-484; miR-718
Final normalisation panel	miR-130b; miR-342-3p	miR-148b; miR-151-3p; miR-18a; miR-28-5p; miR-29c; miR-331-3p	miR-1260; miR-1280; miR-484; miR-718

Detailed overview of the different steps of the establishment of the normalisation panels.

Serum normalisation panel

Papers reporting microarray experiments in serum samples were included until April 2013. In total, three published microarray experiments, consisting of 231 healthy controls, met our inclusion criteria[26–28]. Detailed information on the included studies can be found in Table 1.

In silico analysis revealed that 1 miRNA (miR-197) met the selection criteria for candidate miRNAs in all 3 microarray experiments. Additionally, 9 candidate miRNAs met the inclusion criteria in 2 out of 3 microarray experiments on 2 different platforms. Eight out of 10 candidate normalisation miRNAs were detectable by RT-qPCR..

MiR-1280 was selected by Normfinder as the most stable candidate miRNA, with a stability value of 0.121. The best combination of candidate miRNAs consisted of miR-1280 and miR-1260 and had a stability value of 0.089. Subsequently, geNorm showed miR-1260 was the most stable candidate miRNA. A combination of 4 candidate miRNAs reached a V-value<0.15 (Supplemental Figure 3). Thus, the final normalisation panel consisted of miR-1260, miR-1280, miR-718 and miR-484, including the two miRNAs selected by Normfinder (Table 2).

Panel validation phase

The precision of our normalisation panels was tested via replicate measurements of selected reference miRNAs in an independent cohort for each sample type.

The reference miRNA for whole blood and serum (miR-494 and miR-223 respectively) was selected from in-house microarray experiments. For platelets we selected miR-19b-1-5p, which was identified in a previously conducted study (GSE59421).

For whole blood, there was a higher correlation of reference miR-494 expression between the first and second RT-qPCR run after normalising for the whole blood normalisation panel compared to normalisation for miR-16 (Figure 1, Table 3).

Table 3 Overview of the precision analyses results

Specimen	Whole blood		Platelets		Serum	
	Rho (ρ)	p-value	Rho (ρ)	p-value	Rho (ρ)	p-value
Normalisation for panel	0.68	<0.001	0.91	<0.001	0.98	<0.001
Normalisation for miR-16	0.27	0.01	-0.29	0.28	0.96	<0.001
Normalisation for RNU6B	na	na	na	na	na	na

Detailed overview of the precision analyses results. For each sample type the correlation between two consecutive RT-qPCR runs was calculated after normalising for the normalisation panel and after normalising for miR-16.

Figure 1A

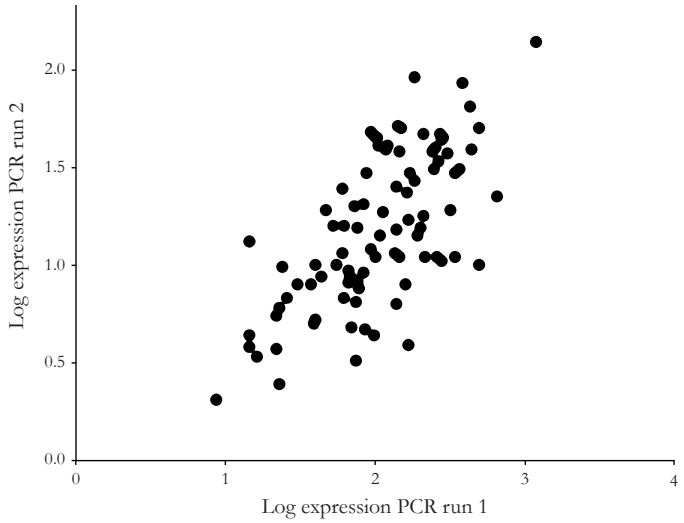


Figure 1B

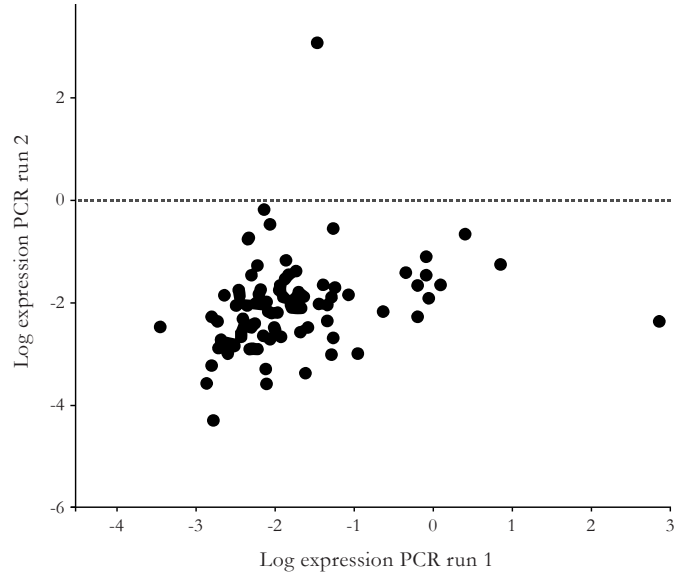


Figure 1 Comparison of the precision of the whole blood normalisation panel and miR-16. **(A)** Comparison of two replicate RT-qPCR runs of miR-494 normalised for our whole blood normalisation panel shows a significant correlation of ρ=0.68. **(B)** Precision of normalisation for miR-16 shows worse results with a correlation of ρ=0.27.

In isolated platelets we observed similar results. There was a higher correlation of reference miR-19b-1-5p expression between the first and the second run after normalisation for the platelet normalisation panel compared to miR-16 (Figure 2, Table 3). Finally, for serum the correlation of reference miR-223 expression between the first and the second RT-qPCR run after normalisation for the serum normalisation panel compared to normalisation for miR-16 were similar (Figure 3, Table 3). Unfortunately, RNU6B could not be detected by RT-qPCR in approximately half of the samples in all sample types.

We recently have performed miRNA microarray experiments on isolated platelets. We measured the expression levels of platelet miRNAs and found a biomarker (e.g. miR-19b-1-5p) for aspirin sensitivity. With the expression data of 25 healthy volunteers, retrieved from this aspirin experiment, we were able to perform accuracy measures only for the platelet normalisation panel. We used data of isolated platelets of 25 healthy volunteers. To assess the accuracy of the platelet normalisation panel we performed RT-qPCR on miR-19b-1-5p, the platelet normalisation panel, miR-16 and RNU6B. Subsequently, we analysed the correlation between miR-19b-1-5p expression and platelet aggregation in both the microarray experiment and the RT-qPCR experiment using all the 3 different normalisation methods (normalisation panel, miR-16, RNU6B) to evaluate which normalisation method used to normalise the RT-qPCR experiment best resembled the correlation as seen in the microarray experiment. Without normalising the data, the observation could not be validated by RT-qPCR (ρ=0.01, p=0.97) (Figure 4A). After normalising for the platelet normalisation panel the correlation observed on the microarray data could be confirmed by RT-qPCR (ρ=0.68, p<0.001) (Figure 4B). On the other hand, normalisation for miR-16 could not confirm the correlation between miR-19b-1-5p expression and platelet aggregation (ρ=-0.26, p=0.22) (Figure 4C). RNU6B could not be used to normalise miR-19b-1-5p expression, since amplification failed in the majority of the samples.

Unfortunately, there were no miRNA microarray experiments available for similar analyses for whole blood and serum, so that we were unable to assess the accuracy of our whole blood and serum normalisation panels.

Figure 2A

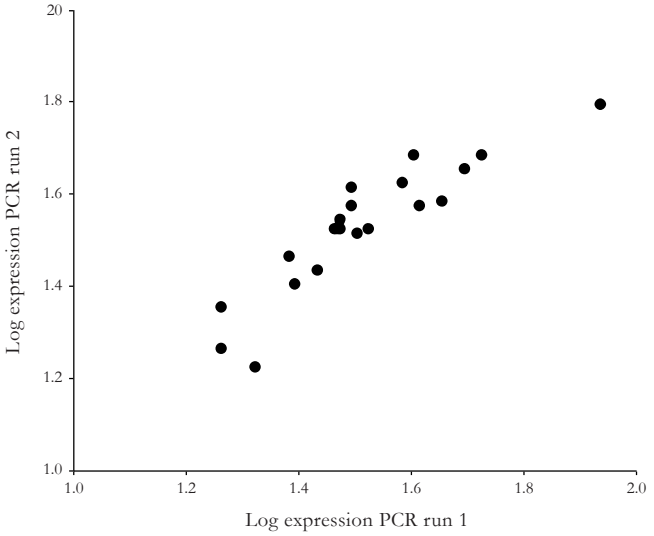


Figure 2B

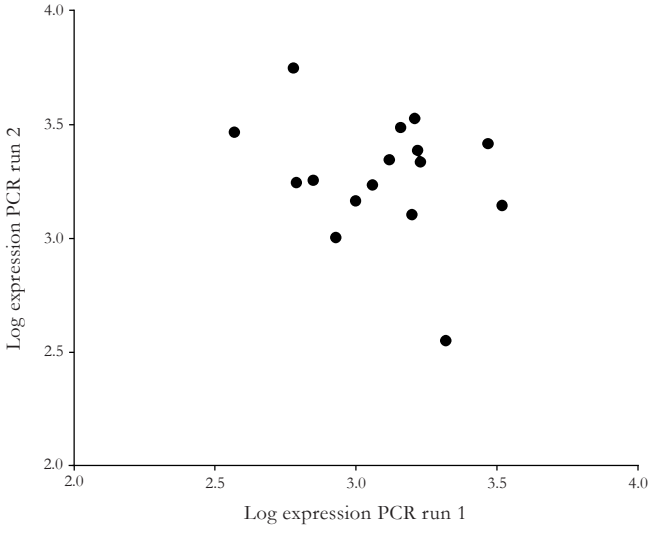


Figure 2 Comparison of the precision of the platelet normalisation panel and miR-16. **(A)** Comparison of two replicate RT-qPCR runs of miR-19b-1-5p normalised for our whole blood normalisation panel shows a significant correlation of ρ=0.91. **(B)** The precision of normalisation for miR-16 shows worse results with a correlation of ρ=-0.29.

Figure 3A

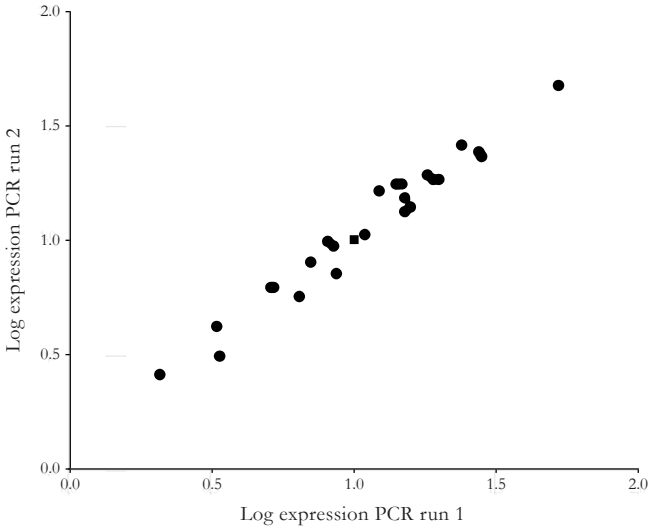


Figure 3B

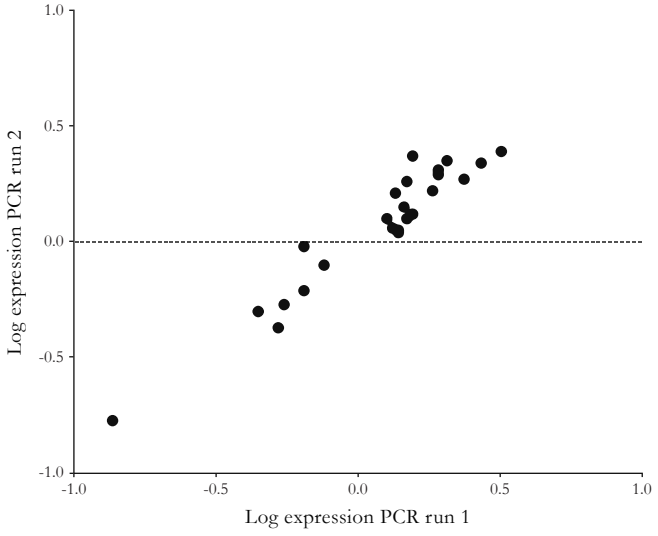


Figure 3 Comparison of the precision of the serum normalisation panel and miR-16.
(A) Comparison of two replicate RT-qPCR runs of miR-223 normalised for our serum normalisation panel shows a significant correlation of ρ=0.98. **(B)** The precision of normalisation for miR-16 shows worse results with a correlation of ρ=0.96.

Figure 4A

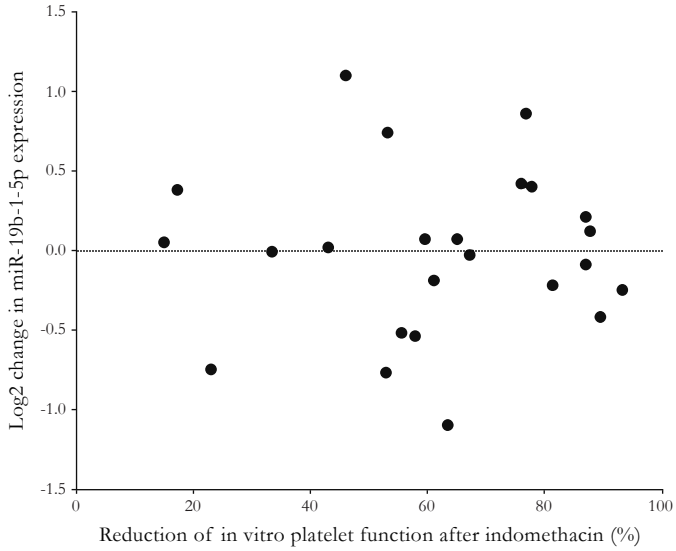


Figure 4B

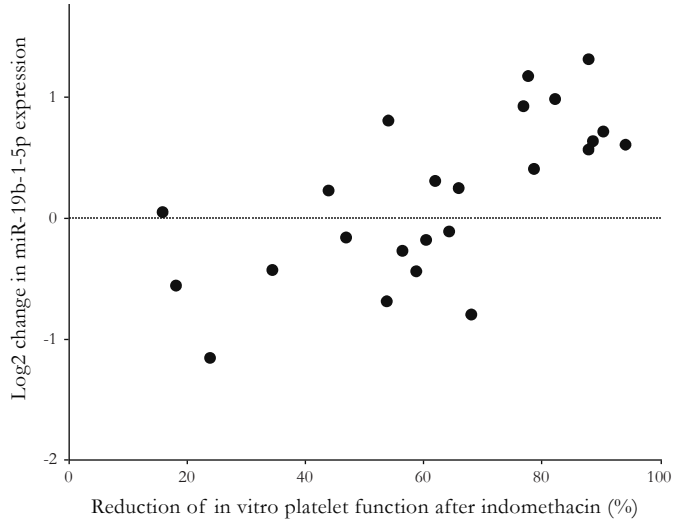


Figure 4C

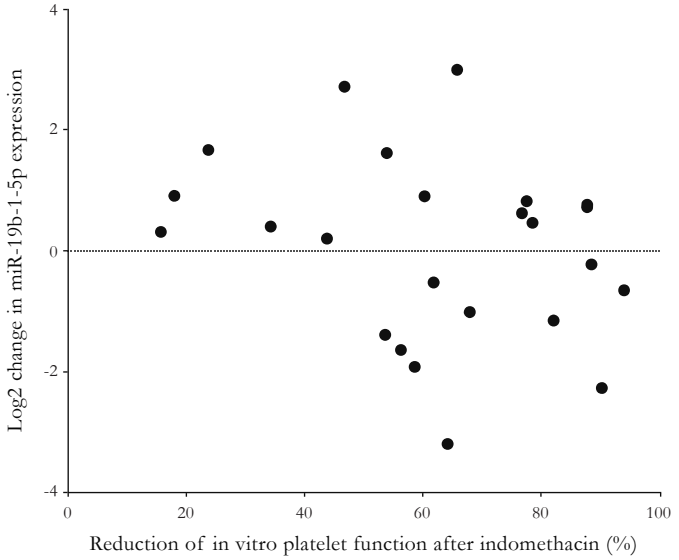


Figure 4 Accuracy analysis of the platelet normalisation panel.
A previous miRNA microarray experiment showed that the percentage reduction in platelet aggregation after indomethacin incubation is positively correlated with the aspirin-induced change in miR-19b-1-5p expression. **(A)** Using RT-qPCR without any normalisation, this could not be confirmed (ρ=0.01). **(B)** When RT-qPCR data was normalised for the platelet normalisation panel the correlation between the reduction in platelet aggregation and the change in miR-19b-1-5p expression could be confirmed (ρ=0.68). **(C)** Normalisation for miR-16 could not confirm the correlation between miR-19b-1-5p expression and platelet aggregation (ρ=-0.26, p=0.22).

DISCUSSION

In the current study we established miRNA normalisation panels for RT-qPCR experiments on whole blood, platelet and serum samples in cohorts of coronary artery disease patients. Candidate normalisation miRNAs with a stable expression were selected from miRNA microarray experiments included in GEO. The stability of these candidate normalisation miRNAs was further assessed on RT-qPCR data using the geNorm and Normfinder algorithms, resulting in a final normalisation panel for each sample type. In additional validation experiments in independent RT-qPCR cohorts, we showed that the precision of these normalisation panels was superior to the precision of normalising for miR-16 or RNU6B. Therefore, these normalisation panels clearly outperformed the strategies most frequently used for normalisation of miRNA RT-qPCR data. Additionally, we could show that normalisation for our platelet normalisation panel is also more accurate compared to normalisation for miR-16 or RNU6B.

The rapid increase in publications reporting the use of circulating miRNAs as biomarker has fuelled the debate on how to properly perform miRNA detection experiments. It is widely accepted that RT-qPCR is the technique of choice for quantification, because of its high specificity and accuracy. However, the accuracy of the test results is highly dependent on proper data normalisation[29,30], and the best method of data normalisation remains a point of discussion. The three most commonly used strategies for normalisation of miRNA RT-qPCR experiments are: normalisation to the geometric mean of all detected miRNAs, normalisation to a single endogenous control (RNU6b, miR-16), and the use of a spike-in. Normalisation to the geometric mean requires the quantification of a large unbiased set of miRNAs, whereas most validation experiments are only performed for a small set of miRNAs. Since we detected only on reference miRNA per sample type, this was not a suitable method to include in our precision analyses. The use of a synthetic spike-in has also been criticized in literature, because of several major drawbacks[31–33]. Although this method starts with an equal amount of spike-in for each sample, it only corrects for either extraction or reverse transcription efficiency, depending on the moment when the spike-in is added to the sample. As such, the use of a spike-in does not correct for remaining experimental variability, and has therefore been considered as a less reliable normalisation method[11]. Since there is consensus that this method should not be used as normalisation method, we did not include it in our study design. Normalisation to a single endogenous control miRNA seemingly overcomes these problems. Therefore, this normalisation method has been discussed in literature as one of the best normalisation methods. However, its reliability completely dependent on which miRNA is selected for normalisation. Initially, small RNAs such as RNU6B were often used, but there is increasing evidence of disease-related regulation of these molecules[34–36]. Furthermore, RNU6B has been reliably detected in serum, but the detection in the circulation is problematic[37] as was also shown in our experiments. Indeed, we show that the use of RNU6B for the normalisation of RT-qPCR data of *circulating* miRNAs is not an option, since RNU6B cannot reliably be detected in the circulation. Therefore, it has been suggested that other specific single miRNAs can be used for normalisation, but these miRNAs were often selected from a single cohort, which could very well have introduced selection bias[38,39]. Thus, this normalisation method cannot be applied to the general population. To overcome this problem we selected data on healthy controls from multiple microarray experiments including many different cohorts. Furthermore, we used a normalisation panel instead of a single normalisation miRNA, which has also been shown to result in superior normalisation in mRNA expression experiments. Exclusion of candidate miRNAs that have been reported to be regulated in any disease and the use of the geNorm and Normfinder algorithms resulted in reliable panels of stably expressed normalisation miRNAs. However, the normalization panels were only tested in a cohort of subject with cardiovascular disease and healthy controls. Since the origin is not known for all miRNAs, it cannot be ruled out that some panel miRNAs might be regulated in other diseases. Therefore, our robust normalisation panels for *circulating* miRNAs should provide more adequate results on miRNA expression levels for studies on cardiovascular disease.

Strengths and Limitations

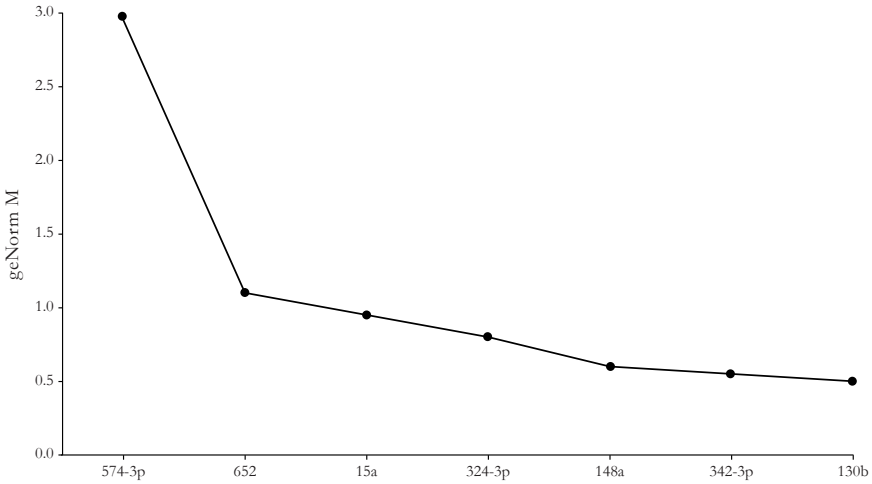
MiR-16 is one of the commonly used single miRNAs for normalisation. In our precision analyses, we showed that normalisation of serum RT-qPCR experiments for miR16 showed similar results as normalisation for the newly constructed normalisation panel. However, in the analysis of the microarray data during the serum panel discovery phase the expression stability of all detected miRNAs, including miR-16 have been evaluated. In these microarray studies the expression of miR-16 did not meet our selection criteria and was therefore not further evaluated within the panel. Furthermore, we excluded all miRNAs which were known to be differentially expressed in any disease. Li et al.[40] recently reported circulating miR-16 as a biomarker for intracranial aneurysms. In addition, this miRNA has been reported to be involved in hepatocellular carcinoma[41] and rheumatoid arthritis[42]. Therefore, the expression of miR-16 cannot be considered to be universally stable. By inclusion of this miRNA in the normalisation panel one would risk ending up with false results due to regulation of (a part of) the normalisation panel. Furthermore, inclusion of miR-16 in the serum normalization did not result in a better performance of this normalization panel. We therefore, chose not to include miR-16 in our normalisation panel. miR-16 was only chosen as a comparative normalization method, since it is often used in literature.

Unfortunately, no accuracy data was available for the whole blood and serum normalisation panels, since no suitable data sets were available. Our platelet normalisation panel showed superior accuracy compared with frequently reported normalisation methods. This underlines the strength of our selection method, in which consecutive steps were used to construct the final normalisation panels. At first, we used all available cohort data in GEO to construct a preliminary panel. Secondly, we tested this preliminary panel in our own cohort and used established algorithms to extract the most stable miRNAs for the final normalisation panels. Finally, we tested the precision of all our panels in a second independent cohort. All our normalisation panels showed superior performance compared to frequently used normalisation methods.

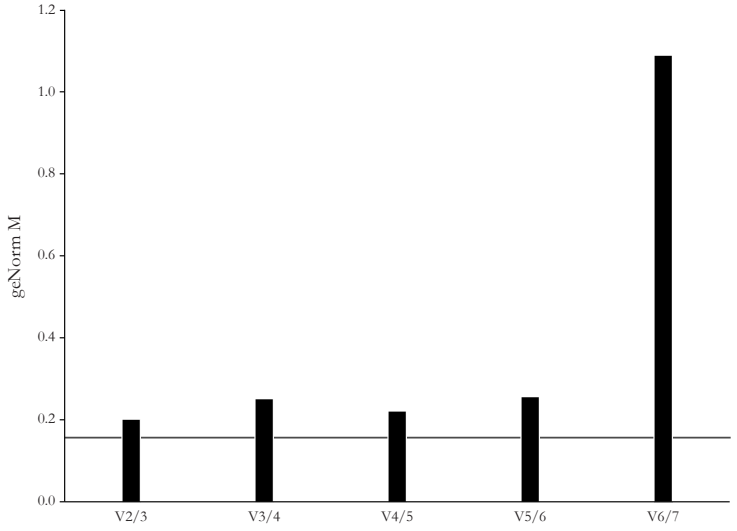
Conclusion

In conclusion, using a standardised method we have constructed reliable normalisation panels for miRNA RT-qPCR experiments in whole blood, platelets and serum in cohorts of CAD patients, which showed superior precision compared to other normalisation methods and should therefore be preferred over other normalization methods in studies on cardiovascular disease.

Supplemental Figure 1A

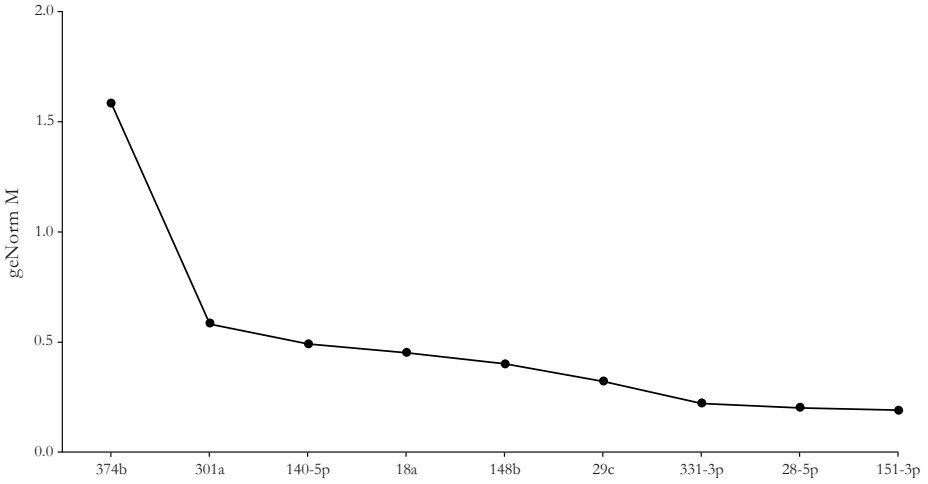


Supplemental Figure 1B

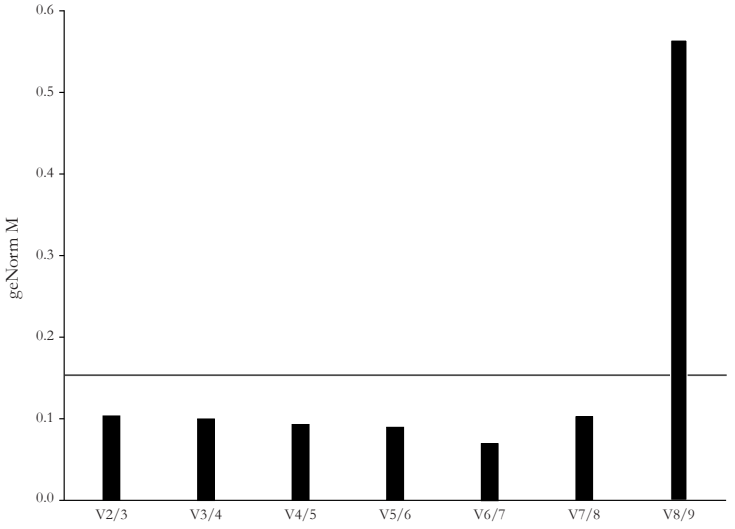


Supplemental Figure 1 geNorm analysis of candidate normalization miRNAs in whole blood **(A)** Ranking of candidate normalization miRNAs according to average expression stability. In a stepwise manner, the least stable miRNAs with the highest M values were excluded until miR-130b and miR-342-3p remained. **(B)** Determination of the optimal number of normalization miRNAs. The optimal normalization panel consists of the number of miRNAs with a V value<0.15 . In this case the optimal V value is achieved when using 2 normalization miRNAs.

Supplemental Figure 2A

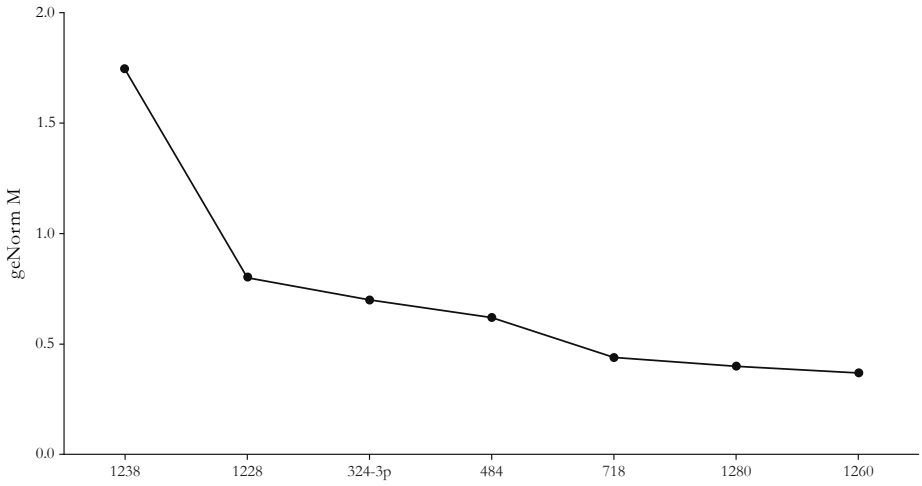


Supplemental Figure 2B

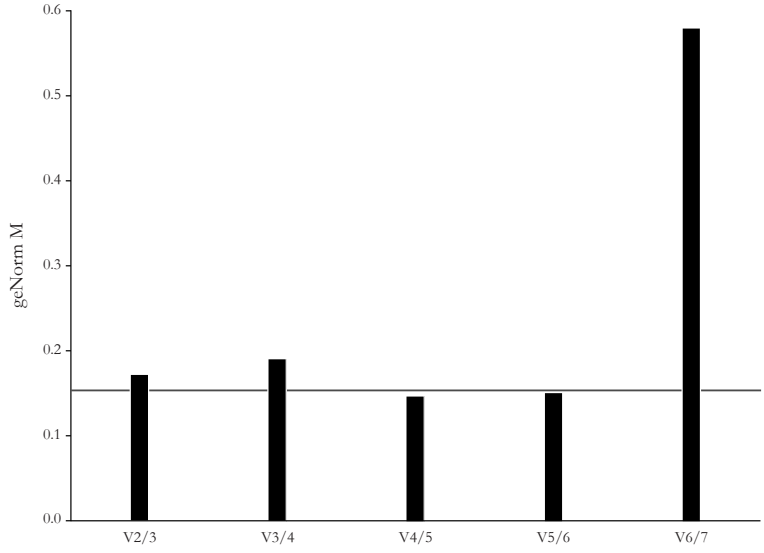


Supplemental Figure 2 geNorm analysis of candidate normalization miRNAs in platelets
(A) Ranking of candidate normalization miRNAs according to average expression stability. In a stepwise manner, the least stable miRNAs with the highest M values were excluded until miR-151-3p, miR-28-5p, miR-331-3p, miR-29c, miR-148b and miR-18a remained. **(B)** Determination of the optimal number of normalization miRNAs. The optimal normalization panel consists of the number of miRNAs with a V-value<0.15. Since in this case geNorm and Normfinder select different normalisation panels we choose to select the panel with the lowest V-value, which corresponds with the Normfinder results.

Supplemental Figure 3A



Supplemental Figure 3B



Supplemental Figure 3 geNorm analysis of candidate normalization miRNAs in serum
(A) Ranking of candidate normalization miRNAs according to average expression stability. In a stepwise manner, the least stable miRNAs with the highest M values were excluded until miR-1260, miR-1280, miR-718 and miR-484 remained. (B) Determination of the optimal number of normalization miRNAs. The optimal normalization panel consists of the number of miRNAs with a V-value<0.15. In this case the optimal V value is achieved when using 4 normalization miRNAs.

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CHAPTER 3

Low miR-19b-1-5p expression in isolated platelets after aspirin use is related to aspirin insensitivity

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ABSTRACT

Worldwide, aspirin is the most commonly prescribed platelet inhibitor after a cardiovascular event. Many patients, however, suffer from re-events that are thought to be due to platelet insensitivity to aspirin. The aim of this study was to investigate whether platelet microRNAs (miRNAs) could be used as a suitable marker for aspirin insensitivity.

We included 15 healthy men between the age of 35 and 65 years and determined miRNA microarray expression profiles in isolated platelets before and after 2 weeks of aspirin use. MiRNA expression levels were compared with in vitro platelet function measured in the same individuals. Aspirin-induced changes in expression of six miRNAs in vivo correlated strongly with the reduction in platelet aggregation after indomethacin incubation as an in vitro measure for aspirin insensitivity. This finding was validated by qPCR in an extended cohort of 25 healthy individuals, which showed that a low miR-19b-1-5p expression was significant correlated with a sustained platelet aggregation in the presence of aspirin ($\rho = 0.68$; $p<0.001$).

In healthy volunteers lower expression of miR-19b-1-5p after aspirin use is associated with the insensitivity of platelets for aspirin. It remains to be established if miR-19b-1-5p expression is a suitable marker to identify patients at risk for re-events.

INTRODUCTION

Aspirin is the most commonly prescribed platelet inhibitor for secondary prevention after a cardiovascular event. A large meta-analysis including > 200,000 patients at high risk of developing cardiovascular events showed that long-term treatment with aspirin significantly reduced the risk of myocardial infarction (MI), stroke and vascular death[1]. However, 10 to 20% of treated patients develop recurrent vascular events[2,3]. It has been postulated that the relatively high incidence of recurrent events is due to insensitivity to aspirin treatment[4]. Indeed, it is known that there are large inter-individual differences in aspirin response[5] and it has been reported that in some individuals platelet function is not decreased by aspirin treatment[6]. Being able to predict which patients respond insufficiently to aspirin therapy and therefore are more prone to develop recurrent events, would be an important step in ongoing efforts to develop more personalized secondary prevention schemes.

Platelets harbour a variety of miRNAs[7]. Activated platelets release miRNAs from intracellular platelet stores[8] and consequently specific miRNAs are up or down regulated in thrombin stimulated platelets[9]. Since aspirin treatment inhibits platelet activation, we hypothesized that platelet miRNA expression profiles after aspirin use differ among healthy individuals and that this resembles differences in platelet aggregation.

METHODS

Microarray cohort

The miRNA microarray experiments were performed in a cohort of 15 healthy Caucasian male volunteers. These healthy volunteers were eligible for participation if they were between the age of 35 and 65 years, did not have a personal or family history of cardiovascular disease and did not use any medication. Inclusion took place between December 2009 and June 2010.

To study the effect of medication on miRNA expression levels, we administered 100 mg of acetyl salicylic acid, once daily, for two weeks. Since this cohort also served as a control group for subjects with CAD, all subjects were also asked to use simvastatin 40 mg, once daily, for 6 weeks, of which the last 2 weeks in combination with the acetyl salicylic acid. Blood samples were collected at baseline in the absence of aspirin and statins and after six weeks of medication use.

PCR cohort

The PCR cohort consisted of the 25 healthy volunteers. Fifteen from the microarray cohort and 10 additional healthy volunteers. Additional participants were selected in a similar manner as the controls in the microarray cohort, using identical inclusion and exclusion criteria. The subjects were also treated with simvastatin 40 mg, once daily, for 6 weeks, of which the last 2 weeks in combination with the administration of acetyl salicylic acid. Inclusion took place between November 2012 and April 2013.

Ethics statement

This study was performed in accordance with the Declaration of Helsinki. The Medical Ethical Committee of the AMC in Amsterdam approved the study protocol and written informed consent was obtained from all participants.

Peripheral blood collection

Venous blood samples were drawn without stasis, using an open system with a 19-gauge needle. Blood samples for platelet isolation were collected in trisodium citrate (each 5 ml containing 0.5 ml 0.105M trisodium citrate). The first sample was discarded. Blood samples for platelet aggregation test were collected in citrate tubes. Samples for the serum thromboxane B2 assay were collected in glass serum tubes.

Platelet isolation

Platelets were isolated as described previously[10]. In short, immediately after withdrawal the samples were centrifuged (180g, 15 min, room temperature) to obtain platelet-rich plasma (PRP). The upper layer of PRP was transferred to a plastic tube to avoid leukocyte contamination. One part of acid–citrate–dextrose (ACD) buffer (0.085 M trisodium citrate, 0.11 M glucose, 0.071 M citric acid) was added to five parts of PRP and then the PRP was centrifuged (800 g, 20 min, room temperature). The platelet-poor plasma was discarded and the platelet pellet carefully resuspended in Tyrode buffer (136.9 mM NaCl, 2.61 mM KCl, 11.9 mM NaHCO3, 5.55 mM Glucose, 2 mM EDTA, pH 6.5). The platelet suspension was centrifuged (800 g, 20 min, room temperature). The supernatant was discarded and the platelet pellet was resuspended in 50 ml sterile phosphate buffered saline (PBS) and stored at -80ºC prior to RNA isolation. The isolated platelets were investigated by fluorescence-activated cell sorting (FACS) using monoclonal antibodies against CD45 (BD Biosciences, Franklin Lakes, NJ, USA), CD235a (DAKO) and CD61 (BD Biosciences, Franklin Lakes, NJ, USA) to identify leukocytes, erythrocytes and platelets. The purity of the isolated platelets was 99.72% by FACS analysis.

RNA isolation

We isolated platelet RNA using the mirVana PARIS kit (Ambion Inc., Foster City, CA, USA), according to the manufacturer's protocol for liquid samples. The protocol was modified such that samples were extracted twice with an equal volume of acid-phenol chloroform.

MiRNA microarray

The integrity of total RNA including microRNAs from platelets was investigated with the BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) using the RNA 6000 Pico kit (Agilent Technologies, Santa Clara, CA USA) and Small RNA kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions.

100 ng of total RNA including microRNAs was dried down in a Centrivap concentrator (Labconco, Kansas City, MO, USA) and dissolved in 2 µl RNase-free water. Sample labeling with Cy3 was performed as described in the miRNA Microarray System with miRNA Complete Labeling and Hyb Kit manual version 2.2 (Agilent Technologies, Santa Clara, CA, USA) with

the inclusion of spike-ins and the optional desalting step with spin columns (Micro Bio-Spin 6, Bio-Rad, Herculer, CA, USA). Labeled samples were hybridized on Human 8x15k miRNA microarrays based on Sanger miRBase release 12.0 containing 866 human and 89 human viral miRNAs (G4470C, Agilent Technologies, Santa Clara, CA, USA) at 55°C and 20 rpm for 20 hours. After washing, the arrays were scanned using the Agilent DNA microarray scanner (G2565CA, Agilent Technologies, Santa Clara, CA, USA). Data was extracted with Feature Extraction software (v10.7.3.1, Agilent Technologies, Santa Clara, CA, USA) with the miRNA_107_Sep09 protocol for miRNA microarrays.

miRNA microarray pre-processing and analysis

A two-step normalisation approach was taken. In the first step, we corrected for systematic technical effects in the raw probe-level data as extracted via the Agilent Feature Extraction software. For this purpose, we fitted a linear mixed-effects model with coefficients for three technical effects (hybridisation block, slide, and slide position, that is, upper or lower half), and patient status using the R/MAANOVA package. Residuals after correcting for the three technical effects were further pre-processed and summarized using a modified version of the robust multi-array average (RMA) method with background correction, as implemented in the AgiMicroRna R package. This pre-processing method has been shown to have better precision than the pre-processing method recommended by Agilent[11]. Quality control was performed using the arrayQualityMetrics R package. Based on arrayQualityMetrics outlier detection and visual inspection of heatmaps, MA-plots, and intensity distributions, 11 arrays were excluded from further analysis. 2 control subjects after medication were excluded because of missing microarray data. Data from the remaining arrays (33 CAD, 37 control without medication and 24 control after medication) were renormalized using the two-stage procedure describing above. Only non-control miRNAs detected on at least one array according to Agilent Feature Extraction software were included in the further analysis. To find miRNAs differentially expressed between healthy controls before and after medication, we employed a paired moderated t-test using the limma R package. Resulting p-values were adjusted to correct for multiple hypothesis testing using the Benjamin-Hochberg false discovery rate. Expression data have been deposited in NCBI Gene Expression Omnibus in a MIAME compliant format and are accessible under GEO Series accession number GSE59421.

qPCR

qPCR was performed with RNA of isolated platelets as previously described[12]. A fixed volume of 8 µl of total RNA was used as input in the reverse transcription reaction. Input RNA was reverse transcribed using the miScript reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The real-time qPCR was performed using High Resolution Melting Master (Roche, Basel, Switzerland). MgCl2 was used in final concentration of 2.5mmol/L and 2 µl of 8 times diluted cDNA was used in a total volume of 10 µl. The forward primers had the same sequence as the mature miRNA sequence with all U's changed into T's. The reverse sequence was GAATCGAGCACCAGTTACGC, which is complementary to the adapter sequence of the RT-primer used to create cDNA. qPCR reactions were performed on a LightCycler480 system

II (Roche, Basel, Switzerland). The candidate miRNAs were normalized to the geometric mean of a previously established miRNA normalization panel for platelet samples consisting of miR-151-3p, miR-28-5p, miR-148b and miR-18a. These normalization miRNAs were selected from independent microarray experiments and were further validated on PCR data using the geNorm and Normfinder algorithms. The original platelet normalization panel consists of 6 miRNAs selected by both algorithms. However, since we did not have enough material to perform PCR on all 6 miRNAs we choose to select the 2 miRNAs that were considered the best normalization panel by the geNorm algorithm and the 2 miRNAs that were selected by Normfinder. Data were analyzed using LinRegPCR quantitative PCR data analysis software, version 11.3[13].

Multiple electrode aggregometry

We assessed platelet function at baseline, using the Multiplate® Analyzer (Roche, Basel, Switzerland) in the absence of both aspirin and statin use according to the manufacturer's instructions. Arachidonic acid is commonly used to analyse the effect of aspirin on platelet aggregation, since it initiates platelet aggregation through the thromboxane pathway. However, in our hands adenosine diphosphate (ADP) most sensitively initiated platelet aggregation in this assay. ADP initiates platelet aggregation through activation of the ADP-receptor, which is not inhibited by aspirin. Activation of the ADP-receptor eventually results in activation of the thromboxane pathway, and therefore aggregation initiated through the thromboxane pathway contributes to the total aggregation measured after initiation by ADP. Since we compared platelet aggregation in the same samples in the presence and absence of indomethacin and did not change any other condition, we could analyse the degree of inhibition of platelet aggregation due to indomethacin inhibition. In short, 300 µl whole blood was diluted with 300 µl 0.9% saline and stirred for 3 minutes at 37 ºC. ADP was added in a final concentration of 2.5 µmol/L to initiate platelet aggregation. Aggregation was measured for 6 minutes and was reported in arbitrary aggregation units plotted against time. Also, the area under the aggregation curve (AUC) was measured. All samples were measured at baseline, before medication use. We measured platelet aggregation in the absence and presence of 200 µmol/L indomethacin (20 min incubation with blood) to mimic the effect of aspirin use. Indomethacin was used instead of aspirin, since aspirin is not soluble in water and needs to be metabolised before it exerts its effect on platelets. Indomethacin on the other hand, is soluble, and causes a direct and irreversible inhibition on cyclo-oxygenase. We calculated the percentage reduction in AUC after incubation with indomethacin as an in vitro measure of the effect of aspirin use on whole blood platelet aggregation.

Serum thromboxane B2 assay

Serum thromboxane B2 (TBX2) was measured at baseline and after 2 weeks of aspirin use to check compliance to therapy. TBX2 was measured in duplicate by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. We calculated the TBX2 concentration by performing a logistic four-parameter fit of the standard

concentrations versus the ratio of absorbance of a particular sample to that of the maximum binding sample. The percentage reduction of serum TXB2 after aspirin use was used to analyse the compliance to aspirin therapy.

Statistical analyses

Student's t-tests and Chi-square tests were used to test for differences in baseline characteristics between the microarray cohort and the 10 additional subjects in the PCR cohort.

Normalized miRNA expression levels from both the microarray and the PCR experiment were log-transformed. Changes in miRNA expression were calculated by subtracting the (log-transformed) expression level of a specific miRNA before aspirin use from its expression level after aspirin use. Spearman's rank correlation of these aspirin-induced expression changes with the percentage of AUC reduction after indomethacin incubation was calculated for both the microarray and the PCR experiment. All analyses were performed using SPSS for Windows 19.0 and the statistical software package R. A p-value < 0.05 (Bonferroni corrected in case of multiple testing) was considered statistically significant.

RESULTS

Microarray cohort

Clinical characteristics of the microarray cohort are reported in Table 1. Subjects were on average 51 years old. 27% of the participants were smokers. Furthermore, they had a normal blood pressure and no cholesterol or glucose abnormalities.

PCR cohort

Clinical characteristics of the PCR cohort are also reported in Table 1. The clinical characteristics of the Microarray cohort and the PCR cohort did not differ.

MiRNA profiling

In total 468 miRNAs were detected in at least one platelet sample.

When analysing miRNA expression profiles before and after aspirin use in the complete cohort only few miRNAs were significantly differentially expressed with modest fold changes at best. However, investigating the medication-induced changes in expression across all detected miRNAs for each individual separately revealed that some individuals showed marked changes in miRNA profiles after medication use, whereas others did not (Figure 1).

This observation suggests that some individuals handle aspirin different than others. Since aspirin has a direct effect on platelet function, we hypothesized that the observed inter-individual heterogeneity of the changes in miRNA expression after medication use were due to a difference in response to aspirin. We invited the subjects of the microarray cohort for a second visit, during

Table 1 Baseline characteristics

	Microarray cohort	PCR cohort
n	15	25
Age, years ± SD	51.4 ± 4.6	53.1 ± 4.6
Gender, male n (%)	15 (100)	25 (100)
Smoking, n (%)	4 (27)	6 (24)
Hypercholesterolemia, n (%)	0	0
Hypertension, n (%)	0	0
Diabetes, n (%)	0	0
BMI, kg/m2± SD	25.5 ± 2.5	25.7 ± 2.7
Systolic blood pressure, mmHg ± SD	128 ± 13	128 ± 12
Diastolic blood pressure, mmHg ± SD	84 ± 8	84 ± 7
Glucose, mmol/L ± SD	5.3 ± 0.6	5.4 ± 0.6
Total cholesterol, mmol/L ± SD	5.4 ± 0.7	5.4 ± 0.7
HDL cholesterol, mmol/L ± SD	1.4 ± 0.4	1.4 ± 0.4
LDL cholesterol, mmol/L ± SD	3.6 ± 0.8	3.5 ± 0.8
Triglycerides, mmol/L ± SD	1.0 ± 0.5	1.2 ± 0.7

Continuous data are expressed as mean ± SD, categorical data as absolute number with (percentages). BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein, n, number; SD, standard deviation.

Table 2 Overview of the microarray results.

miRNA	Medication-induced change in expression		Expression after medication use	
	Correlation	P-value	Correlation	P-value
miR-1225-3p	0.796	0.001	0.618	0.016
miR-1271	0.682	0.007	0.893	< 0.001
miR-1537-5p	0.525	0.047	0.539	0.041
miR-19b-1-5p	0.743	0.002	0.689	0.006
miR-548e	0.657	0.010	0.611	0.018
miR-587	0.593	0.022	0.796	0.001

Overview of the miRNAs that showed a strong correlation (Spearman correlation, nominal p-value<0.05) between the reduction in platelet aggregation after indomethacin incubation in vitro and both the medication-induced change in expression (lefthand side) and the expression after medication (righthand side).

Figure 1A

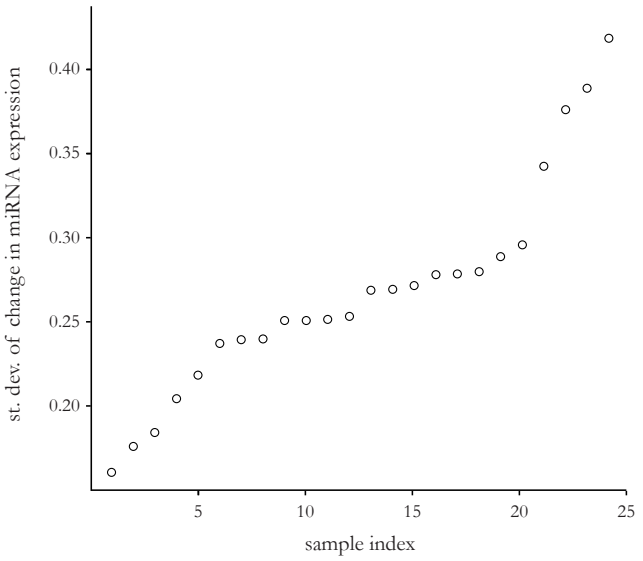

Figure 1B

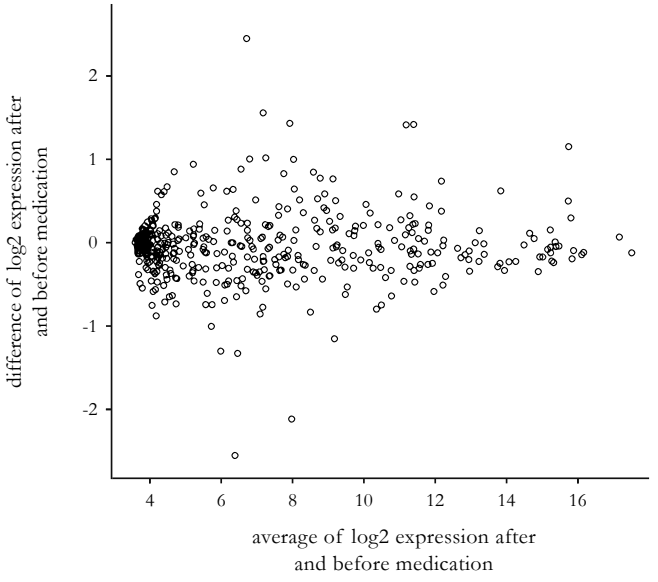

Figure 1C

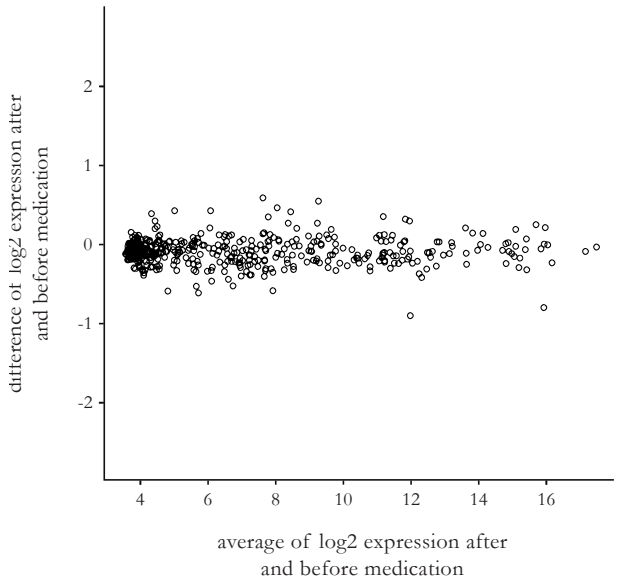

Figure 1 Inter-individual heterogeneity of medication-induced changes in miRNA microarray expression. For each individual and for each miRNA the difference in log2 expression after and before medication use was calculated. **A)** Standard deviation of the resulting changes in miRNA expression across all 468 detected miRNAs showed large inter-individual heterogeneity, **B)** Bland-Altman plot of the change in expression versus the average expression for each miRNA in the individual with highest overall standard deviation (rightmost symbol in panel A), **C)** Bland-Altman plot of the change in expression versus the average expression for each miRNA in the individual with lowest overall standard deviation (leftmost symbol in panel A)

which we performed a platelet aggregation study on whole blood in the absence of medication. We than correlated the medication-induced changes in miRNA expression of each miRNA as observed on the microarray with the reduction in platelet aggregation after incubation with indomethacin. The changes in expression before and after aspirin use of six miRNAs correlated strongly with the extent of platelet aggregation reduction. For all these miRNAs an increase in expression level after aspirin use was correlated with an inhibition of platelet aggregation after indomethacin incubation (Table 2).

Correlation of miRNA expression to in vitro platelet aggregation

To further substantiate this finding, we performed RT-qPCR and platelet aggregation analysis on isolated platelets in a cohort of 25 individuals. We correlated the RT-qPCR expression levels of

the 6 candidate miRNAs to the results of the platelet aggregation assay. We were able to perform RT- qPCR on 3 out of the 6 candidate miRNAs (miR-1271, miR-1537-5p and miR-19b-1-5p). The expression levels of the other 3 miRNAs were below the detection limit of our PCR system. We observed that a lower platelet miR-19b-1-5p expression after aspirin use was significantly associated with a sustained platelet aggregation in the presence of indomethacin, suggesting aspirin insensitivity (ρ = 0.68; $p<0.001$, Bonferroni corrected, Figure 2A). No significant correlation with platelet aggregation could be observed for miR-1537-5p and miR-1271 (ρ = 0.36; p=0.25 and ρ = 0.25; p=0.69, Bonferroni corrected, respectively) (Figure 2B-C). Separate analysis of the microarray cohort (n=15) and the 10 additional healthy volunteers showed similar results (data not shown).

Serum thromboxane B2 assay

Since the observed differences could be due to differences in compliance to aspirin use, we analysed serum TXB2 levels in all individuals. In both the microarray and the PCR cohort serum TXB2 levels were analysed in samples taken at baseline and after two weeks of aspirin therapy. At these time points the samples for the miRNA expression analyses were also taken. The percentage of serum TXB2 reduction after two weeks of aspirin therapy varied among individuals, but all participants had at least a 30% reduction, indicating good compliance of all individuals. When comparing the serum TXB2 levels after aspirin use to the inter-individual differences in platelet aggregation a similar trend was seen (Figure 3).

Figure 2A

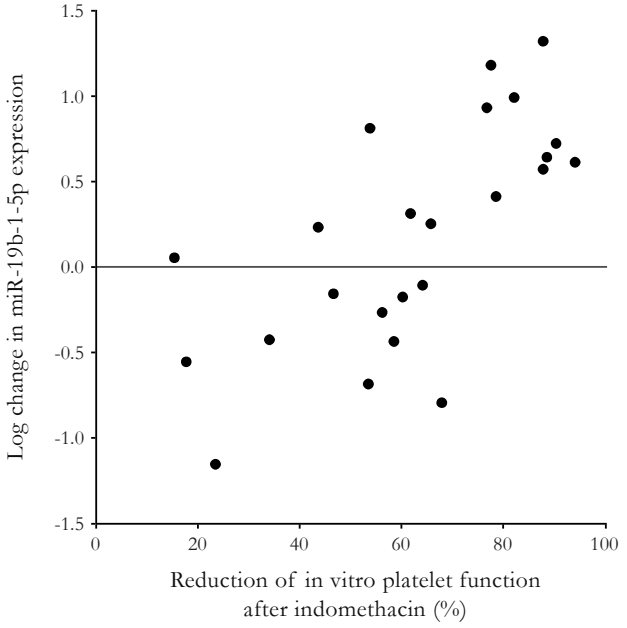

Figure 2B

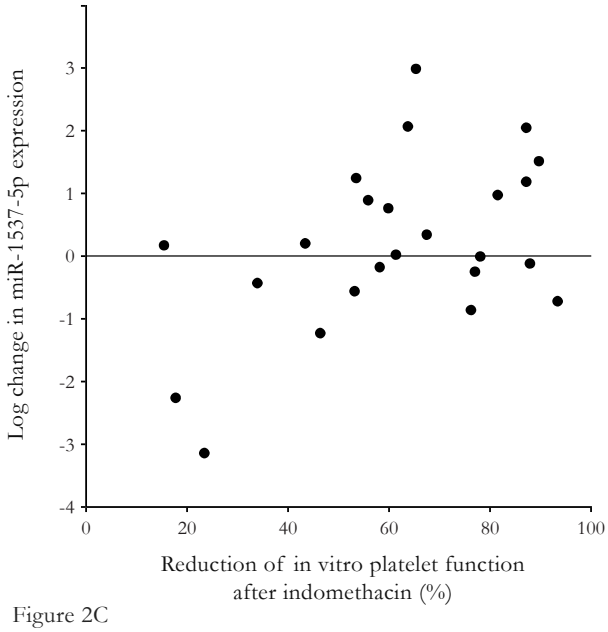

Figure 2C

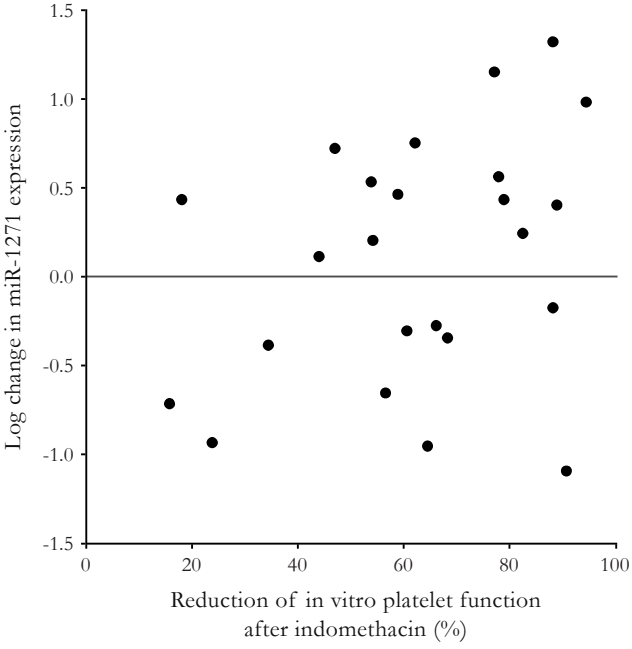

Figure 2 Correlation between the in vivo changes in miRNA expression after aspirin use and in vitro platelet aggregation after indomethacin incubation

Correlation between the change in **A)** miR-19b-1-5p expression, **B)** miR-1537-5p expression and **C)** miR-1271 expression (log fold change of normalised RT-PCR expression after and before medication use) and the sensitivity of the platelets to aspirin as measured by the reduction in platelet aggregation.

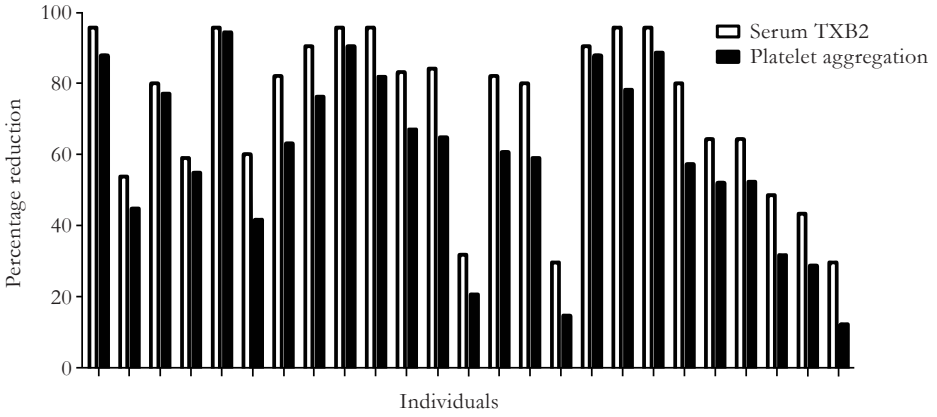

Figure 3 Comparison of the reduction in serum thromboxane B2 levels after aspirin use and the reduction in in vitro platelet aggregation after indomethacin incubation per individual.

DISCUSSION

In the present study we demonstrate that among equally healthy individuals, miRNA expression levels in isolated platelets show a heterogeneous response to medication use in healthy individuals. Some individuals show marked changes in miRNA expression after aspirin use, whereas others do not show any changes. We showed that this heterogeneous response was correlated to the degree of platelet aggregation after incubation with indomethacin. Especially, lower miR-19b-1-5p expression after aspirin use was associated with a sustained platelet aggregation in the presence of indomethacin, suggesting aspirin insensitivity. This is the first study indicating that miRNA expression levels of isolated platelets might be a useful marker for the prediction of aspirin insensitivity.

Multiple studies have focused on possible explanations and diagnostic tools for aspirin insensitivity. Although different groups have found associations between gene polymorphisms involved in platelet function and aspirin insensitivity[14,15], these findings must be interpreted with caution, because of small sample sizes and differences in ethnic origin of the studied populations[16]. Therefore, these polymorphisms are unlikely to be suitable for the identification of subjects susceptible to aspirin insensitivity in a general population. Also platelet function tests fail to correctly measure the antiplatelet effect of aspirin. Comparison of six different platelet function tests revealed that these assays are only moderately concordant and that, based on established cut-off values, the prevalence of aspirin insensitivity varied according to the platelet function test that was used[17]. By analysing the correlation between platelet aggregation data and miRNA expression, we avoided the choice of a cut-off value. We showed that miR-19b-1-5p may be a suitable marker to determine which patients has a sustained platelet aggregation after aspirin use

and might therefore be more prone to develop recurrent events.

Interestingly, in line with a study by Stratz and colleagues[18], in our microarray experiment we only observed subtle differences in the paired comparison of miRNA expression levels before and after aspirin use. This can be explained by the fact that some healthy individuals showed marked changes in miRNA expression after medication use, while others did not, leading to a large inter-individual heterogeneity. In contrast, Willeit and colleagues showed that specific miRNAs were down regulated in platelet poor plasma (PPP) after combined treatment with prasugrel and aspirin[19]. These seemingly conflicting results can possibly be explained by the fact that platelet activation leads to shedding of miRNAs from the platelet stores into the plasma[8]. Therefore, platelet inhibition will reduce miRNA shedding and result in decreased miRNA levels in PPP. However, plasma contains miRNAs derived from a variety of cell types. The presence of miRNAs in PPP can therefore only be a surrogate marker and does not fully reflect processes in the platelet itself, which was investigated in the present study.

Our most striking observation were the large inter- individual differences in reaction to medication use in healthy volunteers. The small sample size of the study by Stratz and colleagues (n=5), could be a reason why they did not observe these inter-individual differences in response. Recently, in a group of non-STEMI patients clopidogrel treatment was shown to alter the expression of two highly abundant platelet miRNAs. One of these miRNAs was miR-223, which significantly correlated with the extent of P2Y12 receptor inhibition[20]. Here we show that a similar phenomenon can be observed after aspirin treatment. The extent of reduction of platelet aggregation, after incubation with indomethacin, was significantly correlated with the change in platelet miR-19b-1-5p expression after aspirin use. Since all individuals were treated in exactly the same way, we concluded that this is an aspirin-induced effect and that the observed differences are due to inter-individual differences in handling aspirin. Presumably, inhibition of cyclooxygenase-1 (COX-1) by aspirin leads to both aggregation inhibition and increased expression of platelet miR-19b-1-5p. Therefore, subjects in whom platelet aggregation is not inhibited, express low levels of miR-b-1-5p. Thus, lower miR-19b-1-5p expression in platelets after aspirin use is associated with aspirin insensitivity and might be a marker suitable for the identification of patients that are less sensitive to aspirin treatment and prone to re-events.

Note that aspirin insensitivity is not a black and white phenomenon. Since there are no validated cut off values for aspirin insensitivity in whole blood platelet aggregation assays, we chose to analyse both the changes in miRNA expression and the reduction of platelet aggregation as continuous variables. Further studies are needed to eventually define a cut-off value for platelet miR-19b-1-5p expression that could be used in a clinical setting to determine whether patients are treated optimally.

Strengths and limitations

A limitation of our study is the relatively small sample size. However, our cohorts consisted of 15 and 25 individuals respectively, which is up to 5 times larger compared to most published

studies[18,19]. Furthermore, we mitigated this problem by performing all measures both at baseline and after aspirin therapy. This enabled us to analyse the effects of aspirin on miRNA expression and the effect of indomethacin on platelet aggregation within each individual. Other studies usually compare a single platelet function measurement in the presence of aspirin amongst responders and non-responders. Moreover, by analysing changes in a continuous manner, we could avoid dividing the cohort in two groups based on a cut-off values on which no consensus exists.

Since we included only healthy individuals to study the relation between miR-19b-1-5p expression and platelet aggregation after indomethacin incubation, it could be questioned whether these results could be extrapolated to patients with CAD. It is known that the effect of aspirin on platelet aggregation is similar in patients with CAD and healthy controls[21]. Furthermore, the expression of the miRNAs studied here is not known to be altered in subjects with CAD compared to healthy controls[10]. Therefore, we think that these findings can be extrapolated to subjects with CAD that are treated with aspirin for secondary prevention.

Whether 2 weeks of aspirin use is long enough to encounter a substantial effect can be argued. It is known that COX-1 is already ineffective after 1 week of aspirin use. Whether this also results in a steady state at the level of miRNA expression is not known, however after two weeks the whole batch of circulating platelets has been renewed. It is likely that the aspirin induced changes are incorporated in the machinery of the new batch of platelets.

In addition to aspirin, all individuals in this study also used statins and it cannot be excluded that statin therapy influences platelet miRNA expression patterns. Indeed, it has been reported that statins inhibit platelet activation[22], whereas other studies do not confirm this pleiotropic effect of statin therapy[23,24]. However, in this study we related the in vivo changes in miRNA expression to in vitro platelet aggregation measurements. In this assay samples were not incubated with statins or statin metabolites. We therefore concluded that the observed changes are more likely a result of the aspirin treatment, than statin influences, but a synergistic effect cannot be ruled out. Further studies are needed to evaluate the effect of statins on miRNA expression.

Conclusion

Lower platelet miR-19b-1-5p expression is related to aspirin insensitivity as measured by sustained platelet aggregation after incubation with indomethacin. Therefore, miR-19b-1-5p might be a suitable marker to identify aspirin insensitivity and thereby patients prone to re-events.

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CHAPTER 4

High miR-124-3p expression in monocytes of smoking individuals is associated with subclinical atherosclerosis

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ABSTRACT

The risk of developing coronary artery disease (CAD) is twice as high among smoking individuals compared to non-smokers. Monocytes play a key role in the development of CAD through their involvement in atherosclerotic plaque formation. We hypothesized that smoking alters the function of circulating monocytes and that this is reflected by changes in monocyte miRNA expression profiles. Furthermore, we studied whether, based on expression levels of specific monocyte derived miRNAs, smokers with an increased risk of developing CAD can be identified.

We performed a miRNA microarray experiment on isolated monocytes of 2 independent cohorts of patients with premature CAD and healthy controls and found that miR-124-3p was heterogeneously expressed among smoking individuals, whereas expression levels were low in non-smokers. Subsequent flow cytometry analysis showed that high miR-124-3p levels were associated with up-regulation of the monocyte surface markers CD45RA, CD29 and CD206, suggesting an altered function of these monocytes, which may contribute to the development of atherosclerosis. Next, we performed a RT-qPCR experiment on whole blood from 2 new independent cohorts and showed that increased miR-124-3p levels were associated with subclinical atherosclerosis in smoking individuals, but not in non-smoking individuals.

We showed that elevated levels of miR-124-3p are predictive for subclinical CAD in smoking individuals and that this miRNA is therefore a candidate biomarker for CAD in smoking individuals.

INTRODUCTION

Tobacco use is the most important avoidable cause of cardiovascular disease in the world[1]. The risk of developing myocardial infarction (MI) is twice as high among smokers compared to non-smoking individuals[2]. However, numerous smokers never develop cardiovascular complaints at all.

The increased risk of developing CAD can be explained by the atherothrombotic effects of smoking. Smoking has been shown to influence all stages of atherosclerotic plaque formation[3]. The exact mechanism by which cigarette smoke triggers atherosclerosis has not been completely unravelled, but many cell types, among which circulating monocytes have been reported to be involved in this process[3]. It is known that circulating monocytes undergo functional changes in the presence of cigarette smoke[4]. For example, monocytes of smoking individuals express more peroxisome proliferator-activated receptor γ (PPARγ), which can promote IL-4 induced M2 macrophage polarization[5]. In the presence of oxidized low density lipoprotein these M2 macrophages will become foam cells that accumulate in the vessel wall and contribute to the development of atherosclerotic plaques.

MicroRNAs (miRNAs) are 18 to 25 nucleotides, noncoding RNAs, that inhibit gene expression by suppression of messenger RNA (mRNA) translation[6]. They are involved in virtually every biological process, including cell differentiation and apoptosis[7]. In the past decade, miRNAs were shown to be involved in many pathophysiological processes and several miRNAs were reported as useful biomarkers for disease[8,9].

Recently, it was shown that smoking alters plasma miRNA profiles in healthy subjects[10,11]. Additionally, it was reported miRNAs, miR-124 and let-7a showed increased expression in smoking individuals compared to controls[12]. Of these, miR-124 has been reported to play a crucial role in the M2 polarization of bone marrow-derived macrophages[13]. Here, we hypothesized that changes in plasma miRNA expression in smoking individuals might be a reflection of altered monocyte miRNA expression profiles and that changes in monocyte miRNA expression are related to changes in the expression of monocyte surface markers. Therefore, we investigated whether smoking is associated with changes in miRNA expression profiles and whether this could be related to subclinical atherosclerosis.

METHODS

Ethics statement

This study complies with the Declaration of Helsinki. The study protocol was approved by the ethical committee of the Academic Medical Center in Amsterdam and all individuals gave written informed consent.

Cohorts

Cohort I consisted of 40 male premature coronary artery disease (CAD) patients and 40 age-matched male controls. Premature CAD was defined as a first cardiovascular event before the age of 51. These individuals might have a genetic predisposition for atherosclerosis, which may be accelerated by smoking. Patients between the age of 35 and 65 years who had a premature cardiovascular event and were willing to participate were included from the outpatient clinic for premature CAD in the Academic Medical Center in Amsterdam between December 2009 and June 2010. Sample collection was performed on average 5.6 ± 3.5 years after the first cardiovascular event. Control individuals were recruited by advertisement and were matched for age. Controls were eligible for participation if they did not have a personal or family history for CAD and did not use any medication. To study the effect of medication on miRNA expression levels, we administered 100 mg of acetyl salicylic acid, once daily, for two weeks. and simvastatin 40 mg, once daily, for 6 weeks, of which the last 2 weeks in combination with the acetyl salicylic acid. Blood samples were collected at baseline in the absence of aspirin and statins and after six weeks of medication use.

Cohort II consisted of 4 families with a high prevalence of premature CAD, of which CAD patients were treated at the outpatient clinic for premature CAD. From these families we included 21 patients with established CAD and 47 apparently healthy family members. All family members were screened at the outpatient clinic and they underwent a coronary CT-scan to assess the coronary calcification score (CAC) as a marker for premature. CAD cases were defined as patients with established atherosclerosis (n=21) and family members with a CAC score > 80th percentile corrected for age and gender (n=6). Controls were defined as healthy family members without any signs of complaints of CAD and a CAC score < 80th percentile (n=41). This group also included subjects under the age of 30 years, in whom no CAC score was measured. Since atherosclerosis is usually not as pronounced at a young age, CAC score determination is not sensitive enough in those individuals and therefore a low CAC score will not rule out the presence of advanced atherosclerosis.

Cohort III To investigate the effect of smoking on monocyte function we performed fluorescence-activated cell sorting (FACS) on isolated monocytes of 20 healthy smoking first degree relatives (FDRs) of patients with premature CAD that visited the outpatient clinic of premature CAD at the Academic Medical Center (AMC) in Amsterdam. All selected subjects were current smokers or had stopped smoking ≤5 years ago, according to the information provided by a questionnaire. To exclude the effect of statins on the monocytes, subjects that used statins were asked to discontinue statin therapy 6 weeks prior to their visit. This was only asked if discontinuation of therapy for a short period of time was medically justified, which was the case in all individuals.

Cohort IV consisted of 71 FDRs of subjects with premature CAD that visited the outpatient clinic for risk assessment between April 2010 and May 2013. We only selected smoking FDRs or FDRs that quit smoking less than five years ago. Of these FDRs, 39 individuals had a CAC score >80th

percentile corrected for age and gender and were considered as having subclinical atherosclerosis. The remaining 32 FDRs had a CAC score of zero and were considered healthy controls.

Cohort V consisted of 69 non-smoking FDRs of subjects with premature CAD. Selection criteria were similar to those in cohort IV. Of these FDRs, 31 individuals had a CAC score >80th percentile corrected for age and gender and were considered as having subclinical atherosclerosis. The remaining 38 FDRs had a CAC score of zero and were considered healthy controls.

Sample collection and processing

In the microarray Cohorts I and II, circulating monocytes were isolated from peripheral blood samples. Non-fasting venous blood was drawn in CTAD tubes (Becton Dickinson, Alphen aan de Rijn, the Netherlands) and centrifuged for 20 minutes at 163 g at 20ºC. The buffy coat was collected and monocytes were positively selected with CD14+ Dynal beads (Invitrogen, Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. Monocytes were incubated in RNA later (Ambion) for 45 minutes. Subsequently, ice-cold PBS was added in a 1:1 volume ratio and the samples were centrifuged for one minute at 5000 g, after which monocytes were obtained and snap-frozen in liquid nitrogen.

Monocyte RNA was isolated using the *mir*Vana PARIS kit (Ambion, Inc.), according to the manufacturer's protocol.

For the flow cytometry analysis in Cohort III monocytes were isolated from fresh whole blood samples through density centrifugation using Lymphoprep™ (Axis-Shield). CD14+ monocytes were isolated using human CD14 magnetic beads (Miltenyi) and MACS® cell separation columns (Miltenyi) according to the manufacturer's instruction.

RNA was isolated from CD14+ monocytes using TRIzol® Reagent (Life Technologies) according to the manufacturer's instruction.

In Cohorts IV and V whole blood samples were collected in EDTA tubes (Becton Dickinson, Alphen aan de Rijn, the Netherlands) and directly stored at -80ºC. Whole blood RNA was isolated using the miREasy Mini kit (Qiagen), according to the manufacturer's protocol.

MiRNA microarray

The integrity of total RNA including miRNAs from monocytes was investigated with the BioAnalyzer (Agilent Technologies) using the RNA 6000 Pico kit (Agilent Technologies) and Small RNA kit (Agilent Technologies) according to the manufacturer's instructions.

100 ng of total RNA including microRNAs was dried down in a Centrivap concentrator (Labconco) and dissolved in 2 µl RNase-free water. Sample labeling with Cy3 was performed as described in the miRNA Microarray System with miRNA Complete Labeling and Hyb Kit manual version 2.2 (Agilent Technologies) with the inclusion of spike-ins and the optional desalting step with spin columns (Micro Bio-Spin 6, Bio-Rad). Labeled samples were hybridized on Human

8x15k miRNA microarrays based on Sanger miRBase release 19.0 containing 866 human and 89 human viral miRNAs (G4470C, Agilent Technologies) at 55°C and 20 rpm for 20 hours. After washing, the arrays were scanned using the Agilent DNA microarray scanner (G2565CA, Agilent Technologies). Data were extracted with Feature Extraction software (v10.7.3.1, Agilent Technologies) with the miRNA_107_Sep09 protocol for miRNA microarrays.

miRNA microarray pre-processing and analysis

Microarrays for Cohort I and II were pre-processed and analysed separately. A two-step normalisation approach was taken. In the first step, we corrected for systematic technical effects in the raw probe-level data as extracted via the Agilent Feature Extraction software. For this purpose, we fitted a linear mixed-effects model with coefficients for two technical effects (slide and slide position, that is, upper or lower half of a slide), and patient status using the R/MAANOVA package. Residuals after correcting for the two technical effects were further pre-processed and summarized using a modified version of the robust multi-array average (RMA) method with background correction, as implemented in the AgiMicroRna R package[14]. Quality control was performed using the arrayQualityMetrics R package. Based on arrayQualityMetrics outlier detection and visual inspection of heatmaps, MA-plots, and intensity distributions, seven arrays were excluded from further analysis for cohort I and one array for cohort II. Data from the remaining arrays were renormalized using the two-stage procedure described above. Only non-control miRNAs detected on at least one array according to Agilent Feature Extraction software were included in the further analysis. Normalized expression values of technical replicate arrays were averaged. To detect miRNAs differentially expressed between smokers and non-smokers, gene-wise linear models were fit with smoking status as explanatory variable corrected for patient status (case/control), BMI and age followed by a moderated t-test (limma R package). Resulting p-values were adjusted to correct for multiple hypothesis testing using the Benjamin-Hochberg false discovery rate.

Flow cytometry analyses

Blood samples were obtained by peripheral venipuncture and collected into K3EDTA BD Vacutainer (BD Biosciences, San Jose, CA) tubes. After red blood cell lysis (eBioscience 1x RBC lysis buffer), cells were washed and incubated with fluorescently labeled antibodies for 20 minutes at room temperature in the dark. We used antibodies against the following surface markers: CD11b, CD11c, CD18, CD29, CD32, CD36, CD45RA, CD49d, CD80, CD86, CD163, CD206, CCR5, CCR7, CX3CR1, SRA and TLR4. After an additional wash, fluorescence was measured with a BD Canto II and analyzed with FlowJo. CountBright Absolute Counting Beads (Life technologies) were used to determine absolute cell counts as de-scribed by the manufacturer. Compensation was performed with Ebioscience OneComp eBeads. Monocytes were gated using a protocol that was adapted from a previous study[15]. Briefly, cells were first plotted on a FCS/SSC plot and a first gate (A) was drawn to exclude the majority of debris, residual red blood cells and granulocytes. These cells were next viewed on a CD14/CD16 plot to gate CD14+ and/or CD16+ cells (B). When cells from gate B were viewed on a CD16/HLA-DR plot, monocytes (gate C) were easily distinguished from contaminating cells ('not monocytes'). The final monocyte

population was viewed again on a CD14/CD16 plot to gate CD14++/CD16-, CD14++/CD16+ and CD14dim/CD16+ monocyte subsets. In addition to the percentage of each subset and their absolute cell count, the expression of various surface markers was assessed and calculated as $\Delta MFI = [\text{median fluorescence intensity}]_{\text{positive staining}} - [\text{median fluorescence intensity}]_{\text{isotype control}}$.

MiR-124-3p mimic experiment

Human monocytes were isolated from Buffy coats (Sanquin) via density centrifugation and CD14+ beads isolation as described above and cultured in IMDM complete (25mM HEPES + 10% Fetal Calf Serum, +1% L-glutamine, +1% penicillin streptomycin) and stimulated with 50ng/ml MCSF (Miltenyi). On day 6, cells were transferred to a 12-wells plate in a density of $1 \cdot 10^6$ cells/ml. Hereafter, monocytes were transfected with 20 μM miRIDIAN hsa-miR-124-3p mimic (Dharmacon, cat# C-300592-05) or 20 μM negative control #1 mimic (Dharmacon, cat# CN-001000-01-05) using the Lipofectamine 2000 transfection reagent (Invitrogen) in triplicate. Additionally, a subset of cells were induced with IL-4 (50ng/ml) for 24 hours after transfection with the negative control mimic. 24 hours after transfection FACS analyses of the cells was performed as described above. We determined expression of CD64, CCR7, CD200R, CD206, CD29 and CD45RA (Supplementary Table 1).

Quantitative PCR

MiR-124-3p specific reverse transcription was performed on 100ng of purified total RNA, including miRNAs, using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium). RT-qPCR reactions were carried out in duplicate, on a LightCycler 480 system II (Roche, Basel, Switzerland). Data were analyzed using LinRegPCR quantitative PCR data analysis software, version 11[16].

MiR-124-3p expression was normalized to the geometric mean of a previously established miRNA normalization panel for whole blood samples consisting of miR-130b and miR-342-3p[17].

Statistical analysis

Results are expressed as mean ± standard deviation, except when indicated otherwise. Student's t-tests and Chi-square tests were used to calculate differences in baseline characteristics. Variables with a skewed distribution, e.g. the miRNA expression levels, were log transformed before they were analyzed. We used Pearson correlation to analyze the correlation between miRNA expression levels and monocyte surface markers.

To analyze differences in monocyte surface marker expression between the miR-124-3p mimic and the negative control treated monocytes, we used one-way ANOVA and Tukey post-hoc tests.

We used a logistic regression model to analyze the relation between miRNA expression levels and the presence of subclinical atherosclerosis in FDRs. This model was adjusted for age, gender and medication use.

All analyses were performed using SPSS for Windows 19.0. A p-value < 0.05 was considered statistically significant.

RESULTS

MiRNA microarrays show elevated miR-124-3p levels in smoking individuals

Clinical characteristics of the individuals included in Cohort I are displayed in Table 1. In this cohort 17.5% of the included individuals were current smokers. Most of these smoking individuals were CAD patients (n=10, 71.4%). Furthermore, hypercholesterolemia and hypertension were significantly more often present in CAD patients compared to healthy controls.

MiRNA microarray analysis of isolated monocytes in Cohort I showed that miR-124-3p was differentially expressed in smokers compared to non-smoking individuals (adjusted $P = 1.22*10^{-12}$). All non-smoking individuals had a low miR-124-3p expression level, whereas miR-124-3p expression was quite heterogeneous in smoking individuals (Fig 1A). In this analysis we did not observe any differences in monocyte miR-124-3p expression between CAD patients and healthy controls. Interestingly, individuals who quit smoking less than 5 years ago (n=13, 16.3%) also had a low expression level, suggesting that the effect of smoking on miRNA 124-3p is relatively short.

Additionally, we analyzed the miRNA microarray results in isolated monocytes of a second, independent cohort. Clinical characteristics of Cohort II are displayed in Table 1. In this cohort 19% (n=13) of the individuals were current smokers. 40% of the included subjects were CAD patients, of which 44% (n=12) were current smokers. In the group of healthy controls 27% of the individuals were current smokers (n=11). Furthermore, CAD patients were significantly older, had more often hypertension and did more often use medication compared to healthy controls (Table 1).

We observed exactly the same effect as for the microarray results of Cohort I. MiR-124-3p expression was low in all non-smokers, whereas it was heterogeneously expressed among smokers (adjusted P=0.0007). No differences were seen in monocyte miR-12-3p expression between CAD patients and healthy controls. Again, individuals who had stopped smoking less than 5 years ago showed low miR-124-3p expression levels (fig. 1B).

Flow cytometry analysis reveals a correlation between miR-124-3p expression and monocyte surface markers

Since miR-124-3p was heterogeneously expressed within the group of smoking individuals in both independent microarray cohorts, we tested whether miRNA 124-3p expression in smoking individuals was associated with the expression of monocyte surface markers.

To investigate this, we performed flow cytometry on whole blood of subjects from Cohort III and gated for monocytes. We correlated the expression of the monocyte surface markers to the miR-124-3p expression in isolated monocytes as determined by RT-qPCR. Baseline characteristics of Cohort III are displayed in Table 1.

In individuals with a high miR-124-3p expression we observed an increase in the monocyte surface marker CD45RA, CD29 and CD206. CD45RA, which is present on activated monocytes[18], was

Table 1 Baseline characteristics

	Cohort I		Cohort II		Cohort III	Cohort IV		Cohort V	
	Premature CAD cases	Controls	Premature CAD cases	Controls		Subclinical atherosclerosis cases	Controls	Subclinical atherosclerosis cases	Controls
N	40	40	27	41	20	39	32	31	38
Age at visit, years ± SD	51.4 ± 4.7	51.0 ± 4.6	59.4 ± 9.8	37.7 ± 11.2 *	51.2 ± 8.3	48.5 ± 9.9	48.7 ± 6.0	48.8 ± 7.9	46.9 ± 7.3
Gender, male (%)	40 (100)	40 (100)	8 (29.6)	20 (48.8)	8 (40)	16 (41.0)	11 (34.4)	15 (48.4)	12 (31.5)
Hypercholesterolemia, n (%)	5 (12.5)	0 *	5 (18.5)	5 (12.2)	8 (40)	12 (30.8)	9 (28.1)	18 (58.1)	11 (28.9) *
Hypertension, n (%)	6 (15)	1 (2.5) *	9 (33.3)	3 (7.3) *	5 (25)	14 (35.9)	7 (21.9)	12 (38.7)	6 (15.8) *
Diabetes, n (%)	0	0	3 (11.1)	2 (4.9)	1 (5)	6 (15.4)	0 *	4 (12.9)	1 (2.6)
Medication use, n (%)	0 (0)	40 (100)	20 (74.1)	2 (4.9) *	6 (30)	14 (35.9)	6 (19)	16 (15.6)	10 (26.3) *
Current smoking, n (%)	9 (22.5)	4 (10) *	12 (44.4)	11 (26.8)	15 (75)	26 (66.7)	18 (56.2)	0	0
Smoking quit <5 years ago, n (%)	9 (22.5)	4 (10) *	2 (7.4))	4 (10.0)	5 (25)	13 (33.3)	14 (43.8)	0	0
Systolic blood pressure, mmHG ± SD	136.8 ± 19.6	128.7 ± 13.4	136.8 ± 24.9	125.1 ± 11.6 *	133.3 ± 24.2	131.4 ± 19.2	124.4 ± 9.7	131.9 ± 18.9	126.4 ± 11.1
Diastolic blood pressure, mmHG ± SD	86.7 ± 12.0	84.0 ± 10.1	137.6 ± 22.7	123.6 ± 10.1 *	79.4 ± 10.5	77.6 ± 10.6	78.7 ± 8.2	78.7 ± 9.1	77.3 ± 6.1

Continuous data are expressed as mean ± SD, categorical data as absolute number with (percentages). (hierna een enter)
N, number; SD, standard deviation. * $p<0.05$ compared to cases

Figure 1A

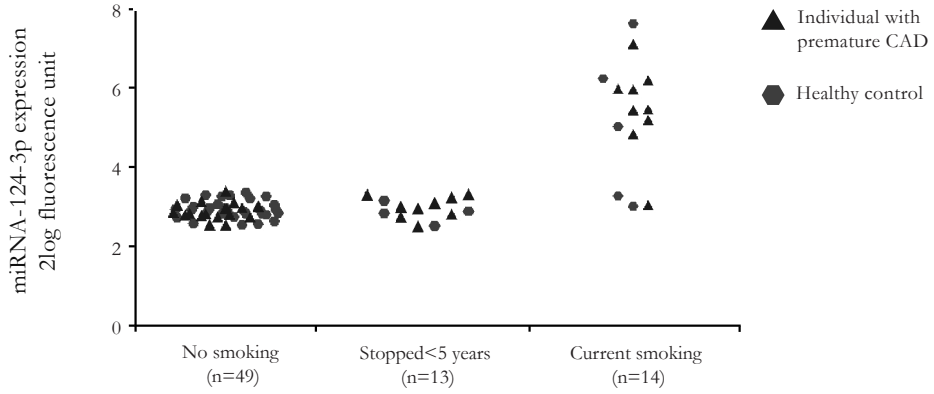


Figure 1B

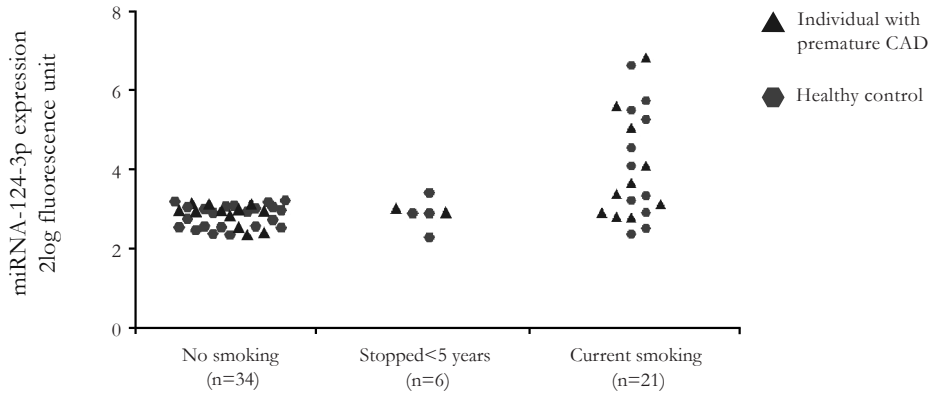


Figure 1 Microarray miR-3p expression in monocytes of smoking and non-smoking individuals. MiR-124-3p expression in non-smokers, individuals that stopped smoking <5 years ago and current smokers in **A.** Cohort I and **B.** Cohort II. Rectangles represent patients with atherosclerosis, whereas hexagons represent healthy controls.

increased in the classical monocyte subtype (ρ=0.57; p=0.008). CD29 was increased in the non-classical monocyte subtype (ρ=0.59, p=0.006). CD29 is part of the ß1 integrin very late antigen-4 (VLA-4) which is also present on activated monocytes[19]. CD206 was increased in both the classical and intermediate monocyte subtype (ρ=0.47, p=0.038 and ρ=0.53, p=0.017 respectively) (Figure 2). CD206 or the mannose receptor, is a marker of the alternatively activated (M2) macrophages[20].

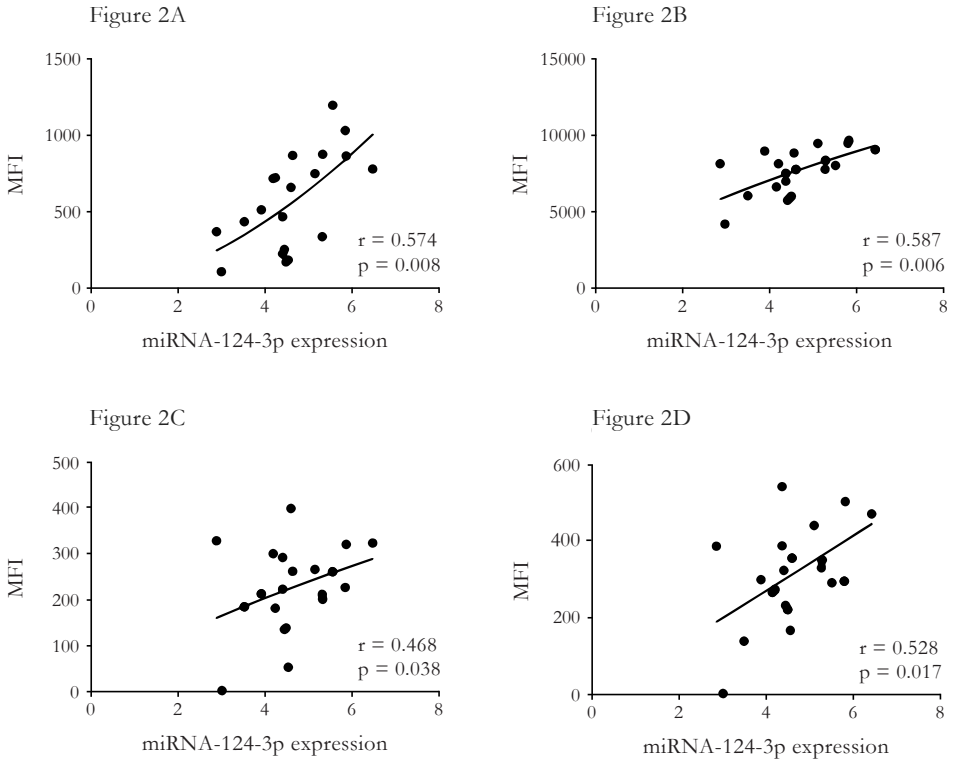


Figure 2 Correlation between miR-124-3p expression and monocyte surface markers
Correlations between miRNA-124-3p and FACS markers (A: CD45RA, classical monocytes; B: CD29, non-classical monocytes; C: CD206, classical monocytes; D: CD29, intermediate monocytes). Pearson correlation coefficient (r) and p-value of the log transformed monocyte surface marker and miRNA expression (log transformed) are displayed in the graphic as well as the log-regression line.

CD206 and CD64 are up regulated after stimulation of human macrophages with a miR-124-3p mimic

To investigate whether up regulation of miR-124-3p indeed leads to an upregulation of the markers CD45RA, CD29 and CD206, we overexpressed miR-124-3p using miRNA mimic transfections in human macrophages. Transfection with the miR-124-3p mimic resulted in a significant increase of CD206 expression (p=0.01) (Figure 3A), which is in line with the flow cytometry results, where high CD206 levels correlated with high miR-124-3p levels. Additionally, we observed a significant increase in CD64 expression after miR-124-3p overexpression (p=0.004) (Figure 3B). Expression of the M1 marker CCR7 and the M2 marker CD200R were not altered after miR-124-3p overexpression (Figure 3C and 3D). The monocyte activation marker CD29 and CD45RA were not expressed on these macrophages.

Figure 3A

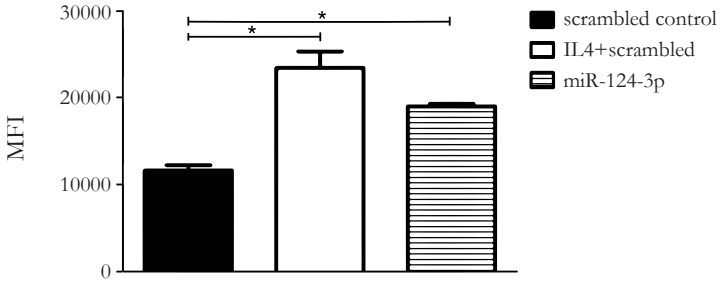


Figure 3B

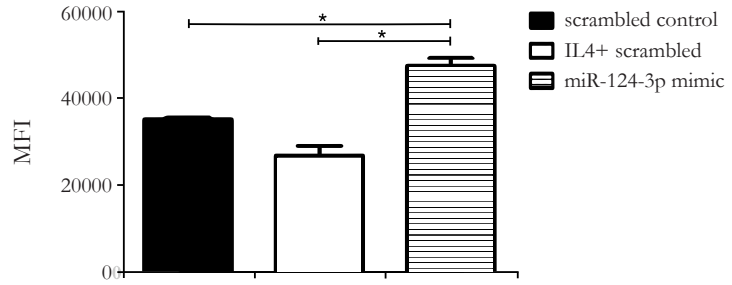


Figure 3 Expression of monocyte surface markers after transfection with a miR-124-3p mimic
Expression of monocyte function markers after human monocyte induction with IL-4 and transfection with a miR-124-3p mimic. (**A:** CD206; **B**: CD64; **C**: CCR7; **D**: CD200R). * =P<0.05

Since it has been reported that IL-4 stimulation of macrophages leads to increased miR-124 expression we investigated whether the monocyte surface markers are upregulated after stimulation with IL-4. As expected, IL-4-induced macrophage polarization resulted in a significant down regulation of the M1 marker CD64 ($p=0.026$) and a significant increase in the expression of M2 markers CD206 ($p<0.001$) and CD200R ($p<0.001$). Expression of CCR7, also a M1 macrophage marker, was not altered by IL-4 induction (Figure 3).

MiR-124-3p expression levels in whole blood predict risk of subclinical atherosclerosis in smokers

Since we showed that miR-124-3p was heterogeneously expressed in circulating monocytes of smoking individuals and that high levels of miR-124-3p correlated with changes in monocyte surface markers, we hypothesized that an increase in miR-124-3p expression is related to subclinical atherosclerosis. Therefore, we related whole blood miR-124-3p expression levels to coronary

Figure 3C

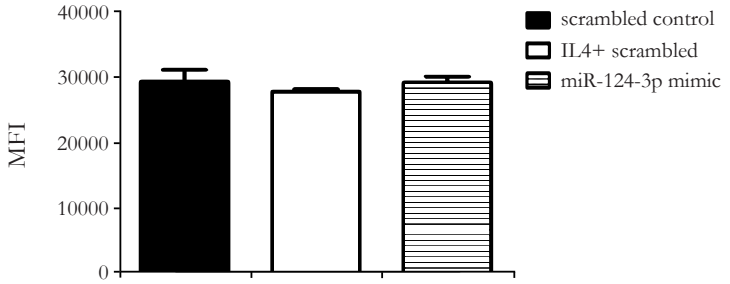
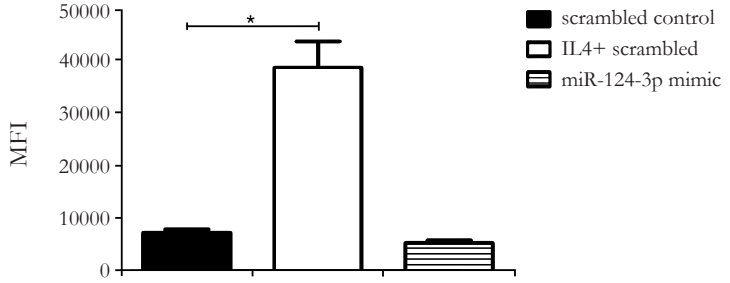


Figure 3D



calcium abnormalities as measured by coronary CT scanning. We choose to analyse this in whole blood samples for practical reasons, since isolated monocytes are not easily obtained in clinical daily practice.

We determined miR-124-3p expression levels by RT-qPCR in Cohort IV, which consisted of 44 smoking FDRs and 27 FDRs that stopped smoking <5 years ago. Of these, 39 had subclinical atherosclerosis and 32 did not. Characteristics of Cohort IV are shown in Table 1. Among the individuals with subclinical atherosclerosis there were significantly more diabetes patients and they more often used medication compared to individuals without subclinical atherosclerosis (Table 1).

The logistic regression analysis showed that, among smoking and former smoking individuals an increase in miR-124-3p expression level was related to a higher risk of subclinical atherosclerosis expression levels (OR 2.36; 95% CI 1.25-4.46; p=0.008). Since individuals that stopped smoking

< 5 years ago from Cohort I and II showed low miR-124-3p expression levels on the miRNA microarray, thereby resembling non-smoking individuals from these cohorts, we repeated the analyses in smokers and individuals that stopped smoking <5 years ago from Cohort IV separately. We found that in currently smoking individuals an even higher risk of subclinical atherosclerosis could be observed with increasing miR-124-3p levels (OR 3.24; 95% CI 1.23-8.46; p=0.016), whereas in individuals that stopped smoking <5 years ago this association could not be observed (OR 1.36; 95% CI 0.42-4.43; p=0.61). This observation underlines the short-lasting effect of smoking on monocytes.

Interestingly, these results suggest that an increase in miR-124-3p expression might identify subjects with subclinical atherosclerosis among smoking individuals. The fact that this was not observed in the microarray experiment could be due to a difference in sample type (monocytes versus whole blood) or to the difference in disease state of the cases (premature CAD versus subclinical atherosclerosis).

To find out whether this observation only holds true for smoking individuals, we repeated the analysis in Cohort V. This cohort consisted of 69 non-smoking FDRs of subjects with premature CAD, of which 31 had subclinical atherosclerosis and 38 did not. Characteristics of Cohort V are shown in Table 1. Individuals with subclinical atherosclerosis had significantly more often hypertension and hypercholesterolemia compared to controls for which they significantly more often used medication (Table 1).

We did not observe a relation between higher miR-124-3p expression levels and subclinical atherosclerosis in non-smoking individuals (OR 1.35; 95% CI 0.43-4.30; p=0.61). This indicates that the observed association between miR-124-3p expression levels and the risk of subclinical atherosclerosis among smoking individuals is likely to be related to smoking, and not to CAD itself.

DISCUSSION

In this study we showed that miR-124-3p expression is heterogeneously expressed in monocytes of smoking individuals in two independent cohorts and that expression of miR-124-3p positively correlates with the expression of the surface markers CD29, CD45RA and CD206, suggesting a potential role for miR-124-3p in the development of atherosclerosis. Finally, we showed that elevated levels of miR-124-3p are associated with subclinical atherosclerosis in smoking individuals.

In two independent miRNA microarray experiments we found that miR-124-3p was heterogeneously expressed in monocytes of smoking individuals, whereas in non-smokers miR-124-3p expression was low. No differences in miR-124-3p expression could be observed between smoking premature CAD patients and smoking healthy controls. This could be due to the small numbers of smoking patients and smoking healthy controls and to the fact that all premature

CAD patients were treated with statin therapy, which is known to attenuate the pro-atherogenic function of monocytes[21]. This might also have influenced miRNA expression in smoking patients with premature CAD.

To investigate a possible relation between elevated miR-124-3p levels and atherosclerosis we performed a flow cytometry analysis of monocyte surface markers which revealed that high miR-124-3p expression levels in smoking individuals were correlated to phenotypic changes in monocytes. Additional qPCR analyses showed that high miR-124-3p levels were associated with subclinical atherosclerosis in smoking individuals.

MiR-124 levels were previously reported to be elevated in plasma of smoking individuals[11]. In this study by Banerjee and colleagues, a dose-dependent effect on miR-124 expression was reported, with an increase in miR-124-3p expression with each additionally smoked cigarette. Here, we report a heterogeneous expression of miR-124-3p in smoking individuals, whereas miR-124-3p expression was genuinely low in non-smokers. A dose-dependent effect of cigarette smoke was not specifically analyzed, but it could very well be that the heterogeneous expression reflects the dose-dependent effect that was reported by Banerjee. Interestingly, analogous to Banerjee's study, we also observed that subjects who quit smoking showed low miR-124-3p levels, suggesting a short, direct effect of smoking on monocyte miR-124-3p expression.

Flow cytometry analyses of monocytes showed that an increase in miR-124-3p expression is associated with an increase of several monocyte surface markers, CD45RA, CD 29 and CD206.

CD45RA (or protein tyrosine phosphate receptor C (PTPRC)), is also considered a pro-inflammatory surface marker. This marker is mainly present on naive T-cells, however, heterogeneous populations of monocytes also express CD45RA as a marker of monocyte activation[18]. *In vitro* studies showed that CD45RA is co-expressed with ß1 integrin very late antigen-4 (VLA-4) and that these cells are most likely to migrate over inflamed endothelial cells. Furthermore, this population of monocytes has been correlated to an atherogenic lipid profile, which in turn forms a risk factor for endothelial inflammation[22]. It has been reported that cigarette smoking contributes to the oxidation of low density lipoprotein, contributing to an atherogenic lipid profile[23]. This might eventually lead to an upregulation of CD45RA expression, which is correlated with increased miR-124-3p expression as seen in smoking individuals.

Together with CD49d, CD29 forms the ß1 integrin very late antigen-4 (VLA-4), which binds to its ligand vascular cell adhesion molecule 1 (VCAM-1) on the endothelium[19]. VLA-4 displays a particular high affinity for inflamed endothelial cells and facilitates monocyte adhesion to and migration over the endothelium[24]. *In vitro* studies have shown that due to cigarette smoke, VCAM-1 expression on endothelial cells is increased[25]. Together with the increase in CD29 expression on monocytes of with high miR-124-3p expression reported here, this may have consequences for monocyte migration into the vessel wall, where they reside and transform into either activated macrophages or lipid-laden foam cells, accelerating the process of atherosclerotic plaque formation.

We also found a positive correlation between the expression of CD206 and miR-124-3p levels. CD206, or the mannose receptor, is a marker present on the alternatively activated (M2) macrophages. Although M2 macrophages are generally known as anti-inflammatory, their presence has been described in various stages of the atherosclerotic plaque[5,26]. It was previously shown that miR-124 up regulation in bone-marrow derived mouse macrophages was associated with a shift from the M1 to the M2 macrophage phenotype[27]. Subsequently, monocytes were stimulated with IL-4 and IL-13 to obtain M2 macrophages. The authors showed that IL4 stimulation of macrophages resulted in high miR-124 expression and upregulation of CD206 expression[13]. Interestingly, after blocking miR-124 using an antimiR, IL4 stimulation did not result in an increase in CD206. The results of our mimic experiment confirmed the causal relation between miR-124-3p and CD206 by showing that transfection with a miR-124-3p mimic induces an increase in CD206 expression. These data together with previously reported findings suggests a role for miR-124-3p in M2 polarization

Besides upregulation of the M2 marker CD206, our miR-124-3p mimic experiment also showed up-regulation of CD64, a marker of the classical activated (M1) macrophages[28]. These inflammatory macrophages produce reactive oxygen species that induce and exacerbate oxidative stress in the atherosclerotic plaque[29]. Furthermore, they secrete the cytokine IL-6 [30] that enhance atherogenesis[31].

The regulation of both M1 and M2 markers on macrophages transfected with a miR-124-3p mimic indicates that up regulation of miR-124-3p does not result in a polarization towards a single macrophage subtype, but rather identifies miR-124-3p as a complex regulator of monocytes/macrophages.

We did not observe an up-regulation of CD29 and CD45RA expression in our mimic experiment. This could be due to the fact that these are markers of activated monocytes rather than macrophages. Indeed, these markers were not detectable in our in vitro cultured macrophages.

Since we showed that miR-124-3p is elevated in monocytes of smoking individuals and that increased miR-124-3p levels are associated with pro-atherogenic changes in monocyte phenotype, we studied whether miR-124-3p expression levels could be used as a biomarker for subclinical atherosclerosis in smoking individuals. For this purpose, we determined miR-124-3p levels in two independent cohorts of smoking and non-smoking individuals. The analysis revealed that in smoking individuals elevated miR-124-3p levels were associated with a 2.36-fold increased risk of having subclinical atherosclerosis, whereas no differences in miR-124-3p expression were observed between subclinical atherosclerosis patients and healthy controls in non-smoking individuals.

Thus, we showed that miR-124-3p was heterogeneously expressed among smoking individuals, whereas high miR-124-3p was associated with a increased risk of having subclinical atherosclerosis in Cohort V. This suggests a susceptibility for the adverse effects of smoking and may explain why several smoking individuals never develop cardiovascular complaints. Therefore, miR-124-3p seems a suitable whole blood biomarker for subclinical atherosclerosis in smoking individuals.

Strengths and limitations

In this study we observed an association between elevated miR-124-3p levels and subclinical atherosclerosis, but not between elevated miR-124-3p levels and premature CAD. This could be due to the fact that individuals with premature CAD in Cohort I and II were treated with statin therapy, whereas patients with subclinical atherosclerosis in Cohort III and IV were not treated yet. Statin therapy is known to attenuate the pro-atherogenic function of monocytes[21]. It could be possible that, in addition to direct effects on monocyte function, miR-124-3p expression in the monocytes is influenced by statin therapy, resulting in reduced miR-124-3p expression levels. Further studies are needed to investigate the effect of statins on miR-124-3p expression. In addition to these experiments, it would also be interesting to study whether monocyte function is altered by an increase in miR-124-3p expression, which cannot be concluded from our experiments. We do show that increased miR-124-3p expression is associated with changes in the expression of some monocyte surface markers, among which CD206. With these findings we confirm previously published experiments in which expression of CD206 was influenced by miR-124-3p expression[13].

Conclusion

We showed that miR-124-3p expression is heterogeneously expressed in monocytes of smoking individuals in two independent cohorts. Phenotypical analyses of these monocytes revealed that elevated miR-124-3p levels are associated with the expression of the pro-atherogenic surface markers CD29 and CD45RA, suggesting a potential role for miR-124-3p in the development of atherosclerosis. Moreover, we showed that elevated levels of miR-124-3p in whole blood are associated with subclinical atherosclerosis in smoking individuals and could therefore be used as a suitable biomarker in these individuals, thus identifying individuals with a susceptibility for the adverse effects of smoking.

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CHAPTER 5

The sample size of miRNA microarray experiments: a common pitfall for the identification of miRNA biomarkers.

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Submitted

INTRODUCTION

Since their discovery, microRNAs (miRNAs) have been reported as important regulators of gene expression. In normal cell conditions, they are involved in crucial biological processes, such as cell differentiation, proliferation and apoptosis[1]. Following the first description of miRNA involvement in cancer[2], many studies have focused on the role of miRNAs in a wide variety of diseases.

miRNAs are short RNA molecules with an average length of 22 nucleotides. These short RNA sequences are processed from a hairpin precursor and subsequently loaded into the Argonaute protein of a miRNA-induced silencing complex (miRISC). Once incorporated, the miRNA pairs with target messenger RNA (mRNA) to direct post-transcriptional repression.

The number of miRNAs encoded by the human genome (approximately 2,000)[3], is limited compared to the estimated 25,000 protein-coding genes. However, a single miRNA can regulate many different mRNAs, and thereby exert an important role in gene regulation.

Circulating miRNAs and biomarker suitability

The first description of human miRNAs originates from cell-based experiments. In 2007, the first study was published that revealed that miRNAs were also present in microvesicles and that these short RNA strands could thus be maintained in the extracellular space[4]. This important finding was quickly followed by the first detection of miRNAs in the circulation, opening up a whole new field of research.

Based on two principal characteristics, circulating miRNAs became a popular research topic. First, the binding of the miRNA to Argonaute stabilizes the RNA strand in the extracellular environment, protecting it from degradation and making it easily detectable. Second, previous studies extensively reported the dysregulation of specific cell-based miRNAs in diseased states. It is thought that, along with the usual cargo, deregulated miRNAs are shed from diseased tissue into the circulation and that miRNA expression levels of specific miRNAs within the circulation could reflect the presence of disease[4]. Taken together, these characteristics led to the hypothesis that circulating miRNAs are suitable biomarker candidates.

miRNA detection and quantification

The first step in the identification of circulating miRNAs as a marker for disease is the accurate detection and quantification of a large panel of miRNAs. During this process several challenges are encountered, resulting from some of the principal characteristics of miRNAs. Profiling methods have to deal with the short length of the miRNAs, low abundance of miRNAs in the circulation, a high degree of sequence homology within miRNA families and the presence of isomiRs, in which sequences of individual miRNAs can differ by a single nucleotide. The most commonly used techniques for a miRNA profiling experiment are miRNA microarrays, polymerase chain reaction (PCR) and next generation sequencing (NGS)[5]. These techniques are all highly sensitive[6], but encounter several difficulties. The short length of the miRNA molecules,

for example, is insufficient for annealing to traditional PCR primers and therefore specific primer design for each individual miRNA is challenging[7]. Furthermore, the combination of short length and variation in miRNA GC content results in a wide variation in melting temperatures for annealing reactions. This is especially problematic when multiple miRNAs are analyzed at the same time as on a miRNA microarray. The incorporation of locked nucleic acids (LNAs) into capture probes can solve this problem, however LNA incorporated arrays have been shown to be subjected to lower signal to noise ratios compared to for example bead-based arrays. This means that background noise might dominate the measured results and lead to less precise miRNA quantification[8]. NGS is not subjected to these issues, but this technique has its own disadvantages, such as the difficulty of robust library preparation to obtain non-biased data. Although much progress has been made over the past years, most popular library preparation protocols being used today may still introduce serious biases in sample composition, which might eventually result in data misinterpretation[9]. Combined with higher costs of NGS experiments and more challenging data analysis, this is one of the reasons that the majority of the published studies have used microarray or PCR experiments for miRNA profiling. Although PCR is the most sensitive of these two methods, it is less appropriate for high-throughput experiments and much more expensive when profiling multiple miRNAs[7]. Costs of microarrays are lower, but so is their sensitivity. For this technique an excellent intra-platform reproducibility has been reported[10], however it showed only limited concordance between platforms, suggesting high false positive and false negative rates[10]. Therefore, validation of microarray results using a different technique is necessary. Despite their disadvantages, microarrays are most suitable as discovery tool and. Moreover, biological significance and plausibility should also be taken into account to report robust findings[6,10,11].

Reproducibility issues

Over the past years multiple groups have searched for circulating miRNAs as biomarker for various diseases. In the field of oncology alone, more than one hundred miRNAs have been described to detect the presence of disease[3]. However, data so far lack specificity and reproducibility[12]. For breast cancer, two reviews on miRNAs as biomarkers, including a total of 32 publications, reported 143 plasma and serum miRNAs to be associated with breast cancer. Only 10 miRNAs had a fold change > 2 in more than one study and only a single miRNA was reported in more than two studies[12,13]. These discordant results are a consequence of several problems. First of all, the studies under investigation all had a different study design, with different in- and exclusion criteria. However, in studies on a specific disease, one might still expect more overlap in differentially expressed miRNAs. Second, a large part of the differences encountered might be due to the use of different measurement platforms, data pre-processing methods and statistical tests for differential expression[14–16]. Furthermore, data are often incompletely annotated and miRNA annotation changes as a consequence of new miRBase versions. These factors probably contributed to the lack of concordance in published results. Finally, much of the discordance between studies could be due to small sample size, which will be discussed below.

The influence of sample size

The quest for new miRNAs as biomarker for disease is often a search for a needle in a haystack. Since miRNA profiling experiments are costly, the initial step of the search is very often performed on only small sample sizes[17]. However, from a scientific perspective it is essential to include enough samples to have sufficient power to detect clinically relevant effect. In underpowered studies three problems occur that contribute to the production of unreliable findings. First, the chance of discovering true effects is low in studies with low power, as a result of a high false-negative rate. Second, along with a decrease in power, the positive predictive value of the discovery will decrease, meaning that many initial discoveries will eventually be rejected as false-positive findings[18,19]. Last, if a true effect is found, the magnitude of the effect is often exaggerated[20]. The consequences of these power-associated problems are particularly problematic when a study is performed to identify high-impact novelties as in the search for biomarkers, since low-likelihood hypotheses might be pursued as groundbreaking discoveries[21]. Several groups have tried to reduce the costs of profiling experiments by pooling samples. Although pooling leads to a decrease in biological variation and therefore a possible gain in power, these studies provide only limited results since outliers cannot be identified and miRNA-specific variance components cannot be determined[22]. A characteristic of underpowered studies is that only large effects can reach statistical significance[23]. A small sample size study that, by chance, discovers such a large effect is more likely to be published and receive more attention and impact than underpowered studies that do not show any effect, resulting in particular example of publication bias known as the small study effect[24,25]. Indeed, the effect size for postulated associations reported by highly cited biomarker studies is often larger than the effect size of that same association in the subsequent meta-analysis[26]. Furthermore, small sample sizes may (partially) explain the lack of concordance between different studies.

The problems associated with small sample size studies described above can be illustrated by the following experiment. Previously, we performed a miRNA microarray experiment on isolated monocytes of a cohort of 61 individuals consisting of 36 male premature coronary artery disease (CAD) patients and 25 age- matched male controls that used simvastatin 40mg once daily for six week and aspirin 100mg once daily during the last two weeks to mimic medication use in cases. MiRNA expression profiles were determined using the Agilent Human 8x15k miRNA microarrays based on Sanger miRBase release 19.0. The exact method of sample collection and data pre-processing can be found in the Supplementary Methods. Analysis of the miRNA expression profiles revealed that none of the detected miRNAs was differentially expressed between premature CAD patients and healthy controls using medication with a, p-value < 0.1 after correction for multiple testing. In fact, the smallest corrected p-value that was obtained in this study was 0.26.

To illustrate the issue of a reduced positive predictive value, we performed additional analyses in smaller subsamples of the cohort. A subsample of five cases versus five controls was selected 1,000 consecutive times and in each subsample differential expression between the groups was analyzed. In 78 out of 1,000 subsamples we found at least one miRNA to be differentially expressed with

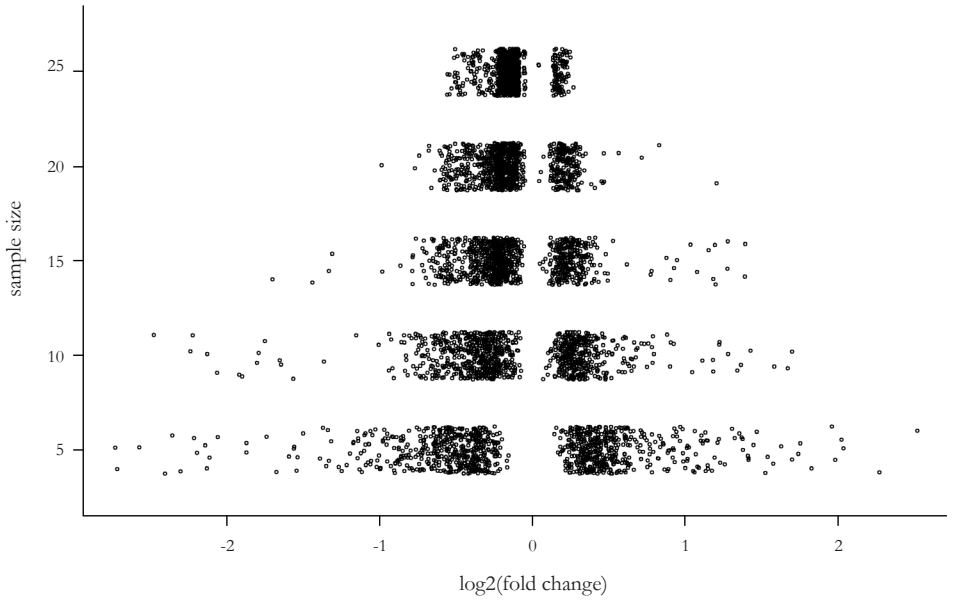

Figure 1 Inflation of the effect size in small sample size studies
Fold changes of the most significant miRNA in each of 1,000 subsamples for five different sample sizes (n = 5, 10, 15, 20, 25). In subsamples of 5 versus 5 individuals the heterogeneity is larger compared to subsamples of 20 versus 20 individuals, with generally higher absolute fold changes.

a corrected p-value < 0.1. In 15 of these subsamples more than 10 miRNAs reached a corrected p-value < 0.1 and in one subsample 165 miRNAs were differentially expressed with a corrected p-value < 0.1. This experiment was repeated using subsamples of 10 cases versus 10 controls. Here, we found 74 out of 1000 subsamples with at least one miRNA with a corrected p-value <0.1. In eight out of these 74 subsamples more than 10 miRNAs were differentially expressed. The maximum number of miRNAs that reached a corrected p-value <0.1 in a single subsample was 121.

Inflation of the effect size in small sample size studies can be illustrated using a similar analysis. Again, we selected 1,000 consecutive subsamples of five cases versus five controls and this time we recorded the fold change of the most significant miRNA in each subsample. This analysis was repeated using 1,000 subsamples of 10 versus 10, 15 versus 15, 20 versus 20 and 25 versus 25 individuals. We found that in small subsamples, fold changes were more heterogeneous and generally larger compared to larger subsamples (Figure 1). In other words, enlarging the size of the subsamples resulted in a regression to the mean. These results underline that the most significant effects found in studies with a small sample size are likely to be larger than those found in larger cohorts.

These analyses show that selection of a rather small cohort, in some cases, can result in the identification of false positive candidate biomarkers, whereas enlargement of the cohort would result in inability to confirm these results, leaving no candidate biomarkers for further validation. Indeed, in several published studies only few of the candidate biomarkers could be replicated in independent cohorts using a different technique[27–30]. On the other hand, the number of false-negative results in small sample size studies should not be underestimated.

Sample size calculation

The question remains what a sufficient sample size is in the search for miRNAs as biomarkers for disease. The identified candidate biomarker should be highly sensitive, with only modest 95% confidence intervals. Many methods of calculating a sufficient sample size have been described[31–35]. Ideally, a pilot study is performed in a cohort with the same characteristics as the intended larger cohort, using the same experimental protocols and measurement platform, before initiation of a large microarray experiment[36,37]. Based on the variability in this pilot study the power of the final study to detect a pre-specified effect size can be calculated. It should be noted that clinical significance of the effect should also be taken into account before initiation of large and costly miRNA microarray experiments.

CONCLUSION

In the search for new biomarkers for disease many miRNAs have been identified, however in replication studies only few of these findings have been reproduced. Part of the replication issues can be related to technical differences and heterogeneity in cohorts and clinical outcomes, but sample size also makes a major contribution discordance of study results. Here we show that a small sample size has a major impact on the replicability of the study, due to high false positive and false negative rates. In most clinical settings to achieve sufficient power while controlling for a low false discovery rate a large sample size is necessary. Omitting this essential point will lead to a high degree of false positive and false negative results and only little concordance between studies. Increasing sample size will result in higher costs of the experiment, but will eventually render more robust results.

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SUPPLEMENTAL METHODS

From the normalized microarray data of Cohort I (Chapter 4), data were selected for 36 male premature coronary artery disease (CAD) patients and 25 age- matched male controls after six weeks of medication use. To detect miRNAs differentially expressed between patients and controls, gene-wise linear models were fit with a patient status as explanatory followed by a moderated t-test (limma R package). Resulting p-values were adjusted to correct for multiple hypothesis testing using the Benjamin-Hochberg false discovery rate.

PART II

Other biomarkers for premature atherosclerosis

CHAPTER 6

Individuals with coronary artery disease at a young age and features of the metabolic syndrome have an increased prothrombotic potential

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ABSTRACT

The relation between coagulation and atherosclerosis has been extensively described, pointing towards a hypercoagulable state in patients with atherosclerosis, especially in young individuals. However, not all studies were conclusive. It is known that the metabolic syndrome (MS), a risk factor for coronary artery disease (CAD), is related to a higher incidence of thrombo-embolic events. We hypothesized that individuals with CAD at a young age and MS have an increased prothrombotic potential.

In this case-control study we analyzed the endogenous thrombin potential (ETP) and related thrombin generation parameters in patients with CAD before the age of 51 in men and 56 in women with and without MS features and their healthy first-degree relatives.

We included 118 CAD patients and 50 first-degree relatives (controls). Parameters of thrombin generation were obtained with calibrated automated thrombinography. An adjusted general linear model (GLM) showed a positive association between the peak thrombin levels and the presence of CAD at a young age. Based on the NCEP criteria we divided our patient group in CAD patients with and without MS, and compared them to the controls without MS. We showed that CAD patients with MS have increased ETP levels, both in comparison with healthy first-degree relatives and with CAD patients without MS. There were no differences in ETP between patients without MS and healthy controls

This study shows that individuals with CAD at a young age and MS features have an increased prothrombotic potential, compared to CAD patients without MS.

INTRODUCTION

The relation between coagulation and atherosclerosis has been extensively described. Different studies showed that hypercoagulable states, for example the presence of procoagulant mutations, are associated with an increased prevalence of myocardial infarction (MI)[1–3]. However, other studies could not confirm the association between these mutations and atherosclerosis[4,5]. In studies investigating coagulation factors or overall in vitro coagulation assays, also conflicting results were obtained. Increased coagulation in patients with coronary artery disease (CAD) has been described[6], whereas other studies did not show any relation between coagulation status and CAD[7]. Yet, what could be observed was, that hypercoagulability is most pronounced in *young* patients with CAD[8].

The metabolic syndrome (MS) is an important risk factor for the development of CAD, but is also known to be associated with a higher incidence of thrombo-embolic events[9,10]. At our outpatient clinic for premature CAD approximately one fourth of the patients met the NCEP ATIII criteria[11] for MS. We hypothesized that individuals with premature CAD and MS have an increased prothrombotic potential. We analyzed this by measuring thrombin generation using calibrated automated thrombinography (CAT) in patients with premature CAD with and without MS, and compared them to their healthy first-degree relatives (FDRs).

METHODS

Subjects

Between June 2009 and June 2011 consecutive patients at the outpatient clinic for premature CAD at the Academic Medical Center in Amsterdam were included. Patients were eligible for participation if they visited the outpatient clinic at least six months after their first premature cardiovascular event. Premature CAD was defined as either an acute MI or coronary atherosclerosis needing revascularisation by percutaneous coronary intervention or coronary artery bypass grafting, before the age of 51 in men and 56 in women. Patients were excluded if they suffered from stroke or peripheral artery disease or when they were using anticoagulant therapy.

Asymptomatic FDRs of premature CAD patients also visited the outpatient clinic for risk assessment. These relatives underwent coronary CT scanning to obtain a coronary calcium score (CAC), as marker for subclinical atherosclerosis. From the relatives seen between June 2009 and June 2011 we selected those that had a CAC score of zero and were above the age of 40 to exclude the possibility of false negative CAC scores in young individuals. Prospective follow-up data showed that asymptomatic subjects with a CAC score of zero had an event rate of 0.6 % per year[12]. Furthermore, subjects were excluded if the used any cardiovascular medication.

This study was approved by the ethics committee of the Academic Medical Center in Amsterdam and all subjects gave informed consent.

Assessment of cardiovascular risk factors

All participants were questioned about their medical history, family history, symptoms of CAD and lifestyle habits. Furthermore, height, weight and waist circumference as well as blood pressure, cholesterol and glucose levels were measured at the outpatient clinic. We defined hypertension and hypercholesterolemia as the use of anti-hypertensive medication or statins respectively, before the first event in patients and at inclusion for FDRs. In individuals that did not use medication, hypertension was defined as a systolic blood pressure >140 mmHg and/or diastolic blood pressure > 90 mmHg. Hypercholesterolemia was diagnosed if a fasting total cholesterol level was above 6.2 mmol/l[11]. Diabetes mellitus was defined as previously diagnosed and treated diabetes mellitus. Subjects were considered smokers if they were current smokers or when they quitted smoking within the last 5 years. In patients these characteristics were determined from data obtained by their treating physician before their cardiovascular event.

Coronary CT scanning

We performed a coronary CT scan in all FDRs to assess CAC, as a marker for the presence of coronary lesions. A 64-slice multidetector CT scanner (Philips Medical Systems, Best, The Netherlands) was used. The scanning protocol was as follows: tube voltage, 120 kV; tube current, 55 mAs; detector collimation, 40x2.5 mm; gantry rotation 420 ms. Data were transferred to a post-processing workstation (Extended Brilliance Workplace; Philips Medical Systems). CAC was recorded for the main arteries; we calculated the total score by summing lesion scores of all sections. For our control group we selected only those first-degree relatives that had a CAC score of zero.

Hemostatic assays

Prothrombin fragment 1+2 (F1+2) was determined by ELISA (Enzygnost F1+2, Siemens Healthcare Diagnostics, Marburg, Germany).

The CAT assays the generation of thrombin in clotting plasma. The assay was carried out as previously described[13] and the Thrombinoscope manual (Thrombinoscope BV, Maastricht, the Netherlands). Coagulation was triggered by recalcification in the presence of 5 pM recombinant human tissue factor (Innovin, Siemens Healthcare Diagnostics), 4 μM phospholipids, and 417 μM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was monitored using a Fluoroskan Ascent fluorometer (ThermoLabsystems, Helsinki, Finland). The thrombin generation parameters (peak thrombin and endogenous thrombin potential) were calculated using Thrombinoscope® software (Thrombinoscope BV), and normalized to pooled human normal plasma[14].

The metabolic syndrome

The metabolic syndrome is a clinical diagnosis that is used for risk stratification for CAD. According to the NCEP guidelines a patient has MS when three or more of the following determinants are present: waist circumference > 102 cm in men and > 88 cm in women, triglycerides > 1.69 mmol/L, HDL-C levels < 1.03 mmol/L in men and < 1.29 mmol/L in women, blood pressure

≥ 130/ ≥ 85 mmHg and fasting glucose > 6.11 mmol/L[15]. Using these criteria, we divided the cohort in subjects with MS and without MS. We excluded FDRs with MS (n=7), since this group was too small to analyse separately.

Statistical analyses

Results are expressed as mean ± standard deviation, except if indicated otherwise. Student's t-tests and Chi-square tests were used to calculate differences in baseline characteristics. Variables with a skewed distribution were log transformed before analysis.

We used an adjusted General Linear Model (GLM) to analyse the relation between the dependent variables peak thrombin and ETP and the independent variable of the presence of CAD at a young age. This model was first adjusted for age and sex and additionally adjusted for smoking and oral contraceptive use. For both GLM models we chose not to correct for other risk factors for CAD, that were significantly different between patients and controls, since these factors may contribute to the development of the disease and are therefore intermediate factors leading to CAD. Additionally, correction for CRP levels did not influence the results and was therefore excluded from the final model.

Similar analyses were performed to analyse the relation between the ETP and the presence of the metabolic syndrome in patients with premature CAD compared to healthy FDRs. All analyses were performed using SPSS for Windows 19.0. A p-value < 0.05 was considered statistically significant.

RESULTS

Subjects

In total 333 subjects visited the outpatient clinic, of which 129 were patients and 204 were FDRs. In the patient group 11 subjects were using anticoagulant therapy and were excluded from the analysis. This left us with 118 patients. Of the FDRs 75 had a positive CAC score, 61 had a CAC score of 0, but were under the age of 40 years and 18 additional subjects were using cardiovascular medication. Therefore, we were left with 50 healthy FDRs eligible for inclusion.

Baseline characteristics are shown in Table 1. Patients were significantly more often male compared to the FDRs. None of the patients suffered from vascular spasms or solely thrombotic occlusions. As expected, classic risk factors for CAD were more often present in patients compared to the healthy FDRs. There were no differences in ETP, peak thrombin and F1+2 between patients and healthy FDRs.

General linear model of patients with premature CAD versus healthy FDRs

After adjusting for possible confounders, GLM analysis showed a positive association between peak thrombin and the presence of premature CAD, with increased peak thrombin levels in patients (β 9.4; 95% CI 0.9–18.0). We did not observe significant differences in ETP and F1+2 levels between patients and healthy FDRs, either with or without adjusting for confounders (models 1 to 3, Table 2).

Table 1 Baseline characteristics of the total cohort

	FDRs (n = 50)	CAD patients (n = 118)
Age, years	47.2 ± 6.3	47.9 ± 7.5
Male gender, n (%)	12 (24) *	77 (65) *
Event type, n (%)		
MI	-	84 (71)
PTCA	-	22 (19)
CABG	-	12 (10)
Smoking, n (%)	11 (22) *	60 (51) *
Hypercholesterolemia, n (%)	9 (18) *	42 (36) *
Hypertension, n (%)	6 (12)	28 (24)
Diabetes, n (%)	1 (2)	9 (8)
Statin use, n (%)	-	104 (88)
Anti-hypertensive therapy, n (%)	-	104 (88)
Anti-platelet therapy, n (%)	-	103 (87)
BMI, kg/m2	26.0 ± 3.8 *	27.6 ± 4.4 *
Waist circumference, cm	94.0 ± 10.5 *	99.8 ± 12.8 *
Systolic blood pressure, mmHg	124.9 ± 16.4	127.4 ± 20.9
Diastolic blood pressure, mmHg	77.2 ± 9.7	78.5 ± 12.3
Oral contraceptive use, n (%)	6 (12) *	2 (2) *
Glucose, mmol/L	5.2 ± 0.7 *	5.8 ± 1.4 *
Total cholesterol, mmol/L	5.7 ± 0.8 *	4.3 ± 1.0 *
HDL-cholesterol, mmol/L	1.6 ± 0.4 *	1.2 ± 0.3 *
LDL-cholesterol, mmol/L	3.5 ± 1.0 *	2.4 ± 0.8 *
Triglycerides, mmol/L	1.2 ± 0.6 *	1.7 ± 2.6 *
CRP, mg/L	2.7 ± 4.3	3.5 ± 6.6
ALAT, mmol/L	23.6 ± 12.3 *	35.1 ± 14.4 *
Gamma GT, mmol/L	23.3 ± 13.7 *	41.6 ± 32.8 *
ETP, %	109.0 ± 19.4	106.6 ± 18.0
Peak thrombin, %	116.4 ± 31.1	113.5 ± 23.7
F1+2, pmol/L	210.8 ± 121.8	221.4 ± 379.4

Continuous data are expressed as mean ± SD, categorical data as absolute numbers with (percentages). * $p<0.05$ compared to controls.

ALAT, alanine aminotransferase; BMI, body mass index; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CRP, C-reactive protein; ETP, endogenous thrombin potential; F1+2, prothrombin fragment 1+2; FDR, first-degree relatives; Gamma-GT, gamma-glutamyltransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction PTCA, percutaneous transluminal coronary angioplasty.

Table 2 General linear model of the association between ETP and the presence of premature CAD compared to healthy FDRs

	ETP β (95%CI)	Peak β (95%CI)	F1+2 β (95%CI)
FDRs	1.0	1.0	1.0
CAD patients			
Model 1	-2.5 (-8.6 - 3.7)	-2.9 (-11.6 - 5.7)	10.6 (-97.8 - 118.9)
Model 2	1.1 (-5.3 - 7.5)	6.3 (-2.4 - 14.9)	46.0 (-70.8 - 162.9)
Model 3	2.5 (-4.1 - 9.1)	9.4 (0.9 – 18.0)*	74.9 (-47.7 - 197.4)

Model 1, crude model; model 2, adjusted for age and sex; model 3, additionally adjusted for smoking and oral contraceptive use. Outcomes are expressed as β with corresponding 95% confidence intervals. The β represents the percentage of change of the outcome variable in the studied population for every unit increase of the outcome variable in the reference population. * $p<0.05$ compared to healthy FDRs.
CAD, coronary artery disease; ETP, endogenous thrombin potential; FDR, first-degree relatives; F1+2, prothrombin fragment 1+2

Metabolic syndrome

Based on the NCEP guidelines we divided our CAD patients in a group of 34 subjects with MS and 87 subjects without MS. We excluded subjects with MS from the control group, which left us with 43 healthy FDRs.

Table 3 shows the differences in classic risk factors and MS determinants between the three groups. The differences between CAD patients and controls could still be observed. Interestingly, hypercholesterolemia was not significantly different between CAD patients without MS and healthy FDRs. Furthermore, higher liver enzyme levels were seen in CAD patients with MS compared to CAD patients without MS and healthy FDRs which might be the result of hepatic steatosis accompanying MS.

General linear model of CAD patients with and without MS versus healthy FDRs

ETP was positively associated with the presence of MS after adjustment for possible confounders. Both in comparison with healthy FDRs (β 13.0; 95% CI 4.5-21.4) and with premature CAD patients without MS (β 12.9; 95% CI 5.9-19.8), ETP was increased in premature CAD patients with MS. No difference in ETP levels was observed between healthy FDRs and premature CAD patients without MS.

The peak thrombin level was positively associated with the presence of MS compared to healthy FDRs (β 17.2; 95% CI 5.8-28.5). We did not observe any differences in peak between patients with and without MS and between premature CAD patients without MS and controls. F1+2 levels were not associated with the presence of MS (Table 4 and Figure 1).

Table 3 Baseline characteristics of premature CAD patients with and without metabolic syndrome features and healthy FDRs

	FDRs (n = 43)	CAD without MS (n = 87)	CAD with MS (n= 31)
Age, years	47.1 ± 6.4	47.5 ± 6.8	48.2 ± 9.5
Male gender, n (%)	12 (28)	55 (63) *	21 (68) *
Event type, n (%)			
MI	-	63 (72)	21 (68)
PTCA	-	15 (17)	7 (23)
CABG	-	9 (10)	3 (10)
Smoking, n (%)	10 (23)	41 (47) *	18 (58) *
Hypercholesterolemia, n (%)	8 (19)	26 (30)	16 (52) * †
Hypertension, n (%)	6 (14)	18 (21)	10 (32)
Diabetes, n (%)	1 (2)	1 (1) *	8 (26) * †
Statin use, n (%)	-	78 (90)	26 (84)
Anti-hypertensive therapy, n (%)	-	76 (87)	28 (90)
Anti-platelet therapy, n (%)	-	76 (87)	27 (87)
BMI, kg/m2	25.8 ± 4.0	26.3 ± 3.7	31.3 ± 4.0 * †
Waist circumference, cm	93.1 ± 9.8	95.8 ± 11.0	110.7 ± 10.8 * †
Systolic blood pressure, mmHg	124.5 ± 16.9	124.3 ± 20.8	136.6 ± 19.2 * †
Diastolic blood pressure, mmHg	76.8 ± 9.8	77.1 ± 12.2	85.5 ± 12.0 * †
Oral contraceptive use, n (%)	8 (19)	7 (8) *	0 (0) *
Glucose, mmol/L	5.0 ± 0.4	5.4 ± 0.6 *	6.8 ± 2.3 * †
Total cholesterol, mmol/L	5.7 ± 0.8	4.1 ± 0.9 *	4.9 ± 1.2 * †
HDL-cholesterol, mmol/L	1.6 ± 0.4	1.3 ± 0.3 *	1.0 ± 0.4 * †
LDL-cholesterol, mmol/L	3.6 ± 0.8	2.3 ± 0.8 *	2.5 ± 0.9 *
Triglycerides, mmol/L	1.1 ± 0.4	1.2 ± 0.6	3.6 ± 4.5 * †
CRP, mg/L	2.6 ± 4.5	2.6 ± 4.1	5.0 ± 9.9
ALAT, mmol/L	24.6 ± 12.8	34.3 ± 15.0	37.7 ± 20.2 *
Gamma-GT, mmol/L	24.4 ± 14.4	36.8 ± 26.1	57.8 ± 45.4 * †
ETP, %	107.2 ± 18.2	103.4 ± 17.7	115.3 ± 16.3 †
Peak thrombin, %	113.1 ± 29.8	111.9 ± 24.5	118.9 ± 20.5
F1+2, pmol/L	207.0 ± 127.0	224.7 ± 421.8	214.2 ± 237.0

Continuous data are expressed as mean ± SD, categorical data as absolute numbers with (percentages).
* $p<0.05$ compared to controls. † $p<0.05$ compared to CAD patients without MS.
ALAT, alanine aminotransferase; BMI, body mass index; CAGB, coronary artery bypass grafting; CAD, coronary artery disease; CRP, C-reactive protein; ETP, endogenous thrombin potential; F1+2, prothrombin fragment 1+2; FDR, first-degree relatives; Gamma-GT, gamma-glutamyltransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction; MS, metabolic syndrome; PTCA, percutaneous transluminal coronary angioplasty.

Table 4 General linear model to assess the association between ETP and the presence of MS in premature CAD patients compared to CAD patients without MS and healthy FDRs

	ETP β (95%CI)	**Peak β (95%CI)**	**F1+2 β (95%CI)**
FDRs	1.0	1.0	1.0
CAD without MS			
Model 1	-3.8 (-10.2 - 2.7)	-1.2 (-10.5 - 8.1)	-0.1 (-0.3 - 0.1)
Model 2	-0.7 (-7.2 - 5.8)	6.5 (-2.6 - 15.5)	0.0 (-0.2 - 0.2)
Model 3	0.1 (-6.6 – 6.8)	8.2 (-0.7 - 17.2)	0.0 (-0.2 - 0.2)
CAD with MS			
Model 1	8.1 (-0.1 - 16.3)	5.8 (-6.0 - 17.6)	-0.1 (-0.3 - 0.1)
Model 2	11.8 (3.7 - 20.0) *	14.6 (3.3 - 25.9) *	-0.1 (-0.3 - 0.2)
Model 3	13.0 (4.5 - 21.4) *	17.2 (5.8 - 28.5) *	0.0 (-0.3 - 0.3)
	ETP β (95%CI)	**Peak β (95%CI)**	**F1+2 β (95%CI)**
CAD without MS	1.0	1.0	1.0
CAD with MS			
Model 1	11.9 (4.7 - 19.2) *	7.0 (-3.4 - 17.5)	0.0 (-0.2 - 0.2)
Model 2	12.5 (5.6 - 19.5) *	8.2 (-1.5 - 17.8)	0.0 (-0.2 - 0.2)
Model 3	12.9 (5.9 - 19.8) *	8.9 (-0.4 - 18.2)	0.0 (-0.2 - 0.2)

Model 1, crude model; model 2, adjusted for age and sex; model 3, additionally adjusted for smoking and oral contraceptive use. Outcomes are expressed as β with corresponding 95% confidence intervals. The β represents the percentage of change of the outcome variable in the studied population for every unit increase of the outcome variable in the reference population. * $p < 0.05$ compared to the reference group
CAD, coronary artery disease; ETP, endogenous thrombin potential; FDRs, first-degree relatives; F1+2, prothrombin fragment 1+2; MS, metabolic syndrome

Figure 1A

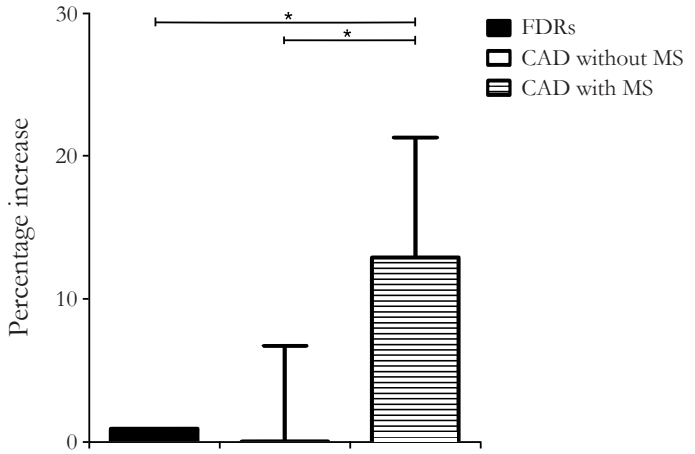

Figure 1B

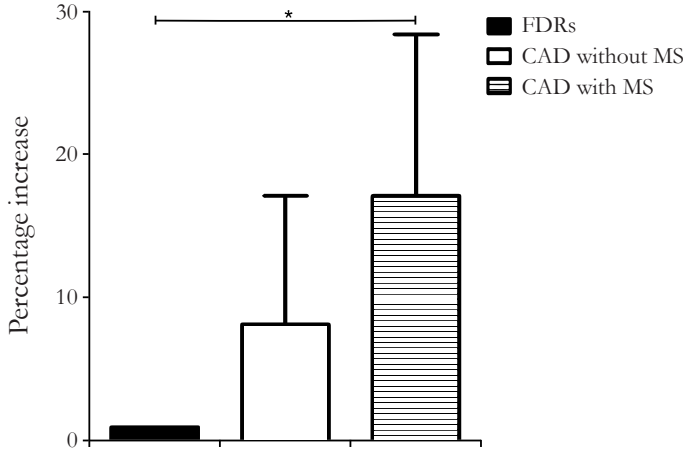

Figure 1 The association between ETP and the presence of MS in premature CAD patients compared to CAD patients without MS and healthy FDRs

The figure shows the increase in ETP **(A)** and Peak thrombin **(B)** for patients with CAD without MS and patients with CAD with MS compared to one percent increase in ETP and Peak thrombin of the FDRs. The results are adjusted for age, sex, smoking and oral contraceptive use.

* $p < 0.05$ compared to FDRs

CAD, coronary artery disease; ETP, endogenous thrombin potential; FDRs, first-degree relatives; MS, metabolic syndrome

DISCUSSION

In this study we show that patients with premature CAD have a higher prothrombotic potential compared to healthy FDRs, only if they have MS. ETP, reflecting total thrombin formation, was significantly higher in CAD patients with MS compared to both CAD patients without MS and healthy FDRs. Besides, also peak thrombin levels were significantly higher in CAD patients with MS compared to healthy FDRs. Our data suggest that MS is an important contributor to the hypercoagulable state in patients with premature CAD.

Clinical studies have shown conflicting results about the relation between thrombophilic defects and CAD. Different meta-analyses show only modest associations of procoagulant mutations with CAD[8,16,17]. Fibrinogen remained the only coagulation factor that, in a meta-analysis of cross-sectional data, was strongly associated with CAD[2].

All previously described studies investigated single factors from the coagulation cascade. Concurrency of single alterations could lead to a shift towards a more procoagulant phenotype in CAD patients. Therefore, several groups have studied markers of active coagulation. It was observed that F1+2 was elevated in patients with proven CAD[19]. Furthermore, plasma levels of F1+2 were shown to be associated with intima-media thickness in healthy controls[20,21]. Thrombin-antithrombin complexes were shown to be elevated in CAD patients. In the same cohort, no difference was seen in ETP[6]. Also, a prospective analysis in an elderly population could not demonstrate a relation between ETP and the development of CAD[7]. However, Smid and colleagues demonstrated that ETP and peak thrombin levels are elevated in the acute phase of a MI and at six months follow-up[22].

The underlying mechanism related to the increased prothrombotic potential in young CAD patients has not been unravelled yet. Previous studies did not look at baseline characteristics between patients and controls that could (partially) explain the differences in coagulation parameters. Risk factors for the development of CAD, especially determinants of the metabolic syndrome, have been described to be associated with hypercoagulability. Obese children have a higher prothrombotic potential than lean children[23,24]. Similar results were seen in obese adults in whom the ETP was shown to decrease after weight loss[25,26]. This observation might be due to the fact that several coagulation factors are associated with MS criteria. Epidemiological data suggests an association between fibrinogen levels and increasing BMI[27,28] as well as reduced HDL-cholesterol levels[27]. Furthermore, plasma fibrinogen levels were reported to be increased in type 2 diabetes[29,30]. Moreover, coagulation factor VII levels increased with increasing triglyceride levels[31,32] and plasma levels were elevated in healthy subjects with MS[33]. Besides, fibrinolysis is impaired in these subjects, due to elevated PAI-1 levels[34–36]. Altogether, these alterations in coagulation factors might contribute to the higher thrombotic event rate observed in individuals with MS[9,10].

We postulate that the observed increase in prothrombotic potential in premature CAD patients compared to healthy FDRs could be explained by the presence of MS. We showed that young CAD patients with MS features have a significantly increased in vitro thrombin generation compared to CAD patients without MS features and healthy FDRs. Surprisingly, in contrast to a previous study[37], we did not observe any difference in F1+2 levels between patients with and without MS. This could be explained by the fact that we did not include healthy subjects with MS, as was done previously. In our study we included patients with established CAD, who used statins for secondary prevention. It is known that statins reduce F1+2 levels[38], which probably influenced our results.

Strengths and limitations

There are some limitations of our study that must be addressed. First, the unadjusted analysis did not show a significant relation. However, there were several confounding factors that distorted the relation between MS features in CAD patients and increased ETP levels, of which gender and oral contraceptive use were the most pronounced. Indeed, after adjusting for these confounders the association became apparent. It is known that both gender and oral contraceptive use have marked influences on ETP[39,40].

Secondly, the thrombin generation assay that was used in this study includes tissue factor as trigger of coagulation. When contact activation is not prevented by corn trypsin inhibitor this may influence thrombin generation[41]. Since corn trypsin inhibitor was not used in this study this may be regarded as a limitation. However, the effect of contact activation on thrombin generation in the presence of 5 pM tissue factor is very small, and will not be different between patients and controls[42]. It is therefore unlikely that this influenced our results and conclusions.

CONCLUSION

In summary this study shows that individuals with CAD at a young age and MS features have an increased prothrombotic potential, compared to CAD patients without MS. Whether this increased prothrombotic potential leads to more recurrent events in patients with MS features needs to be investigated in a prospective study.

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CHAPTER 7

Individuals with subclinical atherosclerosis have impaired proliferation of blood outgrowth endothelial cells, which can be restored by statin therapy

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ABSTRACT

To study the regenerative capacity of the endothelium in patients with coronary artery disease (CAD), we cultured blood outgrowth endothelial cells (BOECs) of patients with premature CAD and their first degree relatives (FDR). Additionally we evaluated the influence of statin treatment on circulating BOEC precursors in subjects with subclinical atherosclerosis.

Patients with premature CAD (men <51yr, women <56yr) and their FDRs were included. Based on coronary calcification (CAC) scores FDRs were divided in a group of healthy subjects (CAC=0) and subjects with subclinical atherosclerosis (CAC>0). We did not observe differences in the number of BOEC colonies and proliferation between premature CAD patients and FDRs. FDRs with subclinical atherosclerosis had lower colony numbers compared with healthy FDRs, however this was not statistically significant, and BOEC proliferation was significantly impaired (OR=0.45, 95% CI 0.21-0.96). Unexpectedly, the number of BOEC colonies and BOEC proliferation were similar for premature CAD patients and healthy FDRs. Since a considerable number of premature CAD patients used statins, we studied the number of BOEC precursors as well as their proliferative capacity in ten individuals with subclinical atherosclerosis, before and after statin therapy. Interestingly, FDRs with subclinical atherosclerosis showed a significant increase in the number of BOEC colonies after statin therapy.

BOEC proliferation of subjects with subclinical atherosclerosis is impaired compared with healthy controls. In these subjects, statin therapy significantly increased the number of circulating BOEC precursors as well as their proliferative capacity, revealing a beneficial effect of statins on endothelial regeneration.

INTRODUCTION

Cardiovascular disease (CVD) represents a major health issue worldwide[1]. Endothelial dysfunction, resulting in atherosclerotic plaque formation and subsequent ischemia is the main underlying cause of this disease[2]. Circulating endothelial progenitor cells (EPCs), have been implicated in vasculogenesis in a large number of experimental studies. Their vasculogenic properties make them attractive for treatment of CVD[3]. Levels of circulating EPCs are lower in patients with coronary artery disease (CAD) than in healthy controls[4] and it has been proposed that circulating EPC levels might be a useful diagnostic tool to identify patients at increased cardiovascular risk[5,6].

EPCs represent a heterogeneous population of circulating mononuclear cells[3], among which two distinct populations can be distinguished[7]: early endothelial progenitor cells (eEPCs) and blood outgrowth endothelial cells (BOECs), also designated as endothelial colony forming cells (ECFCs)[8,9]. Despite their late onset, BOECs display an enhanced proliferative capacity compared with eEPCs[8]. Comparative genetic and phenotypic analyses have firmly established that eEPCs display a gene-transcription profile similar to cells of the hematopoietic lineage, whereas BOECs belong to the endothelial lineage. This implies that the regenerative capacity of the endothelium is crucially dependent on the frequency of these "true EPCs" in the circulation[7,9,10].

At present, limited information exists about the relation between BOECs and cardiovascular risk. We hypothesized that subjects with premature CAD or subclinical coronary atherosclerosis, as assessed by coronary CT-scanning, have lower levels of circulating BOECs and that proliferation of these cells is impaired compared with healthy controls. Besides, since it is known that statin therapy results in a rise in EPC levels[11,12], we additionally wanted to investigate whether this also holds true for circulating BOECs. Therefore, we studied the effect of statin therapy on circulating BOECs in FDRs with subclinical atherosclerosis.

METHODS

Subjects

Between June 2011 and June 2012 all consecutive premature CAD patients that visited the outpatient clinic at the Academic Medical Center in Amsterdam, the Netherlands, were included in this study. Premature CAD was defined as either an acute myocardial infarction or coronary atherosclerosis requiring revascularization by percutaneous coronary intervention or coronary artery bypass grafting, before the age of 51 in men and 56 years in women in line with the GENECARD definition[13]. Asymptomatic FDRs of patients with premature CAD also visited the outpatient clinic for risk assessment. All FDRs over the age of 30 years underwent coronary CT scanning to obtain a coronary calcium score (CAC), as a marker of subclinical atherosclerosis. Based on the CAC score, these subjects were divided in two groups. The first group consisted of subjects with a coronary calcium score of zero and subjects below the age of 30 in whom no CT-scanning was performed. These subjects were considered to be healthy FDRs. The second

group consisted of all subjects with a positive CAC score, representing FDRs with subclinical atherosclerosis. For this study we did not perform a power calculation, since data on BOECs in CAD patients is limited. However, we assume that differences will be similar to those found in levels of early EPCs between CAD patients and healthy controls[14]. Therefore, an inclusion period of one year should be sufficient to reach significant differences between the groups.

Ethics statement

This study complies with the Declaration of Helsinki and was approved by the ethics committee of the Academic Medical Center in Amsterdam, the Netherlands. All subjects gave written consent.

Assessment of cardiovascular risk factors

Clinical risk factors in premature CAD patients and FDRs were assessed at the outpatient clinic. In short, all participants were questioned about their medical history, family history, symptoms of coronary artery disease and lifestyle habits. Hypertension was defined as the use of antihypertensive medication or in untreated individuals as a systolic blood pressure > 140 mmHg and/or a diastolic blood pressure > 90 mmHg. We defined hypercholesterolemia as the use of lipid-lowering medication or a fasting total cholesterol level above 6.2 mmol/L[15]. Both definitions are according to the third report of the National Cholesterol Education Program[16]. Subjects were considered smokers if they were current smokers or when they quitted smoking within the last 5 years.

In patients these characteristics were determined from data obtained by their treating physician before their cardiovascular event. Venous blood was drawn after a 12 hour overnight fast for clinical laboratory measurements.

Coronary CT scanning

We performed a coronary CT scan in all FDRs to assess CAC, as a marker for subclinical atherosclerosis. A 64-slice multidetector CT scanner (Philips Medical Systems, Best, The Netherlands) was used to perform the CT scans. The scanning protocol was as follows: tube voltage, 120 kV; tube current, 55 mAs; detector collimation, 40x2.5 mm; gantry rotation 420 ms. Data were transferred to a post-processing workstation (Extended Brilliance Workplace; Philips Medical Systems). CAC was recorded for the main arteries; we calculated the total score by summing lesion scores of all sections.

BOEC-colony isolation, propagation and cryopreservation.

Heparinized blood samples were collected from all participants and were processed within four hours. BOEC were isolated according to a recently published protocol[17]. Multi-well 48 plates (Thermo-Scientific, Bremen, Germany) pre-coated with collagen type I were seeded with 1.5 x 10^6 peripheral blood mononuclear cell (PBMCs) per well in EGM2 medium. Medium was changed three times per week. Plates were monitored at weekly intervals and wells that were filled with colonies with a cobblestone-like morphology were counted on days 7, 14, 21 and 28. As soon as a colony appeared and became 30-70% confluent, it was transferred to plates with 1.88-cm^2 culture

wells (MW-24, Thermo-Scientific) and upon confluency further propagated in 10 cm^2 and 75 cm^2 culture flasks. Subsequently, cells were harvested by trypsinization. 10 ml of EGM2 was added and cells were transferred to a 50 ml conical tube for centrifugation (5 min. 290*g* at 4^0C). Cells were washed twice with EGM2 and slowly resuspended in fetal bovine serum supplemented with 5% DMSO (vol/vol). Cryovials were deposited in freezing containers (Thermo Scientific) which were stored at -80^0C overnight. Next day cryovials were transferred to containers with liquid nitrogen. A maximum of 3 BOEC colonies per individual were cryopreserved. The ability to cryopreserve BOECs originating from a single colony was considered indicative for its proliferative capacity. Successful cryopreservation of at least one out of three propagated colonies was considered indicative for the proliferative capacity of BOECs derived from a specific subject.

Immunocytochemistry and fluorescence imaging

Immunocytochemistry and fluorescence imaging of fixed cells was performed as previously described[18] using mouse clonal anti-VWF Rag20[19] and rabbit polyclonal anti-VE-cadherin (BMS158, eBioscience, San Diego, CA, USA) followed by goat anti-mouse IgG-Alexa 568 and goat anti-rabbit IgG-Alexa 488 (Molecular Probes, Leiden, The Netherlands).

Intervention study

To study the effect of statin therapy on circulating BOECs we included 10 subjects with the most extended subclinical atherosclerosis. These subjects all had a CAC score above the 80^{th} percentile for age and gender. We compared BOEC colony formation in these subjects at baseline and after at least six months of statin therapy.

Statistical analyses

Results are expressed as mean ± standard deviation (SD), except if indicated otherwise. Student's t-tests and Chi-square tests were used to calculate differences in baseline characteristics between the groups. Variables that did not show a normal distribution were log transformed before they were analysed.

To determine the efficiency of colony isolation we counted the number of wells that were filled with BOEC colonies at day 7, 14, 21 and 28. The number of colonies per ml of blood used for the isolation was calculated for all samples.

We used a logistic regression model to analyse the relation between the dependent variables "successful isolation" and "cryopreservation", as a marker of proliferation capacity and the independent variable of the presence of (subclinical) CAD. This model was first adjusted for age and sex and additionally adjusted for smoking. We chose not to correct for other risk factors for CVD, since these factors may contribute to the development of the disease and are therefore intermediate factors leading to CVD. We used an adjusted General Linear Model (GLM) to analyse the relation between the dependent variables "number of colonies" per time point and the independent variable of the presence of (subclinical) CAD. This model was first adjusted for age and sex and additionally adjusted for smoking. Again, additional risk factors were considered intermediate factors leading to CVD and therefore we chose not to correct for those. From

the cumulative numbers of colonies per time point we calculated the non-cumulative increase in colony number per time point. For each subject we recorded the maximum non-cumulative increase in colony number and the associated time point in two new variables. For subjects that did not provide any colonies we recorded a maximum non-cumulative increase of zero at time point 35 days. The "time to maximum increase" was obtained by dividing the maximum non-cumulative increase in colony number by the associated time point. A similar GLM as described above was used to analyse these variables. Data in all GLM models was log transformed before the analyses; log(colony number day 28 + 0.001), log(maximum increase + 0.001) and log(time to maximum increase + 0.0001), respectively, were used in analyses to account for zero values.

A Wilcoxon signed rank test was used to analyse the number of colonies before and after statin therapy in the intervention study. All analyses were performed using SPSS for Windows 19.0. A p-value < 0.05 was considered statistically significant.

RESULTS

Subjects

In total 227 individuals met the inclusion criteria, of which 70 were patients, 99 were healthy FDRs and 58 were FDRs with subclinical atherosclerosis. Baseline characteristics are shown in Table 1. Patients were significantly more often male compared with FDRs. Furthermore, classical risk factors for CVD were more often present in patients compared with the healthy FDRs. Due to the high percentage of subjects on statin therapy in the patient group, no data on cholesterol levels are provided.

BOECs isolation and proliferation

Colonies were cultured from the majority of subjects. Immunocytochemistry and fluorescence imaging revealed that these colonies were BOECs (Figure 1).

At baseline, proliferative capacity was significantly impaired in FDRs with subclinical atherosclerosis compared with healthy FDRs (53% vs 70%; $p<0.05$, Table 1). Interestingly, patients showed an intermediate phonotype of 61% proliferative capacity, which is not significantly different compared to either the healthy FDRs or the FDRs with subclinical atherosclerosis (Table 1).

An adjusted logistic regression confirmed that the proliferative capacity of BOEC colonies was significantly lower in FDRs with subclinical atherosclerosis compared with healthy FDRs (OR=0.45; 95%CI 0.21-0.96; $p<0.05$, Table 2). Again, patients showed an intermediate phenotype which was not significantly different compared to either the healthy FDRs of the FDRs with subclinical atherosclerosis. Additionally, we did not observe any differences in the success rate of colony isolation between the three groups (Table 2.)

Table 1 Baseline characteristics

	Healthy FDRs	FDRs with subclinical atherosclerosis	Patients
n	99	58	70
Age, years ± SD	41.5 ± 11.2	53.0 ± 9.1 *	55.3 ± 9.8 *
Gender, male (%)	38 (38)	26 (45)	53 (76) * †
Hypercholesterolemia, n (%)	18 (18)	20 (34) *	38 (54) * †
Hypertension, n (%)	13 (13)	23 (40) *	30 (43) *
Diabetes, n (%)	2 (2)	8 (14) *	10 (14) *
Smoking, n (%)	36 (37)	18 (32)	35 (50) †
Statin use, n (%)	11 (11)	19 (33) *	66 (94) * †
BMI, kg/m2 ± SD	25.6 ± 4.2	27.5± 3.7 *	27.8 ± 4.1 *
Systolic blood pressure, mmHg ± SD	126 ± 12	135 ± 16 *	132 ± 17 *
Diastolic blood pressure, mmHg ± SD	77 ± 9	83 ± 9 *	80 ± 12 *
Glucose, mmol/L ± SD	5.0 ± 0.6	5.5 ± 1.2 *	5.9 ± 1.8 *
Total cholesterol, mmol/L ± SD	5.3 ± 1.0	5.7 ± 1.0 *	
HDL cholesterol, mmol/L ± SD	1.4 ± 0.4	1.4 ± 0.4	
LDL cholesterol, mmol/L ± SD	3.4 ± 0.9	3.6 ± 0.9	
Triglycerides, mmol/L ± SD	1.1 ± 0.8	1.5 ± 0.8 *	
Successful isolation, n (%)	79 (80)	42 (72)	52 (74)
Colonies at day 28, n [IQR]	0.07 [0–0.15]	0.06 [0–0.11]	0.07 [0-0.19]
Max increase in colony number, n [IQR]	0.04 [0.04–0.11]	0.04 [0–0.07]	0.04 [0-0.11]
Time point max increase, days ± SD	23 ± 8	24 ± 8	22 ± 8
Time to max increase [IQR]	0.003 [0.001-0.005]	0.002 [0-0.003]	0.003 [0-0.006]
Proliferation capacity, n (%)	69 (70)	31 (53) *	43 (61)

Continuous data are expressed as mean ± SD or median [IQR], categorical data as absolute numbers with (percentages). * $p<0.05$ compared to healthy FDRs. † $p<0.05$ compared to FDRs with subclinical atherosclerosis.

BMI, body mass index; FDRs, first-degree relatives; HDL, high-density lipoprotein; IQR, inter quartile range; LDL, low-density lipoprotein; SD, standard deviation.

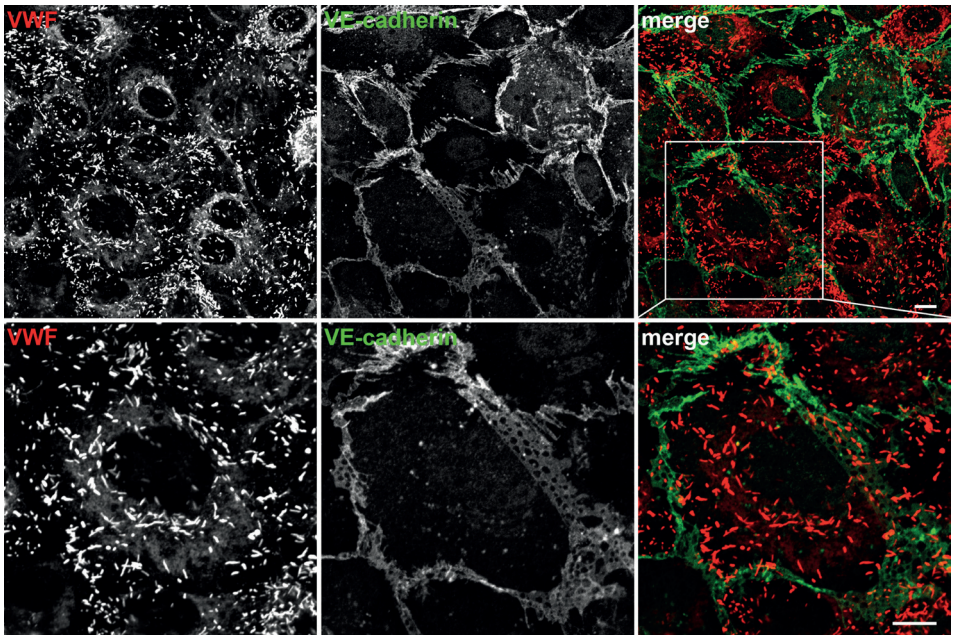

Figure 1 Morphological characterization of blood outgrowth endothelial cells
Paraformaldehyde fixed BOECs were immunostained for VWF (red) and VE-cadherin (green) to delineate Weibel-Palade bodies and endothelial cell boundaries. Magnifications of the boxed region are shown in the bottom row. Scale bars represent 10 μm.

To be able to draw any conclusions about the colony forming rate of BOECs it is important to not only analyse the number of colonies, but also the rate at which the colonies are formed. High numbers of BOEC colonies at an early stage represent a highly viable cell culture. Therefore, we calculated the time to maximum increase, by dividing the maximum non-cumulative increase in colony number of colonies by the time point associated with this maximum increase. After adjusting for age, gender and smoking, GLM analysis showed a positive association between the time point of maximum increase and the presence of subclinical atherosclerosis (B=0.14; 95%CI 0.01-0.27; $p<0.05$, Table 3), meaning that the maximum increase occurs later in FDRs with subclinical atherosclerosis compared with healthy FDRs. Also, we observed a trend towards a negative association between the maximum increase and subclinical atherosclerosis (B=-0.67; 95%CI -1.43 – 0.008; p=0.081, Table 3), with a lower maximum increase in colony numbers in FDRs with subclinical atherosclerosis compared with healthy FDRs. Together, this resulted in a trend towards a negative association between the time to maximum increase and the presence of subclinical atherosclerosis, meaning that the time to maximum increase seems lower in FDRs with subclinical atherosclerosis compared with healthy FDRs. These analyses again showed an intermediate phenotype in patients (Table 3).

Table 2 Relation between colony isolation success rate and proliferation capacity and subclinical or overt CAD

	Colony isolation		Proliferation capacity	
	OR	**95% CI**	**OR**	**95% CI**
Healthy FDRs Patients	1.0		1.0	
Model 1	0.73	0.35 – 1.51	0.69	0.36 – 1.32
Model 2	0.63	0.26 – 1.53	0.68	0.31 – 1.49
Model 3	0.54	0.22 – 1.37	0.63	0.28 – 1.43
FDRs with subclinical CAD				
Model 1	0.67	0.31 – 1.42	0.50	0.26 – 0.98 *
Model 2	0.58	0.25 – 1.35	0.47	0.22 – 0.99 *
Model 3	0.55	0.23 – 1.29	0.45	0.21 – 0.96 *
Patients FDRs with subclinical CAD			1.0	
Model 1	0.91	0.41 – 2.00	0.72	0.36 – 1.46
Model 2	0.93	0.41 – 2.10	0.70	0.34 – 1.45
Model 3	1.01	0.44 – 2.31	0.72	0.34 – 1.51

Model 1, crude model; model 2, adjusted for age and sex; model 3, additionally adjusted for smoking. Outcomes are expressed as odds ratios with corresponding 95% confidence intervals * $p<0.05$. CAD, coronary artery disease; CI, confidence interval; FDRs, first-degree relatives; OR, odds ratio.

Table 3 Association between BOEC isolation parameters and the presence of subclinical or overt CAD

	Colony numbers day 28	Max increase in colony number	Time point of max increase	Time to max increase
Healthy FDRs Patients	1.0	1.0	1.0	1.0
Model 1	-0.30 (-1.00 – 0.40)	-0.32 (-0.97 – 0.32)	0.02 (-0.09 – 0.13)	-0.28 (-0.87 – 0.32)
Model 2	-0.44 (-1.30 – 0.42)	-0.45 (-1.25 – 0.35)	0.03 (-0.11 – 0.17)	-0.38 (-1.11 – 0.35)
Model 3	-0.63 (-1.50 – 0.23)	-0.62 (-1.42 – 0.19)	0.06 (-0.08 – 0.20)	-0.55 (-1.28 – 0.19)
FDRs with subclinical CAD				
Model 1	-0.52 (-1.25 – 0.22)	-0.51 (-1.20 – 0.17)	0.11 (-0.01 – 0.23)	-0.53 (-1.16 – 0.10)
Model 2	-0.65 (-1.47 – 0.17)	-0.63 (-1.39 – 0.13)	0.13 (0.00 – 0.26)	-0.64 (-1.33 – 0.06)
Model 3	-0.70 (-1.51 – 0.11)	-0.67 (-1.43 – 0.08)	0.14 (0.01 – 0.27)*	-0.68 (-1.37 – 0.02)
Patients FDRs with subclinical CAD	1.0	1.0	1.0	1.0
Model 1	-0.22 (-101 – 0.58)	-0.19 (-0.93 – 0.55)	0.09 (-0.04 – 0.22)	-0.26 (-0.93 – 0.42)
Model 2	-0.22 (-1.04 – 0.61)	-0.18 (-0.94 – 0.59)	0.10 (-0.03 – 0.23)	-0.26 (-0.96 – 0.45)
Model 3	-0.07 (-0.89 – 0.76)	-0.05 (-0.82 – 0.72)	0.08 (-0.05 – 0.21)	-0.13 (-0.84 – 0.57)

Model 1, crude model; model 2, adjusted for age and sex; model 3, additionally adjusted for smoking. Outcomes are expressed as B with corresponding 95% confidence intervals * $p<0.05$.
FDRs, first-degree relatives.

Table 4 BOEC isolation parameters and proliferation capacity in subclinical CAD before and after statin therapy

	Before treatment	After treatment
Successful isolation, n (%)	6 (60)	9 (90)
Colonies at day 28, n [IQR]	0.04 [0-0.06]	0.37 [0.13-0.85] *
Max increase in colony number, n [IQR]	0.04 [0-0.05]	0.26 [0.07-0.59] *
Time point max increase, days ± SD	26.60 ± 7.95	21.00 ± 8.08
Time to max increase [IQR]	0.002 [0-0.003]	0.013 [0.005-0.034] *
Proliferation capacity, n (%)	3 (30)	8 (80)

Continuous data are expressed as mean ± SD or median [IQR], categorical data as absolute numbers with (percentages). * $p<0.05$ compared with before treatment.
IQR, inter quartile range SD, standard deviation.

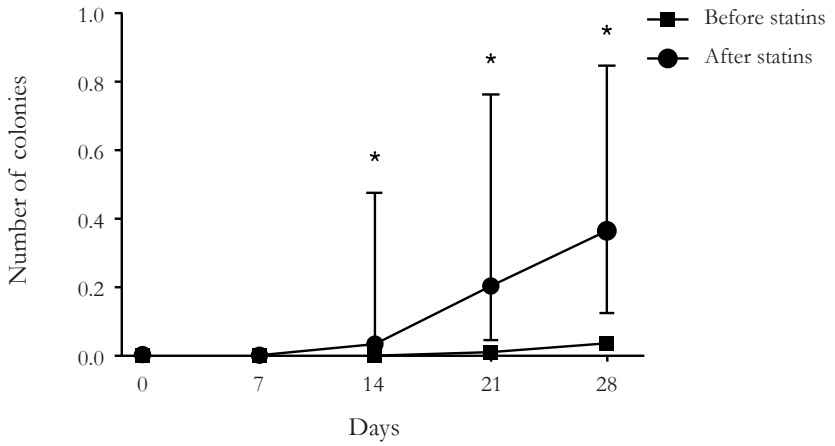

Figure 2 Colony formation curves of FDRs with subclinical atherosclerosis before and after statin treatment
This graph shows the median number of colonies at specific time points before and after statin therapy. Error bars indicate de inter quartile ranges. * P<0.05

Intervention study

To study the effect of statin therapy on circulating BOEC precursors we analysed BOEC colony formation before and after statin therapy in ten subjects with severe subclinical atherosclerosis, as assessed by coronary CT-scanning. From day 14 on, we saw a significant increase in the number of BOEC colonies after statin therapy (Figure 2). Not only the number of colonies at day 28 (0.04 [0-0.06] vs. 0.37 [0.13-0.85]; p<0.05), but also the maximum increase in colony number (0.04 [0-0.05] vs. 0.26 [0.07-0.59]; p<0.05) and the time to maximum increase (0.002 [0-0.003] vs. 0.013 [0.005-0.034]; p<0.05) were significantly increased after statin therapy. Also, the proliferative capacity was increased after statin therapy (30% vs 80%, Table 4), but due to small sample size, this did not reach statistical significance.

DISCUSSION

In this study we show that BOECs isolated from apparently healthy FDRs with subclinical atherosclerosis are less viable, resulting in a decreased proliferative capacity compared with both healthy FDRs and patients with premature CAD. Interestingly, parameters of BOEC isolation and proliferation of premature CAD patients did not differ from either FDRs with subclinical atherosclerosis or from healthy FDRs, indicating an intermediate phenotype. This is interesting, since the both patients and FDRs with subclinical atherosclerosis have atherosclerotic disease, and one would have expected a similar proliferative capacity. On the other hand, statin therapy could have restored the proliferative capacity, as observed in our second part of the study. This however, was not the case. The fact that the proliferative capacity was not completely restored,

could be due to the fact that not all patients adhered to their treatment, which resulted in the intermediate phenotype. Otherwise, subclinical atherosclerosis and full blown CAD are not completely similar, thus the atherosclerotic burden in combination with the restorative capacity of statin therapy might have resulted in an intermediate phenotype. Finally, differences might also have been non-significant due to limited power. However, in our opinion the comparison between both groups of FDRs is the least confounded, since both groups were initially perceived as healthy individuals, and therefore this would be the best comparison.

To further explore the restorative capacity of statin therapy, we additionally analyzed this before and after statin therapy in a subgroup of FDRs with subclinical atherosclerosis. Indeed, we observed a strong increase in BOEC colony formation after statin treatment in FDRs with subclinical atherosclerosis.

In previous studies similar results have been reported for eEPCs, which were shown to be a useful biomarker for CVD[3], with lower levels of eEPCs in CVD patients compared with healthy controls[4,6,20]. Additionally, eEPC levels could independently predict disease progression[14], and already in subjects with subclinical atherosclerosis, as identified by carotid inter media thickness and coronary CT scanning, reduced eEPC levels were detected[21,22]. However, there is a major difference between eEPCs and BOEC cell colonies. eEPCs belong to the haematopoietic lineage, partially retaining their myeloid progenitor activity and explicitly do not resemble endothelial cells. Although these cells can facilitate vasculogenesis *in vivo*, they do not have the ability to form secondary endothelial colonies[23]. On the contrary, BOECs are highly proliferative cells that express solely endothelial cell markers[24] and therefore, these cells have been described as "true" EPCs[25]. Only few studies have assessed characteristics of BOECs in relation to CVD and revascularization. In a recent study it was shown that after an acute coronary event BOECs could be cultured only from a minority of the patients[26]. The ability to culture BOECS from these patients was associated with better outcomes in terms of reduced microvascular obstruction and reduction of infarct size[27]. In contrast, Massa et al. was not able to show a difference in BOEC cultures between CAD patients and healthy controls[28]. However, the presence of subclinical atherosclerosis was not assessed in the controls in this study, which might have confounded the results. Additionally, due to the small sample size, small but clinically relevant differences between patients and controls might have been missed. Here we show that in subjects with subclinical atherosclerosis there is a trend towards lower levels of circulating BOEC precursors and that the proliferative capacity is significantly impaired compared to their healthy relatives. This may possibly affect the potential of vascular regeneration in these subjects, thereby increasing the risk of CVD. Treatment with statins potentially improved BOEC colony formation and proliferation in our group of premature CAD patients, resulting in an intermediate phenotype. Different animal models have shown that statins, apart from there lipid lowering effect, increased mobilization of EPCs[29] and improved neovascularization in experimentally induced myocardial infarction[30]. Now we show that also BOEC colony formation and proliferation significantly increases in sub-

jects with subclinical atherosclerosis after statin therapy. Whether this subsequently results in an improved endothelial repair and a decreased incidence of coronary events in these subjects remains to be investigated.

Strengths and limitations

There are some potential limitations of the design of this study that have to be addressed. First, subjects below the age of 30, in whom no CT-scanning was performed, were considered healthy controls. However, these individuals could already have developed subclinical atherosclerosis which could have negatively influenced the numbers of BOEC colonies in the group of healthy FDRs, resulting in an underestimation of the differences between the study groups. Second, the number of BOEC colonies is generally low and the differences between the study groups are small. As a consequence, our sample size was only marginally sufficient to show significant differences in parameters of BOEC isolation. However, we did show consistent trends towards a decreased viability of BOEC colonies. Therefore, we believe that this is not an incidental finding, but a real phenomenon.

Strength of this study is the division of the FDRs in FDRs with subclinical atherosclerosis and truly healthy FDRs based on coronary CT scanning. By doing so, we made sure that results in our control population are not influenced by the presence of asymptomatic disease. Also, this enabled us to show that, already during the development of atherosclerotic disease endothelial proliferation is impaired. Based on this we hypothesize that circulating BOEC precursors can be used as a biomarker for subclinical atherosclerosis in apparently healthy individuals. Meneveau and co-workers have shown that the level of circulating CD34+/VEGF-R2+ cells correlates with the number of BOEC colonies in patients with acute myocardial infarction. Their findings also suggest that the presence of BOECs correlated with preserved microvascular integrity following an myocardial infarction[27]. Together with the results reported in our study this suggests that circulating BOEC precursors contribute to the regenerative capacity of the vasculature, thereby reducing the propensity of developing CVD.

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CHAPTER 8

Summary and future perspectives

SUMMARY

Atherosclerosis, the underlying pathological mechanisms of cardiovascular disease (CVD), is an extensively studied process. Risk factors that contribute to the origin and the progression of this process are reasonably well established and mathematical models have been built to calculate the exact risk of an individual patient to develop CVD. However, in patients who suffer their first cardiovascular event at an early age (premature CVD), traditional risk factors are usually not as pronounced and other factors seem to contribute to the development of CVD. Since early identification of an increased cardiovascular risk will enable subjects to take preventive measures early in the development of the disease, it is of utmost importance to identify new markers that can be used to predict the risk of premature CVD. It has been postulated that circulating miRNAs could be used as suitable biomarkers for CVD. In this thesis we addressed the question whether the expression of circulating miRNAs could be used to predict premature CVD in specific populations at risk. Furthermore, we investigated whether, apart from miRNAs, different markers could be used to identify specific subpopulations at risk of premature CVD.

Chapter 1 gives an introduction to this thesis. We describe the complexity of premature CVD and the pitfalls of the current risk prediction models. We explain that risk prediction is much more complex for premature CVD, since the traditional risk profile frequently does not fit these patients. Other factors contribute more to the development of the disease, underlining the need of novel markers for premature CVD. We postulate that circulating miRNAs could be suitable biomarkers for this purpose and describe the characteristics of miRNAs on which we based this assumption.

MiRNA research has evolved tremendously over the past decade, providing new insights on these short RNA species. Although many recommendations were provided on how to perform miRNA research, on some topics still no consensus exists. **Chapter 2** deals with one of these topics, namely data normalization of reverse transcription quantitative real-time PCR (RT-qPCR) data. In this necessary step of the analysis, data are corrected for possible processing variation between samples. To date, several normalization methods have been described, but none of them seems ideal. In chapter 2 we used a standardized method to construct normalization panels for RT-qPCR experiments on whole blood, platelets and serum. First, we selected a panel of highly expressed, stable miRNAs from previously performed miRNA microarray experiments. From these panels we excluded miRNAs that were reported to be regulated in any disease. Subsequently, we measured the expression of the miRNAs in a cohort of premature CVD patients and healthy controls using RT-qPCR and used two independent algorithms, namely GeNorm and Normfinder, to determine the stability of the miRNAs. We constructed the final normalization panels of the miRNAs that were found to be most stable by both algorithms. The performance of our normalization panels was further analyzed and compared to normalization for miR-16 and RNU6B. Our self-constructed normalization panels for whole blood, platelets and serum samples showed superior precision and accuracy compared to other normalization methods and should therefore be preferred over other normalization methods in studies on cardiovascular disease.

In **chapter 3** we investigated the influence of aspirin use on platelet miRNA expression profiles. Aspirin is the most commonly prescribed platelet inhibitor for secondary prevention, but although this treatment significantly reduces the risk of a new cardiovascular event, 20% of the patients will develop re-events. This is thought to be due to large inter-individual efficacy of aspirin to inhibit platelet aggregation. In this study we aimed to investigate whether we could predict this so called aspirin insensitivity using platelet miRNA profiles. We performed a miRNA microarray on isolated platelets of 15 healthy volunteers in the presence or absence of aspirin use. Medication-induced changes in expression of each miRNA on the microarray were correlated with the reduction in platelet aggregation after incubation with indomethacin, which mimics aspirin use in vitro. Subsequently the results of these analyses were validated in an extended cohort. We found that the changes in expression of miR-19b-1-5p correlated strongly with the extent of platelet aggregation reduction. In other words, lower platelet miR-19b-1-5p expression after aspirin use was significantly associated with a sustained platelet aggregation in the presence of indomethacin, suggesting aspirin insensitivity. Therefore, miR-19b-1-5p might be a suitable marker to identify aspirin insensitivity and thereby patients prone to re-events.

Chapter 4 discusses risk prediction in a subpopulation of smoking individuals. The risk of developing a cardiovascular event is twice as high among smokers compared to non-smoking individuals, suggesting an influence of cigarette smoke on the atherosclerotic process. Since monocytes play a key role in the development of atherosclerosis, we performed a miRNA microarray on monocyte samples of two independent cohorts of subjects with premature coronary artery disease (CAD) and healthy controls. We found that miR-124-3p levels were genuinely low in non-smoking individuals, whereas in smokers it was heterogeneously expressed. Subsequent flow cytometry analysis of monocytes of smoking individuals showed that high miR-124-3p levels were associated with an up regulation of the monocyte surface markers CD206, CD45RA and CD29, suggesting an altered function of these monocytes which may contribute to the development of atherosclerosis. These correlations led to the hypothesis that an increase in miR-124-3p expression is related to subclinical atherosclerosis. Therefore, we related whole blood miR-124-3p expression levels to coronary calcium abnormalities as measured by coronary CT scanning in both a cohort of smoking individuals and a cohort of non-smoking individuals. We showed that elevated levels of miR-124-3p are predictive for subclinical CAD in smoking individuals, but not in non-smokers. MiR-124-3p levels could therefore be used as a suitable biomarker in smoking individuals, identifying individuals with a susceptibility for the adverse effects of smoking.

In **chapter 5** we discuss a major pitfall of miRNA microarray studies. Since these microarray experiments are costly, they are usually performed on only small sample sizes. However, the accuracy of the detected differentially expressed miRNAs is dependent on the sample size, and reduction of sample size will increase the variance of all parameters, including the outcome parameter, which in this case are the differentially expressed miRNAs. In other words, reducing the sample size will result in an increased false positive discovery rate. This might explain the discordance between several published miRNA experiments, which all propose different miRNAs

as marker for the same disease. In this chapter we show the extensiveness of this phenomenon using data of a self-performed miRNA microarray in a cohort of 40 CAD patients and 40 healthy controls. In random subpopulations of this cohort several miRNAs were found to be differentially expressed, whereas in the complete cohort a regression to the mean could be observed. The differentially expressed miRNAs could not be validated when the complete cohort was studied. To overcome this problem in future experiments we proposed a method of power calculation, that should sufficiently power miRNA microarray experiments and decrease the number of false positive findings.

For **chapter 6** we focussed on the role of the coagulation system in subjects with premature CAD and the metabolic syndrome (MS). Current literature suggests a relation between the coagulation state and CAD, which seems most pronounced in young individuals. Besides an increased risk of CAD, MS patients are known to have a higher risk for thrombotic events. To investigate whether patients with premature CAD and MS can be characterized by a procoagulant state we performed a thrombin generation assay, using calibrated automated thrombinography (CAT) in patients with premature CAD with and without MS, and compared them to their healthy first-degree relatives (FDRs). We found that individuals with CAD at a young age and MS features indeed showed a procoagulant state, whereas CAD patients without MS and healthy FDRs did not. This suggest that MS is an important contributor to a hypercoagulable state in patients with premature CAD.

The focus of **chapter 7** is the regenerative capacity of the endothelium. This is an important factor in the protection against the development of atherosclerotic lesions of the vessel wall. Blood outgrowth endothelial cells (BOECs) resemble the cells that are present in the endothelial lining of the vessel wall and show an extensive regenerative capacity. In this chapter we show that BOECs isolated from subjects with subclinical atherosclerosis, as assessed by coronary calcium scanning, are less viable, resulting in a decreased proliferative capacity compared with healthy individuals, but also compared to patients with established CAD. The major difference between CAD patients and subjects with subclinical atherosclerosis was the statin therapy that was used by CAD patients as secondary prevention. Since statins are known to have beneficial effects on the endothelium we investigated whether statin therapy can improve the regenerative capacity of BOECs. Indeed, we observed a strong increase in BOEC colony formation after statin treatment in FDRs with subclinical atherosclerosis, underlining the usefulness of statins for prevention of cardiovascular disease, even when cholesterol levels are low.

FUTURE PERSPECTIVES

Characterization of premature CVD holds many challenges. The disease profile constitutes of a large heterogeneity of risk factors, of which only few can be identified in the majority of the patients. This underlines the need for further characterization of subpopulations of patients and the identification of different methods of risk prediction for this disease.

In this thesis we postulate that circulating miRNAs could be used as suitable markers for the prediction of CVD risk in specific subpopulations of individuals at risk. However, the main condition for the identification of reliable markers, is the performance of standardized and sufficiently powered experiments. Current research often lacks either one of these requirements. MiRNA microarrays are performed on only small sample sizes or RT-qPCR data are not properly normalized for processing variations. Part of this thesis focused on how to deal with these problems. We propose a method of power calculation that will reduce the identification of false positive results. Furthermore, we constructed novel normalization panels for RT-qPCR experiments on whole blood, platelets and serum samples that reliably correct for processing variations and increase the chance of validating miRNA microarray experiments. The incorporation of these methods in future experiments should result in a decreased identification of false positive markers and an improved concordance between different studies, eventually leading to the identification suitable biomarkers for premature CVD.

Using the above mentioned methods we identified miRNAs that were associated with an increased risk of a cardiovascular event in specific subpopulations. We found that platelet miR-19b-1-5p was associated with an increased risk of re-events in subjects using aspirin for secondary prevention after a premature cardiovascular event. Furthermore, elevated expression of monocyte miR-124-3p levels was associated with an increased risk of premature CAD in smoking individuals. In both studies up regulation of the specific miRNA was associated with functional changes in the investigated cell type, indicating a pathological mechanism behind the discovered associations. But these findings cannot directly be converted to new risk prediction profiles. So far, the associations have been investigated in cross-sectional cohorts. Before incorporation in risk predicting tools, they should be confirmed as useful biomarkers for premature CVD in large prospective cohorts.

Likely, future research will result in the identification of new, prospectively validated, circulating miRNAs as biomarker for premature CVD. These findings should be translated into easily usable risk prediction tools that can be used to predict CVD risk in clinical daily practice. Ideally, this tool would consist of a combination of miRNAs as biomarker, to increase the applicability in the heterogenic population. The first steps are made in the right direction, but before we get to the development of risk prediction tools, new markers should be identified using the proposed standardized methods and previously identified markers should be validated in large prospective cohorts.

APPENDIX I

NEDERLANDSE SAMENVATTING

Hart- en vaatziekten zijn wereldwijd de belangrijkste oorzaak van sterfte. Atherosclerose, of aderverkalking ligt ten grondslag aan de ontwikkeling van deze ziekte. De afgelopen decennia is uitgebreid onderzoek gedaan naar het ontstaan van atherosclerose. Hierbij zijn er vele risicofactoren ontdekt die bijdragen aan de ontwikkeling en de progressie van de ziekte. Deze risicofactoren zijn vervolgens samengevoegd in verschillende rekenkundige modellen, op basis waarvan het individuele risico van een patiënt op het ontwikkelen van hart- en vaatziekten kan worden berekend. In de praktijk worden deze rekenmodellen gebruikt om te onderzoeken welke mensen preventief behandeld moeten worden, omdat zij een verhoogd risico hebben op het ontstaan van hart- en vaatziekten. Echter, bij mensen die op jonge leeftijd hart- en vaatziekten ontwikkelen (premature hart- en vaatziekten), zijn de klassieke risicofactoren waaruit de rekenmodellen zijn opgebouwd vaak niet zo uitgesproken en lijken andere factoren een grotere rol spelen bij het ontstaan van de ziekte.

Voor de behandeling van hart- en vaatziekten is het van groot belang om de ziekte in een vroeg stadium op te sporen. Wanneer een verhoogd risico op hart- en vaatziekten op tijd wordt vastgesteld, kan vroeg in het ontstaan van de ziekte preventieve behandeling worden gestart om de progressie te remmen. Het is daarom van belang dat er nieuwe markers wordt geïdentificeerd, die het risico op premature hart- en vaatziekten kunnen voorspellen.

Een goede marker moet aan een aantal eisen voldoen om te kunnen worden gebruikt voor het opsporen van een verhoogd risico op premature hart- en vaatziekten. Allereerst is het van belang dat de marker specifiek is voor premature hart- en vaatziekten en niet ook verhoogd is bij andere ziekten. Daarnaast moet hij ook sensitief zijn voor premature hart- en vaatziekten. Dit betekent dat de marker te vinden moet zijn bij vrijwel alle mensen met deze ziekte. Om ervoor te zorgen dat de marker in de dagelijkse praktijk ook goed gebruikt kan worden, is het ook van belang dat de aanwezigheid van de marker op een gemakkelijke manier bepaald kan worden.

Men vermoedt dat circulerende microRNAs zeer geschikte markers zouden kunnen zijn voor premature hart- en vaatziekten. MicroRNAs zijn kleine moleculen die overal in het menselijk lichaam voorkomen. Ze spelen een belangrijke rol bij vrijwel alle processen die zich in ons lichaam afspelen en zijn ook betrokken bij het ontstaan van ziekten. Het is bekend dat specifieke microRNAs een rol spelen bij het ontstaan van hart- en vaatziekten. Het idee is dat een deel van deze microRNAs, vanuit cellen die betrokken zijn bij het ontstaan van aderverkalking of vanuit de verkalkte ader zelf, in het bloed terecht komen en zo door het gehele lichaam circuleren. Door te bepalen of en in welke mate deze microRNAs in het bloed voorkomen, zou het, na het afnemen van slechts enkele buisjes bloed, mogelijk kunnen zijn te voorspellen welke mensen premature hart- en vaatziekten ontwikkelen.

In dit proefschrift is onderzocht of microRNAs inderdaad geschikte markers zijn voor premature hart- en vaatziekten. Dit onderzoek kon echter niet zonder slag of stoot uitgevoerd worden. Het onderzoek naar microRNAs staat nog in de kinderschoenen, en er waren nog goede oplossingen

nodig de betrouwbare en reproduceerbare meting van microRNAs in bloed. Een voorbeeld hiervan is de manier waarop wordt omgegaan met de analyse van microRNA bepalingen. In veel studies wordt de expressie van specifieke microRNAs vergeleken tussen patiënten en gezonde controles. Echter, de expressiewaarde van een microRNA wordt niet alleen bepaald door ziekte, maar ook door de manier waarop het bloed verkregen en bewerkt is. In studies kunnen er verschillen zitten in deze stappen die plaatsvinden voor de daadwerkelijke analyse. Om betrouwbare resultaten te krijgen zal hiermee rekening moeten worden gehouden door de waarden corrigeren, ook wel normaliseren, voor de variatie van de voorbereidende stappen. In hoofdstuk 2 is dit probleem onderzocht en wordt een methode beschreven waarin gebruik gemaakt wordt van nieuw geïdentificeerde controle microRNAs die beter betrouwbare resultaten levert in vergelijking tot eerdere beschreven methoden.

Een ander probleem dat zich kan voordoen bij het onderzoek naar microRNAs is het feit dat de meeste onderzoeken uitgevoerd zijn bij kleine groepen patiënten. De belangrijkste reden hiervoor is dat microRNA onderzoek erg duur is en hoe meer patiënten onderzocht moeten worden, des te hoger de kosten. De consequentie van een kleine groep patiënten is dat er een grotere risico bestaat dat de resultaten van het onderzoek op toeval berusten. De kans dat een microRNA toevallig bij 2 patiënten een hoge expressie heeft is nu eenmaal veel groter dan dat dit microRNA bij 20 mensen een hoge expressie heeft. In hoofdstuk 5 wordt dit probleem beschreven en wordt met behulp van een experiment aangetoond hoe groot het effect van een kleine groep patiënten daadwerkelijk is.

Uiteraard is er ook onderzocht of er microRNAs geïdentificeerd konden worden die geschikt zijn als marker voor premature hart- en vaatziekten. Hiervoor is onderzoek gedaan in specifieke groepen mensen met een verhoogd risico. Hoofdstuk 3 richt zich op mensen die al premature hart- en vaatziekten hebben. Deze mensen worden behandeld met aspirine om een volgende uiting van hart- en vaatziekten te voorkomen. Er zijn echter mensen bij wie de behandeling met aspirine geen effect heeft. Van tevoren is het echter slecht te voorspellen om welke mensen dit gaat en wie er een verhoogd risico loopt op een tweede uiting van hart- en vaatziekten. Uit de experimenten in dit hoofdstuk bleek dat miR-19b-1-5p een goede marker is om te voorspellen welke patiënten een verhoogd risico hebben. Bij patiënten met een lage miR-19b-1-5p expressie in bloedplaatjes na behandeling met aspirine, was de functie van deze bloedplaatjes niet voldoende geremd, waardoor zij een verhoogd risico hadden op een volgende uiting van hart- en vaatziekten.

Mensen die roken vormen een andere belangrijke risicogroep voor hart- en vaatziekten. Het risico op een hartinfarct is zelfs twee keer zo groot bij rokers vergeleken met niet-rokers. Er zijn echter ook voldoende rokers die nooit hart- en vaatziekten krijgen. In hoofdstuk 4 is onderzocht of er een marker is die het risico voor premature hart- en vaatziekten kan voorspellen onder rokers. Hiervoor is gekeken naar de expressie van microRNAs in monocyten, omdat deze cellen een belangrijke rol spelen bij het ontstaan van hart- en vaatziekten. Er werd ontdekt dat miR-124-3p expressie over het algemeen laag is onder niet-rokers, terwijl de expressie van dit microRNA zeer wisselend is onder rokers. Verdere analyse liet zien dat een verhoogde miR-124-3p expressie in

monocyten van rokers geassocieerd was met veranderingen van deze monocyt, die bijdragen aan de ontwikkeling van aderverkalking. Daarom werd in een nieuwe groep patiënten onderzocht of een verhoging van miR-124-3p in het bloed van rokers kon fungeren als een marker voor premature hart- en vaatziekten. Dit bleek het geval te zijn, rokers met een verhoogde miR-124-3p expressie hadden een grotere kans op het ontwikkelen van premature hart- en vaatziekten dan rokers met een lage miR-124-3p expressie.

Naast microRNAs zijn er nog verscheidene andere markers voor premature hart- en vaatziekten. In hoofdstuk 6 hebben we onderzoek gedaan naar de rol van de bloedstolling bij patiënten met premature hart- en vaatziekten en het metabool syndroom. Het metabool syndroom bestaat uit een combinatie van overgewicht, een verminderde gevoeligheid voor insuline, een verhoogde bloeddruk en afwijkingen van het cholesterol. Dit syndroom is een belangrijke risicofactor voor het ontstaan van hart- en vaatziekten. Onderzoek in dit hoofdstuk liet zien dat mensen met premature hart- en vaatziekten en het metabool syndroom een verhoogde stollingsneiging hebben in vergelijking met premature hart- en vaatziekten patiënten zonder het metabool syndroom en gezonde controles. Dit suggereert dat het metabool syndroom een belangrijke factor is voor de verhoogde stollingsneiging bij patiënten met premature hart- en vaatziekten.

Een andere belangrijke factor bij het ontstaan van hart- en vaatziekten is de capaciteit van de bloedvatwand om zich te herstellen. Beschadiging van deze wand kan ontstaan als gevolg van bijvoorbeeld een verhoogde bloeddruk of een verhoogd cholesterol, beide risicofactoren voor het ontstaan van aderverkalking. Wanneer de bloedvatwand gemakkelijk herstelt heeft dit een beschermende werking tegen het ontstaan van hart- en vaatziekten. In hoofdstuk 7 is bij mensen met bewezen aderverkalking onderzoek gedaan naar de herstellende capaciteit van de cellen waaruit de bloedvatwand bestaat en dit vergeleken met gezonde controles. Het bleek dat cellen van de bloedvatwand van mensen met bewezen aderverkalking zich veel minder goed herstellen dan die van gezonde controles. Ook werd ontdekt dat behandeling met statines, medicijnen die het cholesterol verlagen, een gunstig effect hebben op het herstel van de cellen van de bloedvatwand. Dit benadrukt dat, ook wanneer het cholesterol laag is, statines zeer bruikbaar zouden kunnen zijn voor de preventie van hart- en vaatziekten.

Samenvattend hebben we in dit proefschrift verschillende markers geïdentificeerd, die geassocieerd zijn met een verhoogd risico op premature hart- en vaatziekten. Voordat deze markers gebruikt kunnen worden in de dagelijkse praktijk zullen ze verder moeten worden onderzocht in grote cohorten. Indien de voorspellende capaciteit wordt bevestigd, vormen het zeer veel belovende markers voor premature hart- en vaatziekten en kunnen deze opgenomen worden in de huidige rekenmodellen voor een nog nauwkeurigere risico inschatting bij met name de jonge patiënten.

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PORTFOLIO

Courses	Year	ECTS
Web of science	2011	0.2
Cardiovascular disease: Vascular biology	2011	1.5
Practical biostatistics	2012	1.1
Computing in R	2012	0.4
Good Clinical Practice	2012	0.2
Basic course in legislation and organization for clinical researchers	2012	0.9
Oral presentation, Niek Vink Training and Consultancy B.V.	2012	2.0
Advanced topics in biostatistics	2013	2.1
AIO course arterial thrombosis (NVTH)	2013	2.0
Selection of presentations		
International Symposium on Atherosclerosis (Sydney, Australia)	2012	
Oral presentation Title: Platelets in patients with premature coronary artery disease exhibit upregulation of miR-340* and miR-624*		0.5
Poster presentation Title: Specific platelet miRNAs associated with subclinical atherosclerosis		0.5
European Lipoprotein Club (Tutzing, Germany)	2012	
Poster presentation Title: Subclinical atherosclerosis is associated with specific changes in miRNA profiles		0.5
Nederlandse Vereniging voor Trombose en Hemostase (Koudekerke, the Netherlands)	2013	
Oral presentation Title: Differences in platelet miRNA profiles after aspirin use are associated with whole blood platelet aggregation and might identify aspirin resistance		0.5
International Society of Thrombosis and Haemostasis (Amsterdam, the Netherlands)	2013	
Oral presentation Title: Differences in platelet miRNA profiles after aspirin use are associated with whole blood platelet aggregation and might identify aspirin resistance		0.5

Selection of presentations	Year	ECTS
Poster presentation Title: Individuals with coronary artery disease at a young age and features of the metabolic syndrome have an increased prothrombotic potential		0.5
European Council on Cardiovascular Research (Nice, France) Oral presentation Title: Changes in miRNA expression in isolated platelets after aspirin use in vivo is related to aspirin insensitivity.	2013	0.5
Poster presentation Title: Statin therapy restores proliferation of blood outgrowth endothelial cells, which is impaired in subjects with subclinical atherosclerosis		0.5
Seminars and workshops		
Weekly department research seminars	2010-2014	4
Weekly laboratory research seminars	2010-2014	4
Lipid literature debate	2011-2014	2
Teaching		
Supervision of medical student Silvia Nieuwburg	2011-2012	2

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