Recurrent miscarriage and the subsequent risk of cardiovascular disease

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Recurrent miscarriage and the subsequent risk of cardiovascular disease

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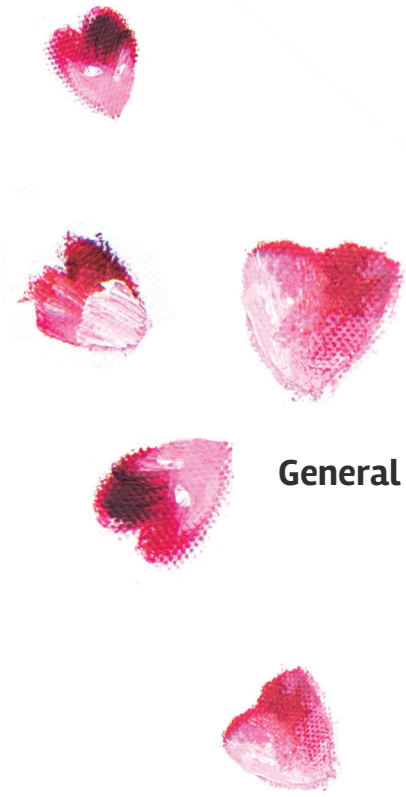
Voor mijn vader,

“Hoe zou je nòg gelukkiger,
nòg trotser kunnen worden?”

Contents

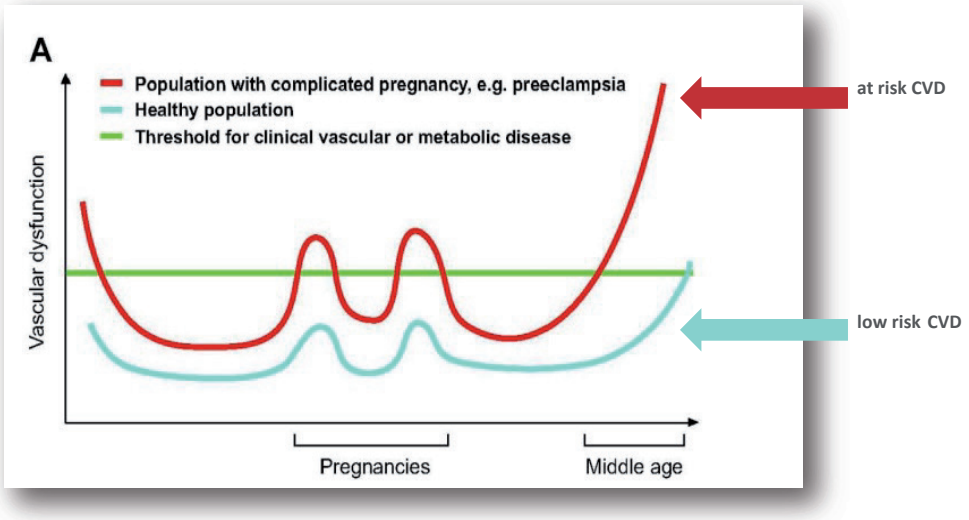


1

General introduction and outline of this thesis

Approximately 15% of clinically recognized pregnancies fail to result in a live birth (1). A miscarriage can be defined as a pregnancy that ends spontaneously before the fetus has reached a viable gestational age. Most miscarriages occur before 12 weeks, frequently caused by sporadic fetal chromosome abnormalities. It has been estimated that only 2–3% of pregnancies end spontaneously in the second trimester(2). Recurrent miscarriage is commonly defined as three or more consecutive pregnancy losses before 22 weeks of gestation(3). It is a problem affecting 0.5-3% of all fertile couples(4).
Recurrent miscarriage is a highly heterogeneous condition. Whenever the diagnosis 'recurrent miscarriage' is established, an underlying cause is identified in only 25-50% of cases(5, 6). Possible etiologic factors include genetic disorders, uterine anomalies, endocrine factors, maternal autoimmune disorders, metabolic disorders, maternal thrombophilia, obesity and toxic factors such as smoking and alcohol consumption.

Cardiovascular disease is the leading cause of death in women in the Western world(7). Clinical manifestation of cardiovascular disease in women is different compared to men(8, 9). Historically, cardiovascular risk factors in women have been insufficiently recognized, diagnosed and treated(10). Most data used to develop prevention guidelines came from trials that enrolled few or no women. In 2004, the American Heart association published evidence-based guidelines for cardiovascular prevention in women(11). In the most recent guideline, pregnancy complications as a history of pregnancy induced hypertension or preeclampsia, and gestational diabetes are mentioned as important risk factors for cardiovascular disease(12). Recurrent miscarriage is not mentioned in current guidelines as a risk factor for cardiovascular disease. A woman is classified as 'at risk for cardiovascular disease' when she is having one or more risk factor(s). Other risk factors are, for example; cigarette smoking, high blood pressure and a family history of premature cardiovascular disease in a first-degree relative. The guidelines further call for new research to identify events during 'periods of potential vulnerability' such as pregnancy, across a woman's lifespan that might influence her cardiovascular disease risk(12).
Pregnancy provides a unique opportunity to estimate a woman's risk because of its exceptional cardiovascular and metabolic stress. Cardiac output increases enormously, there is a decrease in maternal systemic vascular resistance; the renin-angiotensin-aldosterone system is significantly activated; and the heart and vasculature undergo remodeling. These changes are already significant in the first weeks of gestation(13). Pregnancy can be considered as a vascular "stress test" (14) and can unmask early or preexisting endothelial dysfunction and vascular or metabolic disease. This suggests that women at high risk of future cardiovascular disease are identifiable during pregnancy. (Figure 1)



Based on: J.W.Rich-Edwards et al. Hypertension 2010;56:331-334.

Figure 1. Pregnancy as a vascular 'stress test'.

Women at high risk of future cardiovascular disease, indicated by the red line in figure 1, are crossing the threshold for clinical vascular disease during pregnancy. Women at low risk of future cardiovascular disease (blue line) will pass this test with an uncomplicated pregnancy.

Since the cardiovascular and metabolic changes are already significantly present in the first trimester, recurrent miscarriage might be a first sign of subsequent cardiovascular disease in women. As earlier stated recurrent miscarriage is a highly heterogenous condition. Several hypotheses are possible for an association between recurrent miscarriage and cardiovascular disease; shared common risk factors such as obesity and smoking(15), thrombophilia, and a genetic predisposition is assumed(16).

Epidemiologic studies are showing an association between both conditions; a recently published meta-analysis found an association between recurrent miscarriage and coronary heart disease: pooled odds ratio 1.99, 95%CI (1.13-3.50)(17). Though, clinical heterogeneity between studies was evident. An assessment of the association between recurrent miscarriage and cerebrovascular disease was not possible due to the small number of studies available.

In this thesis, we further want to examine the association between recurrent miscarriage and cardiovascular disease, since current research is sparse. Therefore, in *chapter 2* we report a large retrospective cohort study with a long follow-up which assessed whether consecutive miscarriage is (independently) associated with an increased risk of cardiovascular disease later in life, including ischemic heart disease and cerebrovascular disease.

Worldwide multivariable risk assessment tools are developed to detect apparently healthy individuals at high risk for cardiovascular disease and to effectively implement prevention strategies(18). At present the most common externally validated risk model is the Framingham risk score(19). We hypothesize that women with a history of recurrent miscarriage have a more unfavorable cardiovascular risk profile already at a young age compared to women with no miscarriage. If so, women with recurrent miscarriage represent an ideal target population for preventive strategies. In *chapter 3* we conducted a follow-up study to determine cardiovascular risk factors and predict the long term cardiovascular disease risk using Framingham risk scores in women with a history of recurrent miscarriage compared to women with no miscarriage.

In addition to classic cardiovascular risk factors, there is a wide variety of novel cardiovascular biomarkers associated with future cardiovascular disease. Biomarkers regarding inflammation, thrombosis, lipid metabolism, renal function and myocardial damage. For example; high-sensitivity C-reactive protein (HsCRP), an inflammatory biomarker, lipoprotein(a) (Lp(a)), a lipid related biomarker and homocysteine, a thrombosis biomarker, which alters the process of hemostasis. Hyperhomocysteinemia is associated with recurrent miscarriage and with cardiovascular disease(20, 21). These novel cardiovascular biomarkers might contribute in linking recurrent miscarriage to cardiovascular disease and therefore can lead to a better understanding of the association. In *chapter 4* we conducted a follow-up study to determine novel cardiovascular biomarkers in women with a history of recurrent miscarriage compared to women with no miscarriage.

We hypothesize that the association between miscarriages and cardiovascular disease indicates shared common acquired risk factors, such as smoking and antiphospholipid syndrome, and shared heritable (genetic) risk factors. A Scottish retrospective cohort study found an increased incidence of ischemic heart disease in the parents of women who experienced multiple miscarriages before their first birth, which supports the hypothesis of shared genetic factors(22). A family history of (premature) cardiovascular disease is an independent predictor of myocardial infarction and cardiovascular disease(23). We conducted a matched case-control study to investigate whether a family history of premature cardiovascular disease was more common in women who experienced recurrent miscarriage compared to women with no miscarriage, which is described in *chapter 5.*

As earlier stated, research suggests a multifactorial etiology in recurrent miscarriage with a role for genetics. A familial predisposition is described in literature. An increase in the risk of spontaneous miscarriage was seen in family of women with recurrent miscarriage compared to control women(23-26). In addition, a case-control study showed that women with two or more unexplained recurrent miscarriage more often had a family history of recurrent miscarriage compared to healthy control subjects, RR 3.2 (95%CI 1.3-8.1)(27). However, it is still unclear what specific genes are involved

and what individual variants contribute to the development of recurrent miscarriage. Polymorphisms have been investigated in almost 90 different genes(28). We hypothesize that there are shared genetic polymorphisms which are associated with recurrent miscarriage and cardiovascular disease. Therefore, a systematic overview of the genetic variants associated with recurrent miscarriage was described in *chapter 6*. A meta-analysis was performed to assess the pooled effect of the genetic variants that are repeatedly investigated and that are significantly associated with recurrent miscarriage in at least two of the performed studies (and find possible genetic links between recurrent miscarriage and cardiovascular disease).

Other pregnancy complications, for instance, pregnancy induced hypertension, preeclampsia, intrauterine growth restriction and pre-term delivery may increase the risk of cardiovascular disease later in life(29, 30). It is unclear whether these pregnancy complications are on the causal pathway between miscarriage and cardiovascular disease(31, 32), they are possibly a confounding factor. Women with recurrent miscarriage might have more complicated pregnancies prior and after their recurrent miscarriage. Not all women with recurrent miscarriage will have an ongoing pregnancy, so will not have the chance to develop pregnancy complications. Approximately 40% of the women with recurrent miscarriage have a previous ongoing pregnancy and are diagnosed with secondary recurrent miscarriage(4). Women who did not have an ongoing pregnancy prior to their recurrent miscarriage are diagnosed with primary miscarriage. The chance of a live birth in the subsequent pregnancy after recurrent miscarriage is reported to be 60-90%(33-35). A review identified an increased risk for placenta previa, premature preterm rupture of membranes, preterm delivery, intrauterine growth restriction, low birth weight and congenital abnormalities in the pregnancy subsequent to the recurrent miscarriage(36). Little is known about pregnancy outcome prior to recurrent miscarriage(5, 37, 38). Endothelial dysfunction has been hypothesized as the underlying link between recurrent miscarriage, preeclampsia, intrauterine growth restriction and future cardiovascular events(16). Other pathophysiological links could be inherited and acquired thrombophilia such as antiphospholipid syndrome. As knowledge of obstetric details regarding the pregnancy prior to miscarriage may contribute to our understanding of the development of recurrent miscarriage, or cardiovascular disease and their possible link, in *chapter 7* a retrospective cohort study was performed to assess if women with secondary recurrent miscarriage have a more complicated first pregnancy compared to control women.

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2

Association between miscarriage and cardiovascular disease in a Scottish cohort

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Abstract

Objective To assess if miscarriage, whether consecutive or not, is associated with an increased risk of subsequent cardiovascular disease.

Methods A cohort study was performed using women with at least one miscarriage or live birth recorded from 1950 - 2010 in the Aberdeen Maternity and Neonatal Databank. The exposed groups consisted of women with non-consecutive, two consecutive or ≥ three consecutive miscarriages; the unexposed group consisted of all women with at least one live birth and no miscarriages. Women were linked to Scottish Morbidity Records for hospital admissions for cardiovascular conditions, cardiac surgery and death registrations. Main outcome measures were ischemic heart disease, cerebrovascular disease and a composite outcome of any disease of circulatory system. A sensitivity analysis was performed dividing the women into those who had one, two or ≥ three miscarriages irrespective of these were consecutive or not.

Results After excluding women with pre-existing hypertension, type one diabetes mellitus, kidney disease and 'disease of circulatory system', 60105 women were analysed; 9419 with non-, 940 with two, 167 with ≥ three consecutive miscarriages and 49579 with no miscarriage. In the multivariate analyses a significant association was found between ischemic heart disease and women with two {Hazard Ratios (HR) 1.75 (95% confidence interval (CI) 1.22-2.52-1.72)} or ≥ three {HR 3.18 (95%CI 1.49-6.80-4.51)} consecutive miscarriages. Similar patterns of risk were observed in the sensitivity analysis.

Conclusions Women with a history of two or more miscarriages, irrespective of whether consecutive or not, appear to have an increased risk of ischemic heart disease.

INTRODUCTION

Globally, cardiovascular disease(CVD) is a major cause of premature mortality in women[1]. There is increasing evidence that women with adverse pregnancy outcomes are at increased risk of CVD[2]. A history of preeclampsia, gestational diabetes or pregnancy-induced hypertension is mentioned as a major risk factor for cardiovascular disease in women in the American Heart Association guideline[3]. The association between recurrent miscarriage and CVD is less clear[4 5].

Approximately 15% of clinically recognized pregnancies fail to result in a live birth[6]. Recurrent miscarriage is commonly defined as three or more consecutive pregnancy losses before 22 weeks' gestation[7] and affects 0.5 to 3% of fertile couples[8]. Recurrent miscarriage is a highly heterogeneous condition. Possible etiologic factors include genetic disorders, uterine anomalies, endocrine factors, maternal autoimmune disorders, metabolic disorders, maternal thrombophilia and lifestyle factors[9]. An underlying cause may be identified in 25-50% of cases.

CVD and recurrent miscarriage share risk factors such as smoking and obesity[10]. Further endothelial dysfunction has been hypothesized as the underlying link between recurrent miscarriage, preeclampsia, IUGR and future cardiovascular events[11]. It is possible therefore that recurrent miscarriage is a first sign of subsequent CVD in women. Early identification of women at increased risk of CVD from their reproductive history may enable them to benefit from screening and preventive interventions[12].

A recently published meta-analysis found an association between recurrent miscarriage and coronary heart disease: pooled odds ratio 1.99 (1.13 to 3.50)[4]. However clinical heterogeneity between studies was evident. The effect of recurrent miscarriage on cerebrovascular disease could not be pooled due to the small number of primary studies. A large cohort study, not included in the meta-analysis, reported an association[5].

We report here a retrospective cohort study with a long follow-up which assessed if miscarriage (consecutive or not) is associated with an increased risk of subsequent CVD.

METHODS

Data sources and record linkage: The Aberdeen Maternity and Neonatal Databank (AMND) has recorded and stored information on all pregnancy-related events occurring in a geographically defined population living in Aberdeen Scotland from 1950 to the present date. Data were extracted for all women with at least one singleton live birth or miscarriage from 1950 until 2010. These women were linked using probabilistic record linkage to Scottish Morbidity Record (SMR 01) to identify any hospital admissions (SMR data available since 1968) for cardiovascular conditions and to the National Register of Scotland (NRS) for death registrations (up to 2013). After linkage the dataset was anonymised.

To ensure confidentiality, the linked dataset was managed by the Grampian Data Safe Haven, a facility providing a secure environment for the safe linkage, analysis, management and storage of datasets containing non-consented clinical data.

Study design: In this retrospective cohort study, the women were grouped according to their reproductive history into four mutually exclusive groups: no miscarriage, non-consecutive miscarriage, two consecutive miscarriages and three or more consecutive miscarriages. Women with miscarriages could have had one or more live births prior to, or after, their consecutive or non-consecutive miscarriages. The non-consecutive miscarriage group consisted of women with one miscarriage or more than one miscarriage that were not consecutive. Women experiencing miscarriage(s) formed the exposed cohorts while those without a history of miscarriage and at least one live birth comprised the unexposed cohort (reference group).

The AMND uses the International Classification of Disease-version 9 (ICD-9) to code events. Miscarriage in this study refers to spontaneous loss of pregnancy before 24 weeks of gestation.

Outcome: The primary outcomes of interest were arterial CVD identified by admission to hospital or death from ischemic heart disease (ICD-9 411, 413-414, ICD-10 I20-I25), cerebrovascular disease (ICD-9 430-438, ICD-10 I60-I69, G45), or a composite outcome of any disease of the circulatory system, defined as admission to hospital or death due to diseases of the circulatory system ((ICD-9 390-459 ICD-10 I00-I99, G45) or cardiac surgery (OPCS4 Classification of Surgical Operations and Procedures: K40 saphenous vein graft replacement of coronary artery, K51 diagnostic transluminal operations on coronary artery, K65 catheterisation of heart, K75 percutaneous transluminal balloon angioplasty and insertion of stent into coronary artery).

In a separate sensitivity analysis we divided the women into those who had one, two or three or more miscarriages irrespective of whether these events were consecutive or not. Subgroup analyses were performed dividing the women into those with consecutive and non-consecutive miscarriages (2 or more) and primary and secondary consecutive miscarriages. Primary miscarriages were defined as miscarriages without a prior live birth. Secondary miscarriages as miscarriages after a prior live birth. For all analyses women with pre-existing cardiovascular related disease were excluded. Pre-existing disease was defined as disease before entry time in the cohort (see statistical analysis below); divided into pre-existing hypertension, pre-existing type one diabetes, kidney disease and any disease of the circulatory system.

Definition of covariates:

Baseline characteristics and covariates were obtained from AMND. Gravidity, parity and primary versus secondary miscarriages were retrieved. Birth dates were collected, as well as dates of pregnancy events and dates of the cardiovascular outcome. Maternal age was defined as age at the time of last pregnancy event or age at first, second consecutive

or third consecutive miscarriage, depending on the reproductive history. Self-reported smoking habits are coded in AMND at the time of the first antenatal clinic visit. As a covariate 'ever' or 'never' smoked were used. Socio economic deprivation is coded in the AMND using the husband/ partner's social class according to the Registrar General's occupation based social class[13]. The social class was divided into two categories: class 1-3a- 'non manual', class 3b to 5- 'manual'. Body mass index (BMI) was measured at first antenatal visit. For each woman a mean BMI (using information from all pregnancies) was calculated.

Statistical analyses

All data were extracted, linked and entered into SPSS (Statistical Package for Social Science; SPSS Inc., Chicago, IL) version 21.0. Statistical comparisons of baseline characteristics between the exposed and unexposed groups were done using independent samples t-test, the χ^2 test or Fisher's exact test, as appropriate. P-values of 0.05 or less were used to indicate statistical significance. Kaplan-Meier's curves of survival from cardiovascular events of interest were constructed for each of the exposure groups, including log rank tests. To calculate the event-free survival, univariate and multivariate analyses were done by Cox regression analysis. Hazard ratios (HR) were calculated with 95% confidence intervals (CIs). Time since exposure in years was used as underlying time variable. Entry time was defined as the year of the last pregnancy event for women in the unexposed cohort. Depending on their reproductive history, the entry time for women in the exposed cohort was year of first miscarriage, second consecutive miscarriage or third consecutive miscarriage. End time was defined as the date of the cardiovascular event, or in case no event occurred January 2013. The following covariates were included in the adjusted analyses: maternal age at entry time and BMI (as continuous variables), social class and smoking (as categorical variables). All of the covariates used for adjustment were significantly associated with CVD. A complete case analysis was performed, as well as a Cox regression using multiple imputation for the missing values (creating five different 'complete' datasets using maternal age, BMI, social class, smoking).

Power calculation

A power calculation was performed *a priori* in nQuery advisor using the smallest exposed cohort. The prevalence of CVD in women aged 55-64 in Scotland is 15.3%[14]. Assuming the same prevalence in the unexposed cohort, using a 2 sided test with 90% power at 5% significance level, to detect a clinically relevant HR of 2.0 we would need 177 in the smallest exposed cohort and 705 in the unexposed group.

RESULTS

We identified 65227 women who had a pregnancy ending in miscarriage and/or live birth between 1950 and 2010 (Figure 1). A total of 5122 women had some pre-existing cardiovascular related disease.

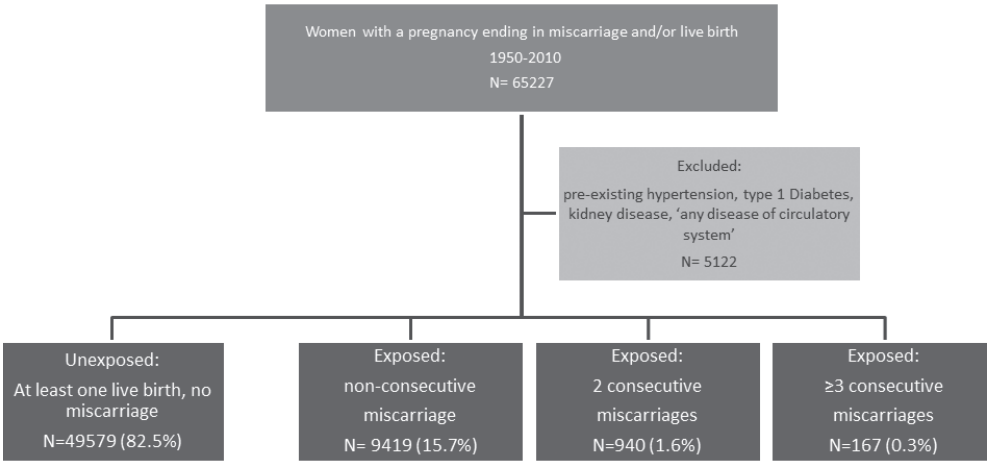


Figure 1 Flow chart selection of cohort

Table 1 Pre–existing morbidity in 65227 women; divided in women experiencing consecutive miscarriages and women with live birth and no miscarriages

	no miscarriages	non-consecutive miscarriage	2 consecutive miscarriages	≥3 consecutive miscarriages
	N=53646	N=10297	N=1083	N=201
Pre-existing hypertension (%)	2182 (4.1)	512 (5.0) (p<0.01)	77 (7.1) (p<0.01)	21 (10.4) (p<0.01) (p=0.10)[a]
Pre-existing type one diabetes mellitus 1 (%)	328 (0.6)	67 (0.7) (p=0.64)	11 (1.0) (p=0.21)	3 (1.5) (p=0.20) (p=0.55)[a]
Pre-existing kidney disease (%)	936 (1.7)	167 (1.6) (p=0.38)	24 (2.2) (p=0.33)	4 (2.0) (p=0.85) (p=0.84)[a]
Pre-existing diseases of the circulatory system (including cardiac surgery) (%)	866 (1.6)	177 (1.7) (p=0.44)	38 (3.5) (p<0.01)	10 (5.0) (p<0.01) (p=0.31)[a]

p- values refer to comparisons between miscarriage group and no miscarriage group.
[a]Comparison between 2 and ≥3 consecutive miscarriages

The prevalence of pre-existing hypertension was higher in women with miscarriages compared to women with no miscarriages, the prevalence of pre-existing diseases of the circulatory system was higher in women with consecutive miscarriages compared to women with no miscarriages (Table 1). Women with pre-existing disease were excluded from all analyses, leaving 60105 women; 9419 women with none, 940 women with two and 167 women with three or more consecutive miscarriages, and 49579 women with at least one live birth and no miscarriages.

The exposed and unexposed groups differed at baseline on maternal age; women in the non-consecutive miscarriage group being younger and women in the two and ≥ three miscarriage groups older than the no miscarriage group (Table 2).

Table 2 Comparison of characteristics between women experiencing consecutive miscarriages and women with live birth and no miscarriages

	no miscarriages	non-consecutive miscarriage	2 consecutive miscarriages	≥3 consecutive miscarriages
	N=49579	N=9419	N=940	N=167
Maternal age mean(SD)[a]	27.92(5.43) N=49532	27.51(6.51) (p<0.01) N=9127	29.75(6.52) (p<0.01) N=938	31.50(6.71) (p<0.01) N=167
BMI mean(SD)	24.52(4.39) N=47862	24.92(4.63) (p<0.01) N=6043	24.81(4.62) (p=0.09) N=708	24.64(4.06) (p=0.77) N=124
RGCS non manual (%)[b] missing (%)	17242 (43.3) 9789 (19.7)	2325 (43.9) (p=0.40) 4128 (43.8)	338 (51.9) (p< 0.01) 289 (30.7)	48 (43.2) (p=0.99) 56 (33.5)
Smoking (ever) (%)[b] missing (%)	14896 (36.6) 8883 (17.9)	2603 (38.7) (p<0.01) 2695 (28.6)	325 (40.3) (p=0.03) 134 (14.3)	54 (37.8) (p=0.77) 24 (14.4)
Gravidity mean(SD)	1.78 (0.92)	2.60(1.46) (p<0.01)	3.96(1.56) (p<0.01)	4.90(1.52) (p<0.01)
Parity mean(SD)	1.59 (0.72)	1.27(1.15) (p<0.01)	1.50(1.13) (p=0.03)	1.29(1.05) (p<0.01)
Primary miscarriages (%)	-------	6552 (69.6)	609 (64.8)	117 (70.1)
At least one continuing pregnancy (%)	49579 (100)	6219 (66.0) (p<0.01)	725 (77.1) (p<0.01)	123 (73.7) (p<0.01)

p- values refer to comparisons between miscarriage group and no miscarriage group.
[a]maternal age at last pregnancy or miscarriage, depending on exposure
[b]percentage without missing
BMI= body mass index; RGSC= Registrar General Social Class

Mean BMI was higher in the exposed groups, although only significantly so in the non-consecutive miscarriage group. Women in the miscarriage groups were more likely to be 'ever smokers' than those with no miscarriages, significantly so for the non-consecutive and two consecutive miscarriage groups. Significantly more women in the two consecutive miscarriage group were of 'non-manual' socio-economic status than those in the no miscarriage group, although this variable was missing for a large proportion of women in each group.

Median follow-up time was 17 years (range 0-62 years). An association was found on univariate analysis between two or three consecutive miscarriages and most cardiovascular endpoints examined (tables 3-5).

Table 3 Survival analysis Ischemic Heart Disease

	no miscarriages	non-consecutive miscarriage	2 consecutive miscarriages	≥3 consecutive miscarriages
	N= 49579	N=9419	N=940	N=167
Events N (%)	1440 (2.9)	272 (2.9)	30 (3.2)	7 (4.2)
Person-years[a]	1006	192	15	3
Univariate HR (95% CI)	1.0	0.92 (0.81-1.05)	2.24 (1.56-3.22)	4.00 (1.90-8.42)
Multivariate[b,c] HR (95% CI)	1.0	1.28 (0.96-1.72)	2.40 (1.28-4.51)	3.80 (0.94-15.33)
Missing[d] (%)		(38.5)	(31.3)	(30.8)
Multivariate[b,e] HR (95% CI)	1.0	0.99 (0.87-1.13)	1.75 (1.22-2.52)	3.18 (1.49-6.80)

[a]Person-years in thousands
[b]Adjusted for maternal age, body mass index, social class, smoking
[c]Complete case analysis
[d]Percentage of cases with missing covariates; exposed and unexposed group together
[e]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval

Table 4 Survival analysis Cerebrovascular Disease

	no miscarriages	non- consecutive miscarriage	2 consecutive miscarriages	≥3 consecutive miscarriages
	N= 49579	N=9419	N=940	N=167
Events N (%)	826 (1.7)	139 (1.5)	14 (1.5)	3 (1.8)
Person-years[a]	1010	193	15	3
Univariate HR (95% CI)	1.0	0.82 (0.68-0.98)	1.71 (1.01-2.90)	2.53 (0.81-7.86)
Multivariate[b,c] HR (95% CI)	1.0	1.12 (0.77-1.64)	1.27 (0.47-3.41)	--
Missing[d] (%)		(38.5)	(31.3)	(30.8)
Multivariate[b,e] HR (95% CI)	1.0	0.88 (0.73-1.05)	1.30 (0.77-2.22)	--

[a]Person-years in thousands
[b]Adjusted for maternal age, body mass index, social class, smoking
[c]Complete case analysis
[d]Percentage of cases with missing covariates; exposed and unexposed group together
[e]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval

Figure 2 shows the event free survival for ischemic heart disease for the unexposed and exposed groups. In the multivariate Cox regression analysis between consecutive miscarriages and ischemic heart disease (Table 3), the HR in the ≥three miscarriage group was 3.18 (95%CI 1.49-6.80) (multiple imputation model). Results from the Cox regression analysis between consecutive miscarriages and cerebrovascular disease can be found in Table 4. We were unable to perform multivariate analyses for women experiencing ≥ three consecutive miscarriages because of small numbers.

Table 5 Survival analysis Diseases of the Circulatory System (including cardiac surgery)

	no miscarriages	non- consecutive miscarriage	2 consecutive miscarriages	≥3 consecutive miscarriages
	N= 49579	N=9419	N=940	N=167
Events N (%)	6841 (13.8)	1207 (12.8)	126 (13.4)	23 (13.8)
Person-years[a]	957	184	14	2
Univariate HR (95% CI)	1.0	0.88 (0.83-0.94)	1.53 (1.28-1.82)	1.70 (1.13-2.56)
Multivariate[b,c] HR (95% CI)	1.0	1.19 (1.08-1.31)	1.29 (1.00-1.66)	1.32 (0.73-2.40)
Missing[d] (%)		(38.5)	(31.3)	(30.8)
Multivariate[b,e] HR (95% CI)	1.0	0.97 (0.91-1.03)	1.34 (1.12-1.60)	1.38 (0.91-2.09)

[a]Person-years in thousands
[b]Adjusted for maternal age, body mass index, social class, smoking
[c]Complete case analysis
[d]Percentage of cases with missing covariates; exposed and unexposed group together
[e]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval

Table 6 Survival analysis Ischemic Heart Disease – groups divided by total number of miscarriages

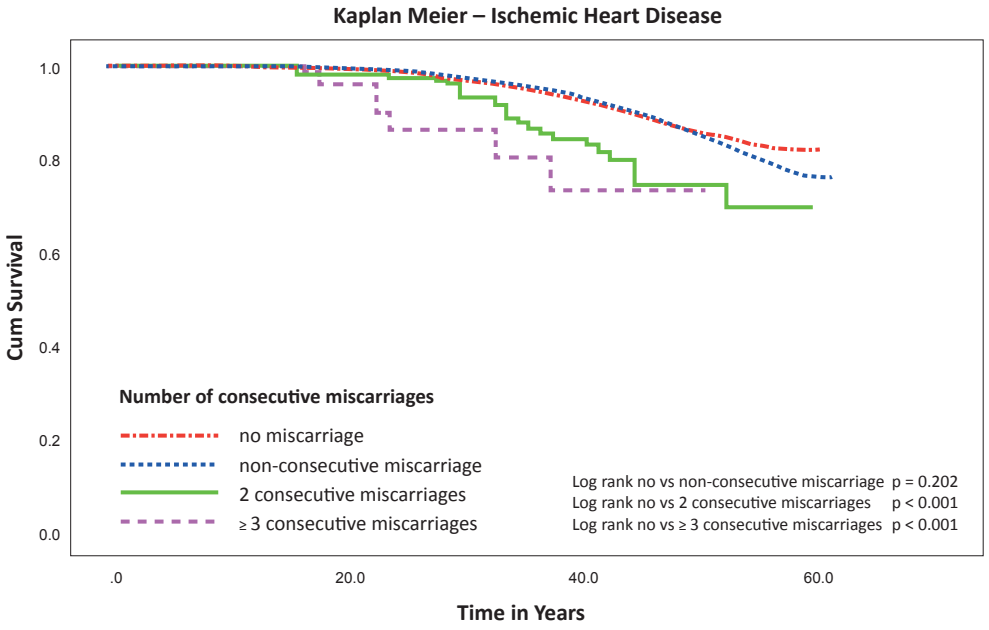
	no miscarriages	1 miscarriage	2 miscarriages	≥3 miscarriages
	N= 49579	N=9022	N=1222	N=282
Events N (%)	1440 (2.9)	262 (2.9)	36 (2.9)	11 (3.9)
Person-years[a]	1006	184	19	4
Univariate HR (95% CI)	1.0	0.90(0.79-1.03)	2.38(1.71-3.32)	4.35(2.40-7.89)
Multivariate[b,c] HR (95% CI)	1.0	1.22(0.90-1.67)	2.16(1.21-3.84)	5.65(2.32-13.78)
Missing[d] (%)		(38.3)	(31.4)	(30.8)
Multivariate[b,e] HR (95% CI)	1.0	0.97 (0.85-1.11)	1.82 (1.30-2.54)	3.18 (1.76-5.78)

[a]Person-years in thousands
[b]Adjusted for maternal age, body mass index, social class, smoking
[c]Complete case analysis
[d]Percentage of cases with missing covariates; exposed and unexposed group together
[e]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval

In the model looking at diseases of the circulatory system, HRs remained significantly elevated after multivariate analyses in the two consecutive miscarriage group; whereas statistical significance was lost in the ≥ three consecutive miscarriage group (Table 5).

Sensitivity analysis: A sensitivity analysis was performed using data whereby women were allocated to groups according to the total number of miscarriages, irrespective of whether these were consecutive or not (Table 6, supplementary data). In this analysis there were 9022 women in the one miscarriage group, 1222 in the two miscarriages group and 282 in the ≥three miscarriages group. The results were consistent with those described above; univariate analyses found increasing HR's by number of miscarriages, for each cardiovascular endpoint examined. This association remained significant in the multivariate analysis for ischemic heart disease (Table 6) and diseases of the circulatory

system.



Life Tables for Ischemic Heart Disease

Consecutive miscarriages

TIME	0 YEARS	10 YEARS	20 YEARS	30 YEARS	40 YEARS	50 YEARS	60 YEARS
no miscarriage	49579	34927	21458	12116	6544	4047	492
non-consecutive miscarriage	9419	6831	3397	2110	1394	813	196
2 consecutive miscarriages	940	605	239	130	64	24	1
≥ 3 consecutive miscarriages	167	115	42	17	9	2	-

Figure 2 Kaplan–Meier estimate: event–free survival for ischaemic heart disease

Subgroup analyses (supplementary data): Hazard ratios of ischemic heart disease and diseases of the circulatory system were lower in the women with consecutive miscarriages compared to the women with 2 or more non-consecutive miscarriage in the multivariate (multiple imputation) model. No significant difference was found in the subgroup analysis comparing women with primary and secondary consecutive miscarriages, for each cardiovascular endpoint examined.

DISCUSSION

Main findings

Our data suggest that women with a history of two or more miscarriages, irrespective of whether consecutive or not, have an increased risk of ischemic heart disease.

Strengths and limitations

To our knowledge, this study has the longest follow-up compared to other studies on this topic [5 15 16], giving time for CVD to develop. Another strength was the ability to exclude women with relevant pre-existing morbidity (type one diabetes, hypertension and kidney disease) from the analysis. Unfortunately information about type 2 diabetes was not available. Data about exposure was recorded prospectively, and information about outcome was collected from two national datasets, thereby eliminating recall bias. The quality of data collected from hospital admissions is periodically assessed, the accuracy of SMR 01 data was 88% for Main Condition [17]. Inaccuracy of outcome and wrong linkage (possibility of 3%) [18], is unlikely to introduce bias as collection of these data and linkage was done blind to the exposure status of the women. We are confident that complete reproductive histories of the women were captured in the AMND thereby minimising the possibility of misclassification according to the number of miscarriages. Previous studies have found that data from the AMND is more than 90% accurate regarding the studied exposure variables of miscarriage assessed by case note review[19] (www.abdn.ac.uk/amnd). The covariates like BMI were collected at time of exposure, by health care personnel who were blind to any outcome at that time, adding validity to the measurement.

Since the patient population is exclusively from Scotland our findings may not be applicable to other populations, for example as a result of genetic differences. Misclassification of exposure could have occurred if miscarriages were underreported (more likely to occur during the earlier years of the Databank before the widespread use of ultrasound or improved pregnancy tests). If this is the case, women who experienced miscarriages could have been placed in the no miscarriage group, and our risk estimation could have been underestimated. Bias could have been introduced due to missing data about smoking habits and social class. Women who have had an ongoing pregnancy were less likely to have missing data, because variables were better recorded at their antenatal visits. To reduce the risk of bias due to missing data we performed both complete case analysis and multiple imputation.

Each of the univariate HRs for different endpoints in the non-consecutive miscarriage group was less than one. This is likely to have been because these women were younger at exposure (first miscarriage) than women in the unexposed group (last pregnancy). When age was adjusted for the direction of effect changed. We defined maternal age in

the unexposed group as age at last pregnancy because from this point women could not become part of the exposed groups; they were no longer at risk for miscarriage.
Some women experience a complication during pregnancy, such as preeclampsia, placental abruption, IUGR or pre-term delivery, events which may increase their risk of CVD later in life[2 20]. A substantial proportion of the women in the miscarriage groups had no ongoing pregnancy, so did not have the chance to develop pregnancy complications. We did not adjust for history of complications of pregnancy since it is unclear whether these events are on the causal pathway between miscarriage and CVD[19 21]. If they are not, and if there are important differences in the prevalence of these complications, the effect of not making an adjustment will be to bias the effect towards null and therefore any effect seen in our analysis is likely to be an underestimate.

Interpretation
Our findings concur with the meta-analysis by Oliver-Williams et al[4], which included 10 studies investigating the association between miscarriage and coronary heart disease However, comparability between case-control studies in the meta-analysis was moderate and 6 of the included studies had no or minimal adjustment for confounding factors[4]. Due to the small number of studies available, the meta-analysis was unable to examine the association between recurrent miscarriage and cerebrovascular disease[4]. Our data suggest (on univariate analysis) an increased risk of cerebrovascular disease for women with two and ≥ three consecutive miscarriages. An association between miscarriage and cerebral infarction was seen in a large cohort study[5]. Although our estimates are based on relatively small numbers they can contribute to future meta-analyses.
Any higher risk of CVD later in life in women with consecutive miscarriages is probably multifactorial in aetiology. It is noteworthy that we observed an association between consecutive miscarriages and pre-existing cardiovascular related disease such as; hypertension, and diseases of circulatory system (including cardiac surgery). This suggests that miscarriage and CVD share either common risk factors or mechanism(s) of effect or both. One of the mechanisms involved may be related to the metabolic syndrome, as research suggests an association between miscarriage and insulin resistance and obesity[22]. Antiphospholipid syndrome is known to be a risk factor for women experiencing miscarriage, as well as CVD[23]. Another contribution to aetiology could be genetic; several papers describe a higher risk of miscarriage in women with a family history of miscarriage[24 25] and Smith et al 2011[26] found an increased risk of CVD in parents of women who had recurrent miscarriage.
Most of previous studies has focused on whether total number of miscarriages, rather than the consecutive nature of the events, is associated with future risk of cardiovascular disease.
We focused on the consecutive nature, as in the clinic recurrent miscarriage is mostly defined as three or more consecutive miscarriages. Also from a pathophysiological point

of view consecutive miscarriages are interesting, since it is more likely that a maternal factor plays a role[9]. Ranthe described an increased risk of cerebral infarction and renovascular hypertension in women with consecutive compared to non-consecutive miscarriages[5]. In our paper similar patterns of risk were observed when the data were examined by number of consecutive miscarriages or total number of miscarriages, irrespective of whether consecutive or not. In subgroup analyses, risks for ischemic heart disease and diseases of the circulatory system were lower in women with consecutive miscarriages compared to women with 2 or more non-consecutive miscarriages. This suggests that the number of miscarriage (2 or more) is more important than the consecutive nature of events. Although, it is important to recognise that these subgroup analyses had more limited statistical power than our main analysis. The American Heart Association advises monitoring and control of risk factors postpartum in women with a history of hypertensive complications of pregnancy[3]. As the HR for ischemic heart disease in women with two or more miscarriages is comparable with the HR for CVD in women with hypertensive disorders of pregnancy[20] we think that a comparable approach for women with two or more miscarriages is justified.

Conclusion

Women who have experienced miscarriages appear to have an increased risk of ischemic heart disease, suggesting its importance as an independent risk indicator. Miscarriage may also be an important risk indicator for other cardiovascular outcomes. We suggest that women who have experienced two or more miscarriages, irrespective of whether consecutive or not, should be made aware of an increased cardiovascular risk and advised appropriate risk factor modifications. Work is needed to determine whether women with such a history will benefit from these screening and preventative interventions.

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Supplementary data

Sensitivity analyses; groups divided by total number of miscarriages:

Table 7 Survival analysis Cerebrovascular Disease – groups divided by total number of miscarriages

	no miscarriages N= 49579	1 miscarriage N=9022	2 miscarriages N=1222	≥3 miscarriages N=282
Events N (%)	826 (1.7)	138 (1.5)	13 (1.1)	5 (1.8)
Person-years[a]	1010	185	19	4
Univariate HR (95% CI)	1.0	0.83(0.69-0.99)	1.38(0.80-2.39)	2.90(1.20-6.98)
Multivariate[b,c] HR (95% CI)	1.0	1.15 (0.79-1.69)	1.18(0.49-2.88)	--
Missing[d] (%)		(38.5)	(31.3)	(30.8)
Multivariate[b,e] HR (95% CI)	1.0	0.89 (0.74-1.06)	1.03 (0.59-1.77)	2.07 (0.86-5.01)

[a]Person-years in thousands
[b]Adjusted for maternal age, body mass index, social class, smoking
[c]Complete case analysis
[d]Percentage of cases with missing covariates; exposed and unexposed group together
[e]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval.

Table 8 Survival analysis Diseases of the Circulatory System (including cardiac surgery) – groups divided by total number of miscarriages

	no miscarriages N= 49579	1 miscarriage N=9022	2 miscarriages N=1222	≥3 miscarriages N=282
Events N (%)	6841 (13.8)	1155 (12.8)	152 (12.5)	45 (16)
Person-years[a]	957	177	18	4
Univariate HR (95% CI)	1.0	0.87 (0.82-0.93)	1.53 (1.30-1.79)	2.23 (1.66-2.99)
Multivariate[b,c] HR (95% CI)	1.0	1.19 (1.08-1.32)	1.22 (0.97-1.53)	1.68 (1.13-2.49)
Missing[d] (%)		(38.5)	(31.3)	(30.8)
Multivariate[b,e] HR (95% CI)	1.0	0.95 (0.89-1.01)	1.32 (1.12-1.55)	1.72 (1.28-2.31)

[a]Person-years in thousands
[b]Adjusted for maternal age, body mass index, social class, smoking
[c]Complete case analysis
[d]Percentage of cases with missing covariates; exposed and unexposed group together
[e]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval

Subgroup –analyses:

Table 9 Survival analysis – Consecutive miscarriages vs Non–Consecutive miscarriages

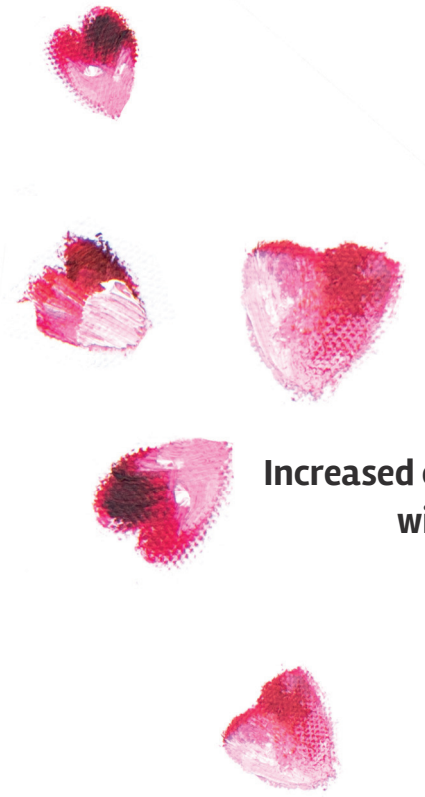
		Non-consecutive miscarriages (2 or more) N= 397	Consecutive miscarriages (2 or more) N= 1107
Ischemic Heart Disease	Events N (%)	10 (2.5)	12 (3.3)
	Univariate HR (95% CI)	1.0	0.74 (0.37-1.50)
	Multivariate[a,b] HR (95% CI)	1.0	0.47 (0.15-1.49)
	Missing[c] (%)		(33.0)
	Multivariate[b,d] HR (95% CI)	1.0	0.45 (0.21-0.97)
Cerebrovascular Disease	Events N (%)	1 (0.3)	17 (1.5)
	Univariate HR (95% CI)	1.0	3.70 (0.49-28.00)
	Multivariate[a,b] HR (95% CI)	1.0	1.19 (0.10-14.70)
	Missing[c] (%)		(33.0)
	Multivariate[b,d] HR (95% CI)	1.0	2.71 (0.34-21.48)
Diseases of the Circulatory System (including cardiac surgery)	Events N (%)	48 (12.2)	149 (13.5)
	Univariate HR (95% CI)	1.0	0.79 (0.57-1.09)
	Multivariate[a,b] HR (95% CI)	1.0	0.72 (0.45-1.14)
	Missing[c] (%)		(33.0)
	Multivariate[b,d] HR (95% CI)	1.0	0.63 (0.44-0.89)

[a]Adjusted for maternal age, body mass index, social class, smoking
[b]Complete case analysis
[c]Percentage of cases with missing covariates; groups together
[d]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval

Table 10 Survival analysis – Primary vs Secondary consecutive miscarriages

		Primary consecutive miscarriages N=726	Secondary consecutive miscarriages N= 381
Ischemic Heart Disease	Events N (%)	25 (3.4)	12 (3.1)
	Univariate HR (95% CI)	1.0	1.18 (0.59-2.38)
	Multivariate[a,b] HR (95% CI)	1.0	1.07 (0.32-3.60)
	Missing[c] (%)		(36.9)
	Multivariate[b,d] HR (95% CI)	1.0	1.25 (0.61-2.56)
Cerebrovascular Disease	Events N (%)	14 (1.9)	3 (0.8)
	Univariate HR (95% CI)	1.0	0.61 (0.17-2.19)
	Multivariate[a,b] HR (95% CI)	1.0	0.73 (0.05-10.84)
	Missing[c] (%)		(36.9)
	Multivariate[b,d] HR (95% CI)	1.0	0.61 (0.17-2.19)
Diseases of the Circulatory System (including cardiac surgery)	Events N (%)	95 (13.1)	54 (14.2)
	Univariate HR (95% CI)	1.0	1.30 (0.93-1.82)
	Multivariate[a,b] HR (95% CI)	1.0	1.21 (0.75-1.94)
	Missing[c] (%)		
	Multivariate[b,d] HR (95% CI)	1.0	1.24 (0.88-1.74)

[a]Adjusted for maternal age, body mass index, social class, smoking
[b]Complete case analysis
[c]Percentage of cases with missing covariates; groups together
[d]Multiple imputation model, model includes maternal age, body mass index, social class, smoking
HR: Hazard ratio; CI: confidence interval



3

Increased cardiovascular disease risk in women with a history of recurrent miscarriage

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Abstract

Background Cardiovascular disease is the leading cause of death in women. Observational studies suggest that women with a history of recurrent miscarriage have an increased risk of cardiovascular disease.

Methods Women who visited the recurrent miscarriage clinic at Leiden University Medical Centre (between 2000-2010) and had their third consecutive miscarriage < 31 years, were invited to participate in this follow-up study (between 2012-2014). The reference group consisted of women with at least one uncomplicated pregnancy and no miscarriage, matched on zip code, age, and date of pregnancy. All women were invited for risk factor screening, including physical examination and blood collection. Main outcome measures were the (extrapolated) 10- and 30-year cardiovascular risk scores using the Framingham risk score. A sub analysis was performed for women with idiopathic recurrent miscarriage.

Results 36 women were included in both groups. Mean follow-up was 7.5 years. Women with recurrent miscarriage had a significantly higher extrapolated 10-year cardiovascular risk score (mean 6.24%, SD 5.44) compared to women with no miscarriage (mean 3.56%, SD 1.82, p=0.007) and a significantly higher 30-year cardiovascular risk score (mean 9.86%, SD 9.10) compared to women with no miscarriage (mean 6.39%, SD 4.20, p=0.04). Similar results were found in women with idiopathic recurrent miscarriage (n=28).

Conclusions Women with a history of recurrent miscarriage differ in cardiovascular risk profile at young age compared to women with no miscarriage. The findings support an opportunity to identify women at risk of cardiovascular disease later in life and a possible moment for intervention.

Introduction

Cardiovascular disease(CVD) is the leading cause of death in women in the western world[1]. Women have a unique risk profile for CVD compared to men[2]. There is increasing evidence that women with adverse pregnancy outcomes are at increased risk of premature CVD. Pregnancy can be considered as a "stress test" unmasking underlying cardiovascular defects[3]. A history of gestational diabetes, preeclampsia or pregnancy induced hypertension is mentioned as a major risk factor in women for developing CVD in the American Heart Association Guidelines[2]. Miscarriages are not considered in this guideline.

Recurrent miscarriage is commonly defined as three or more consecutive pregnancy losses before 22 weeks of gestation[4] and affects 0.5-3% of all fertile couples[5]. Recurrent miscarriage is a highly heterogeneous condition. Possible etiologic factors include genetic disorders, uterine anomalies, endocrine factors, maternal autoimmune disorders, metabolic disorders, maternal thrombophilia and lifestyle factors[6]. An underlying cause may be identified in 25-50% of cases. Observational studies suggest that also women with a history of recurrent miscarriage have an increased risk of CVD[7-10]. Several hypotheses are possible for the association between both diseases; shared common risk factors such as obesity and smoking[11], endothelial dysfunction[12], and a genetic predisposition is assumed[13].

Determining cardiovascular risk factors in women with recurrent miscarriage could be an opportunity to identify women at high risk for future CVD at a young age. Worldwide multivariable risk assessment tools are developed to detect apparently healthy individuals at high risk for CVD and to effectively implement prevention strategies[14]. At present the most common externally validated risk model is the Framingham risk score[15].

We conducted a follow-up study to determine cardiovascular risk factors and predict the long-term CVD risk using Framingham risk scores in women with a history of recurrent miscarriage.

Methods

Study design

Follow-up study.

Exposed

Women who visited the recurrent miscarriage clinic at Leiden University Medical Centre between 2000 and 2010 and had their third consecutive miscarriage below the age of 31 years were invited to participate in this follow-up study. Recurrent miscarriage was defined as ≥ 3 consecutive miscarriages before 22 weeks of gestation. All women had

a routine recurrent miscarriage work-up to identify possible causes for the recurrent miscarriage: a standardised history of the couple was performed, karyotyping of the couple (this was offered routinely before 2005 to all couples, after 2005 this was only offered in presence of low maternal age and/or positive family history for recurrent miscarriage)[16], presence of uterus anomalies by ultrasound or hysteroscopy and presence of acquired and heritable thrombophilia was assessed. Acquired thrombophilia: antiphospholipid syndrome was defined as the presence of anticardiolipin antibodies or lupus anticoagulant in repeated samples taken 3 months apart and at least 10 weeks after delivery[17], after revision of the classification criteria the presence of anti-β2 glycoprotein-I was added to the work-up[18]. Hyperhomocysteinemia was evaluated. Heritable thrombophilia was defined by the presence of a factor V Leiden mutation, factor II (prothrombin) gene mutation, protein C or S deficiency or antithrombin deficiency. Women with primary miscarriage (no live birth before miscarriage) and secondary miscarriage (live birth(s) before miscarriage) were included. The time interval between the diagnosis recurrent miscarriage and the time of follow-up had to be at least 2 years.

Unexposed

Women with one or more uncomplicated pregnancy(ies) and no miscarriages were enrolled (reference group). In the Netherlands, it is common practice that independent community midwives are taking care of low-risk women (with no medical or obstetrical history) during pregnancy and child birth. The zip code of each woman with recurrent miscarriage was used to contact the nearest midwifery practice to take the impact of socio-economic status into account. Women with the same zip code, the same age (difference in birthdate maximal 1 year) and of which the time of first delivery was close to the time of the third miscarriage of the matched exposed woman (maximum 6 months before or 6 months after) were asked to participate. Women with recurrent miscarriage were included in the study before the matched controls were invited to participate, a small difference in follow-time was therefore expected. In both groups, pregnant and lactating women (within the last 3 months) were excluded. Enrolment took place between 2012 and 2014.

Procedures and definitions

After enrolment, all women were asked to fill out a web based questionnaire and were invited for risk factor screening. The questionnaire included general information, medical history, family history of CVD, use of medication, intoxications and obstetric history. Information about medical history, use of medication, intoxications and pregnancy outcome was cross checked in obstetrical records to overcome recall bias. Gestational diabetes was defined as a glucose intolerance resulting in hyperglycaemia with onset during pregnancy[19]. Preeclampsia was defined as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg combined with proteinuria[20],

pregnancy induced hypertension as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg or higher measured on two occasions(after 20 weeks' gestation)[21], preterm birth as a delivery between 24 and 37 weeks gestation, intra-uterine growth restriction as birth weight below the 10th percentile for gestational age and sex according to the Netherlands Perinatal Registry birth weight percentiles[22]. Assessment of classic cardiovascular risk factors was performed by trained research nurses or doctors at the Leiden University Medical Centre or at the participants' home. Blood pressure was measured manually with a validated sphygmomanometer in sitting position at the left upper arm with the appropriate cuff size, the mean of two measurements was taken. Length and weight was measured wearing light clothes and without shoes; length was measured to the nearest 1 cm and weight to the nearest 1 kg. Body mass index(BMI) was calculated as weight/ length^2, Venous blood samples were collected after an overnight fast and assayed for classical risk factors of CVD (glucose, insulin, HbA1c, total cholesterol, HDL cholesterol, triglycerides), Insulin resistance was assessed by the homeostasis model assessment (HOMA)[23]. The blood samples were centrifuged, separated and frozen at -80°C within 2 hours. Routine chemistry analyses were performed on a Roche Modular P800 chemistry analyser using reagents of Roche Diagnostics (Mannheim, Germany). Analytical variation of all analytes was well below 5%. Insulin was analysed on an Immulite 2000 Xpi immunoanalyser of Siemens Healthcare Diagnostics (Tarry town, NY, USA). Analytical variation varied between 5% and 8%. HbA1c was analysed using a Boronate affinity chromatographic system (Primus Ultra^2, Trinity Biotech, Bray Ireland). Analytical variation was well below 2%. All analyses were performed by technicians blinded for obstetrical history. Family history of premature myocardial infarction (MI) and/or stroke was defined as having at least one parent with MI and/or stroke before the age of 60.

10- and 30-Year CVD risk by the Framingham score[24, 25] was calculated using information on age, systolic blood pressure, antihypertensive treatment, smoking, diabetes and lipid spectrum (total cholesterol and HDL cholesterol) or BMI (a simpler model of the risk score). Both models, using lipids and using BMI, were applied. CVD was defined as coronary death, MI, coronary insufficiency, angina pectoris, ischemic stroke, haemorrhagic stroke, transient ischemic attack, peripheral artery disease and heart failure. The 10-Year CVD risk score was calculated twice; using current age and subsequently estimating the risk as if the woman was 60 years of age (due to the young age of our participants, the estimated absolute 10-year CVD risk was likely to be low). This approach has been recommended in the cardiovascular risk factor management guidelines for young women with elevated risk factor levels [26]. During our study period a new guideline was published[27], which revised the approach to CVD in the young in using 'cardiovascular risk age'. This method was not applicable to our young cohort (age < 40 years) and we decided to follow the afore-mentioned approach. The risk estimation was repeated in a subgroup analysis including women with idiopathic recurrent miscarriage. Recurrent miscarriage was defined as idiopathic when the work-up for causes of recurrent miscarriage showed no abnormalities.

Sample size considerations

The calculation was based on results of the Hyras study: the (extrapolated) 10-year CVD risk was 4,4% (SD 1.9) in women with uncomplicated pregnancies[28]. We planned to include women in 1:1 ratio, i.e. a woman who had recurrent miscarriage matched to 1 control subject. A relative risk of 1,5 or higher was considered to be clinically relevant. A sample size of 68 women (34 exposed, 34 non-exposed) was sufficient, with a 10% drop-out rate (two sided alpha .05. power 90%).

Statistics

Data were analysed using SPSS software version 22.0 (Statistical Package for Social Science; SPSS, Chicago, Illinois, USA). Comparisons of normal distributed data were performed using paired T- test. Comparisons of continuous data were performed using the McNemar's test. For all tests, a p-value < 0.05 is indicated statistical significant.

Ethics

Approval from the medical ethics committee of Leiden University Medical Centre (P04-020; October 3, 2012) was obtained and all participants gave informed consent. The study was registered with the Dutch trial registry NTR3408. This study is part of the REMI (REcurrent MIscarriages) studies, studies which investigate consequences and causes of recurrent miscarriages.

Results

A flowchart of the inclusion of the participants is shown in Figure 1. Of the 76 included women, 4 women were excluded from analysis, because they did not meet the inclusion criteria for this study as described in Figure 1. Leaving 36 matched pairs.

Women with recurrent miscarriage had a significantly higher gravidity and lower parity than those in the no miscarriage group (Table 1). Women with recurrent miscarriage were more often smokers during pregnancy (p=0.05). On all other variables groups were comparable.

78% of the women with recurrent miscarriage (n=28) were diagnosed with idiopathic recurrent miscarriage. Parental chromosomal abnormality was found in one case, antiphospholipid syndrome in one case, hyperhomocysteinemia in three cases and heritable thrombophilia was found in three cases.

Mean follow-up time was 6.8 years (SD 3.0) in women with recurrent miscarriage and 8.1 years in women with no miscarriage (SD 2.9), (p<0.001). Classical cardiovascular risk factors are described in Table 2. Women with recurrent miscarriage were slightly younger at time of follow-up than women with no miscarriage (p<0.001). Values of classical cardiovascular risk factors were higher in women with recurrent miscarriage compared to no miscarriage, although only significant for systolic blood pressure.

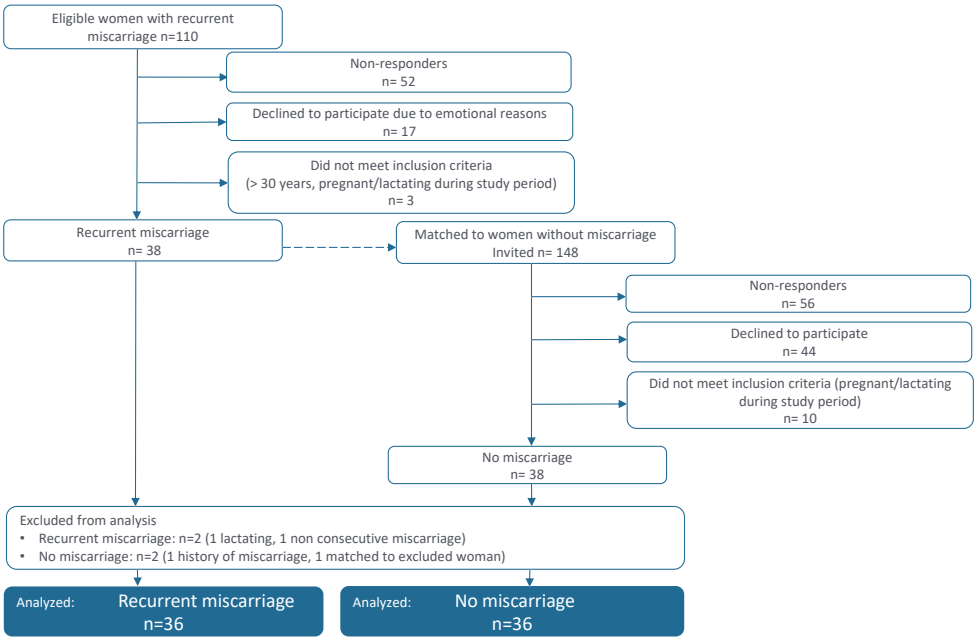

Figure 1. Flow chart Selection of participants

Table 1. Characteristics of participants

	No miscarriage n=36	Recurrent miscarriage n=36	p-value
Maternal age at index pregnancy[a]	26.36 (2.65)	26.47 (2.69)	0.70
Caucasian (%)	32 (88.9)	30 (83.3)	0.63
University level education (%)	16 (44.4)	8 (22.2)	0.08
Gravidity	2.28 (0.62)	7.11 (2.07)	<0.001
Parity	2.25 (0.60)	1.64 (0.83)	0.001
Primary miscarriages (%)	--	27 (75.0)	--
At least one continuing pregnancy[b] (%)	36 (100)	35 (97.2)	0.31
Smoking during pregnancy[c] (%)	5 (13.9)	14 (38.9)	0.05
Gestational diabetes[c] (%)	0 (0)	0 (0)	--
Preeclampsia/Gestational hypertension[b,c] (%)	0 (0)	3 (8.3)	0.08
Preterm birth[c] (%)	1 (2.8)	4 (11.1)	0.38
Intra uterine growth restriction[c] (%)	4 (11.1)	4 (11.1)	1.00

Data are presented as mean (SD)
[a]Age at first pregnancy for unexposed women, age at third consecutive miscarriage for exposed women
[b]Chi-squared test. Mc Nemars test not possible (at least one variable in each 2-way table is a constant)
[c]In at least one continuing pregnancy

Table 2. Classical cardiovascular risk factors

	No miscarriage n=36	Recurrent miscarriage n=36	p-value
Maternal age at follow-up	34.50 (3.59)	33.28 (3.51)	<0.001
Smoking at follow-up (%)	5 (13.9)	10 (27.8)	0.23
BMI at follow-up	23.78 (3.49)	25.89 (7.08)	0.09
Systolic blood pressure mmHg	101.11 (10.72)	111.11 (13.06)	<0.001
Diastolic blood pressure mmHg	67.22 (7.62)	70.64 (9.54)	0.08
Antihypertensive medication use[a] (%)	0 (0)	3 (8.33)	0.08
HOMA score	2.28 (1.95)	3.40 (6.20)	0.31
HbA1c mmol/mol Hb	29.89 (2.45)	32.25 (8.36)	0.13
Total cholesterol mmol/L	4.89 (0.76)	4.76 (0.68)	0.46
HDL cholesterol mmol/L	1.71 (0.39)	1.59 (0.49)	0.25
Triglycerides mmol/L	0.98 (0.29)	1.12 (0.61)	0.24
Family history of premature MI and/or stroke (%) ***Missing=1***	9 (25.7)	8 (22.9)	1.00

Data are presented as mean (SD)
[a]Chi-squared test. Mc Nemars test not possible (at least one variable in each 2-way table is a constant)
BMI body mass index, HOMA homeostasis model assessment, HbA1C haemoglobin A1c, HDL high-density lipoproteins, MI myocardial infarction

Women with recurrent miscarriage had significantly higher mean CVD risk scores compared to women with no miscarriage (Table 3), independent of using the lipids or the BMI model. In the subgroup analysis including women with idiopathic recurrent miscarriage comparable results to the total group were found (Table 3).

Discussion

Main findings

In this follow-up study an increased (extrapolated) 10 and 30-year CVD risk was found in women with a history of recurrent miscarriage compared to women with no miscarriages, calculated by Framingham risk scores (lipids and BMI model). Women with recurrent miscarriage had an increased systolic blood pressure compared to women with no miscarriage at time of follow-up.

Interpretation

The Framingham risk score is the most externally validated risk score and is widely used in North American countries[15]. It is the only model which can estimate the 10 and 30-year CVD risk (mortality and morbidity) and is therefore useful to estimate risks for a young population. European guidelines advise the use of SCORE, which assesses only mortality risk and therefore is less useful in our young population[29]. Overestimation of the risk of CVD is possible using the Framingham score in a European cohort[15]. If so,

Table 3 Cardiovascular disease risk estimation

		No miscarriage n=36	Recurrent miscarriage n=36	Mean difference (95% CI)	p-value	No miscarriage n=28	Idiopathic recurrent miscarriage n=28	Mean difference (95% CI)	p-value
10 year Framingham risk score (%)	lipids	1.06 (0.68)	2.05 (2.45)	0.99 (0.13-1.85)	0.03	1.06 (0.73)	2.28 (2.72)	1.21 (0.11-2.31)	0.03
	BMI	1.12 (0.65)	2.03 (2.42)	0.91 (0.10-1.71)	0.03	1.12 (0.68)	2.16 (2.66)	1.04 (0.01-2.07)	0.05
10 year Framingham risk score (%) (extrapolated to 60 years)	lipids	3.56 (1.82)	6.24 (5.44)	2.68 (0.78-4.58)	0.007	3.59 (1.99)	6.73 (6.00)	3.14 (0.72-5.57)	0.01
	BMI	4.67 (2.13)	8.57 (7.85)	3.90 (1.22-6.58)	0.006	4.74 (2.31)	9.07 (8.65)	4.33 (0.91-7.75)	0.02
30 year Framingham risk score (%)	lipids	6.39 (4.20)	9.86 (9.10)	3.47 (0.25-6.70)	0.04	6.54 (4.52)	10.68 (10.00)	4.14 (0.02-8.27)	0.05
	BMI	7.31 (4.08)	11.9 (12.1)	4.56 (0.53-8.56)	0.03	7.46 (4.41)	12.43 (13.27)	4.96 (-0.16-10.1)	0.06

Data are presented as mean (SD)

an overestimation of the risk occurred in both groups and therefore is not changing the direction of effect. Due to the young age of our participants we calculated the 10-year risk scores as if the women were 60 years of age according guidelines for young women with elevated risk factor levels[26]. The new method of 'cardiovascular risk age'[27] was not applicable to our young cohort (age < 40 years). A disadvantage of this method, extrapolating to an age of 60 years, is that the real risk could be underestimated assuming that levels of cardiovascular risk factors will increase without prevention or intervention. Perhaps this is the reason why we found a quite large difference between the 10-Year CVD risk after extrapolating the age to 60 years and the 30-Year CVD scores, in women with recurrent miscarriage mean risk 6.24% and 9.86%, respectively.

Only few studies have been performed regarding cardiovascular risk factors in women with recurrent miscarriage. Our findings are inconsistent with the results of the study from Mahendru et al[30], which found no difference in cardiovascular function and risk factors between women with unexplained recurrent miscarriage (n=26) and women with uncomplicated pregnancy before a subsequent pregnancy. Explanations for this may be a lack of power, short follow-up time (median 8 months in study from Mahendru et al) or the difference in selection of the women with recurrent miscarriage; we used an age criterion; recurrent miscarriage ≤ 30 years versus unexplained recurrent miscarriage irrespective of age in the other study. Our findings are in line with the report by Germain et al[12] who found that women with recurrent pregnancy loss (n= 29), defined as ≥ 2 consecutive miscarriages, have an altered cardiovascular risk profile compared to women with uncomplicated pregnancy (significant for total cholesterol). Their methods differed from ours as they excluded all women with: overweight, chronic hypertension, diabetes, renal and CVD at index pregnancy, smokers and women with thrombophilia except for antiphospholipid syndrome (APS), introducing a high level of selection bias. The explanation for this is that they investigated the hypothesis that endothelial dysfunction could be the link between miscarriage and CVD (and these factors alter markers of endothelial function). We performed a subgroup analysis including only women with idiopathic recurrent miscarriage (n=28) (Table 3), which showed comparable results to the results of the total group. Therefore, in this study the increased risk of CVD in women with recurrent miscarriage cannot be explained by the presence of known acquired and heritable thrombophilia.

In Table 2 we described the individual classical risk factors. Only systolic blood pressure was significantly higher in women recurrent miscarriage compared to no miscarriage. Since we did not perform a sample size analysis based on individual risk factors, a lack of power is likely when investigating the individual risk factors. It would be interesting to investigate these risk factors in a larger study group to answer the question which risk factor is contributing the most to the elevated CVD risk in women with recurrent miscarriage. As we were only able to look at cardiovascular risk factors in women after they experienced recurrent miscarriage we are not answering the question about cause

and effect. Although pre-existing CVD risk factors are associated with an increased risk of developing miscarriages, it is not known whether miscarriages merely unmask risk or contributes directly to future CVD. Miscarriages could trigger a pathophysiological mechanism or cascade that in turn leads to CVD, potentially via interactions with classical risk factors.

Strengths/limitations

To our knowledge this is the first study which investigated and calculated CVD risk scores in women with a history of recurrent miscarriage. A strength of our research is the unique, well defined cohort. Recurrent miscarriage is a highly heterogenic condition, to strive to more homogeneity, in present study we included only women who had their third consecutive miscarriage below the age of 31. A younger age at diagnosis makes a maternal cause of recurrent miscarriage more plausible and reduces the change of miscarriages due to foetal abnormalities[31]. Another strength is the availability of a wide range of covariates in both groups (Table 1). We decided not to adjust for any covariates in our CVD risk estimation as both groups were comparable at baseline, except for gravidity and parity what is a direct consequence of the exposure (recurrent miscarriage). Besides, some covariates have an effect at our outcome of interest. For example, it wouldn't make sense to adjust for BMI or smoking as both are included as variables in the risk estimation[32]. Some women experienced a complication during pregnancy, such as gestational diabetes and preeclampsia which may increase their risk of CVD later in life[2, 33]. We did not adjust for history of complications of pregnancy since these events are possibly on the causal pathway between miscarriage and CVD[34]. If we assume that they are not on this pathway and that these events are confounding factors we should have adjusted for these pregnancy complications. Therefore, we repeated the risk calculations for women who did not have a pregnancy complicated by gestational diabetes, preeclampsia, pregnancy induced hypertension and/or preterm birth (online supplementary data). Women with recurrent miscarriage (N=30) had still a significantly higher extrapolated 10-year cardiovascular risk score (using lipids: mean 5.31%, SD 3.96) compared to women with no miscarriage (mean 3.59%, SD 1.94, p=0.03). Comparable results were found in women with idiopathic recurrent miscarriage (N=24) (online supplementary data). Therefore, we can conclude that the elevated risk scores in women with recurrent miscarriage cannot be explained solely by other pregnancy complications known to be a risk factor in women for developing CVD.

Limitations of our study should be acknowledged. The risk for CVD in women with recurrent miscarriage could be underestimated due to the following reasons: At first selection bias may have been introduced. Women declined to participate due to emotional reasons or did not respond on the invitation for this follow-up study. It is imaginable that the 'worst' cases, women without a live birth, were more likely to decline or not respond (only one woman in our study group had no live birth). Secondly, women received lifestyle advice

during their consultations at the recurrent miscarriage clinic. Individual risk factors may have been changed which could decrease the risk profile. And finally, bias could have been introduced since women with recurrent miscarriage were included in the study before the matched controls were invited to participate resulting in a small difference in age (1.2 years) at follow-up. In case this small difference will influence the results, the increased risk in women with recurrent miscarriage would be an underestimate because risk factors are likely to increase with age. On the other hand, since the unexposed group consisted of women who had at least one uncomplicated pregnancy, this may have resulted in a healthier cohort compared to a population based cohort. Selection bias is also possible in the unexposed group; probably women with a higher education are more likely to participate (although no significant difference was found for university level education between both groups). Another limitation is the relatively small sample size. A preliminary calculation was performed based on the 10-year risk score with age extrapolated to 60 years, which showed that 34 women in both groups would be sufficient. Though it is possible that, especially for the subgroup analyses, our study may partly be underpowered and we should be cautious drawing conclusions.

Perspectives

In present study, we show that women with a history of recurrent miscarriage, irrespective whether idiopathic or not, differ in cardiovascular risk profile at a young age compared to women with no miscarriage. Our study provides intriguing data which support the need for more research to find out if women with a history of recurrent miscarriage should be offered screening and counselling for cardiovascular risk factors.

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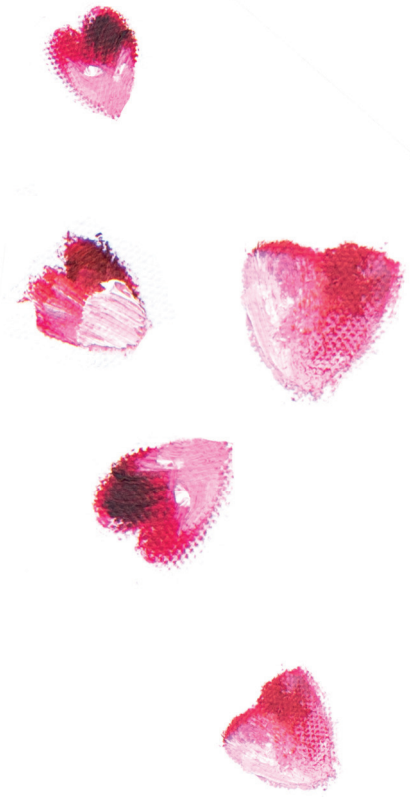
Table 4 Cardiovascular disease risk estimation, in women with recurrent miscarriage without other pregnancy complications

		No miscarriage n=30	Recurrent miscarriage n=30	Mean difference (95% CI)	p-value	No miscarriage n=24	Idiopathic recurrent miscarriage n=24	Mean difference (95% CI)	p-value
10 year Framingham risk score (%)	lipids	1.06 (0.71)	1.44 (1.25)	0.39 (-0.12-0.90)	0.13	1.10 (0.78)	1.58 (1.35)	0.48 (-0.16-1.11)	0.13
	BMI	1.09 (0.66)	1.65 (1.73)	0.57 (-0.10-1.23)	0.09	1.12 (0.71)	1.82 (1.90)	0.70 (-0.13-1.52)	0.10
10 year Framingham risk score (%) (extrapolated to 60 years)	lipids	3.59 (1.94)	5.31 (3.96)	1.72 (0.14-3.29)	0.03	3.70 (2.12)	5.75 (4.28)	2.04 (0.09-3.99)	0.04
	BMI	4.59 (2.23)	7.38 (6.31)	2.79 (0.37-5.21)	0.03	4.77 (2.42)	8.03 (6.91)	3.25 (0.24-6.27)	0.04
30 year Framingham risk score (%)	lipids	6.50 (4.45)	8.33 (6.59)	1.83 (-0.90-4.57)	0.18	6.79 (4.80)	9.04 (7.12)	2.25 (-1.15-5.65)	0.18
	BMI	7.27 (4.27)	10.13 (9.44)	2.87 (-0.71-6.44)	0.11	7.50 (4.63)	10.92 (10.41)	3.42 (-1.05-7.88)	0.13

Data are presented as mean (SD)



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Assessment of novel cardiovascular biomarkers in women with a history of recurrent miscarriage

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Abstract

Objectives A history of recurrent miscarriage is associated with future cardiovascular disease. The aim of this study was to determine novel cardiovascular biomarkers in women with a history of recurrent miscarriage as this might lead to a better understanding of the association.

Study design Women who visited the recurrent miscarriage clinic at Leiden University Medical Centre (between 2000 – 2010), and had three consecutive miscarriages ≤ 30 years were invited to participate in this follow-up study (between 2012-2014). The reference group consisted of women with at least one uncomplicated pregnancy and a history of no miscarriage, matched on zip code, age, and date of pregnancy.

Main outcome measures Cardiovascular biomarkers were determined, classified into; inflammation (HsCRP, lipoprotein-associated phospholipase A2), thrombosis (homocysteine, folate, anti-cardiolipin antibodies and anti-ß-2-glycoprotein antibodies), lipid metabolism (lipoprotein(a)), renal function (creatinine, microalbuminuria), myocardial damage (N-terminal pro-brain natriuretic peptide, high sensitive TroponineT) and multiple mechanisms (albumin, vitamin D).

Results In both groups, 36 women were included. Women with recurrent miscarriage had a significantly higher median HsCRP (1.49mg/L) compared to women with no miscarriage (1.01mg/L, p=0.03) and a significantly lower mean albumin (46.0 vs 47.6g/L, p=0.004) and vitamin D (55.6 vs 75.4nmol/L, p=0.007), respectively. Differences remained after adjustments for classic cardiovascular risk factors (BMI, smoking, diabetes mellitus, and hypertension).

Conclusions Our findings suggest a proinflammatory state in women with a history of recurrent miscarriage, which suggests a less optimal health, compared to women with no miscarriage. More research (observational and intervention) is warranted to investigate the association with vitamin D.

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in women in the western world[1]. There is increasing evidence that women with adverse pregnancy outcome are at increased risk of future cardiovascular disease, most well established for preeclampsia[2, 3]. Recent epidemiological research suggests that also women with a history of recurrent miscarriage have an increased risk of cardiovascular disease later in life[4-6]. Recurrent miscarriage is commonly defined as three or more consecutive pregnancy losses before 22 weeks of gestation[7]. It is a very heterogeneous condition and affects 0.5-3% of all fertile couples[8]. Several hypotheses are possible for the association between recurrent miscarriage and cardiovascular disease: shared risk factors and underlying pathology may lead to both diseases or alternatively, miscarriage could trigger a mechanism or cascade (second hit) that in turn leads to cardiovascular disease. Possibly via interactions with well-known risk factors. Two small studies are published concerning classical cardiovascular risk factors in women with a history of recurrent miscarriage with inconsistent results[9, 10]. An altered cardiovascular risk profile in women with recurrent miscarriage was found in the first study[9], although in the second study no difference in cardiovascular function and risk factors was described[10]. In addition to classic cardiovascular risk factors, there is a wide variety of novel biomarkers strongly associated with future cardiovascular disease in general [11, 12]. For example, high-sensitivity C-reactive protein (HsCRP), an inflammatory biomarker and lipoprotein(a) (Lp(a)), a lipid related biomarker. The most recent European guideline for the prevention of cardiovascular disease states that biomarkers may be useful in specific subgroups[12]. Knowledge about these markers might contribute to a better understanding of the association between miscarriage and future cardiovascular disease. Therefore, we conducted a follow-up study to determine novel cardiovascular biomarkers in women with a history of recurrent miscarriage compared to women with no miscarriage.

Methods

Study design

Follow-up study.

Exposed

Women who visited the recurrent miscarriage clinic at Leiden University Medical Centre between 2000 and 2010 and had their third consecutive miscarriage before the age of 31 years were invited to participate in this follow-up study. Recurrent miscarriage was defined as ≥ 3 consecutive miscarriages before 22 weeks of gestation. Women with

primary miscarriage (without birth ≥ 22 weeks of gestation before miscarriage) and secondary miscarriage (with a birth ≥ 22 weeks of gestation before miscarriage) were included. All women had a routine recurrent miscarriage work-up to identify possible causes for the recurrent miscarriage: a standardized history of the couple was performed, karyotyping of the couple (this was offered routinely before 2005 to all couples, after 2005 this was only offered in presence of low maternal age and/or positive family history for recurrent miscarriage[13], presence of uterus anomalies by ultrasound or hysteroscopy and presence of acquired and heritable thrombophilia was assessed. Acquired thrombophilia: Antiphospholipid syndrome was defined as the presence of elevated anticardiolipin antibodies or lupus anticoagulant in repeated samples taken 3 months apart and at least 10 weeks after a delivery [14], after revision of the classification criteria an elevated level of anti-β2 glycoprotein-I was added to the work-up [15]. Homocysteine levels were determined to exclude hyperhomocysteinemia. Heritable thrombophilia was defined by the presence of a factor V Leiden mutation, factor II (prothrombin) gene mutation, protein C or S deficiency or antithrombin deficiency. Enrolment took place between 2012 and 2014.The time interval between the diagnosis recurrent miscarriage and the time of follow-up had to be at least 2 years.

Unexposed matched group

For the reference group women with one or more uncomplicated pregnancy(ies) and no miscarriages were enrolled. In the Netherlands, it is common practice that independent primary care midwives are taking care of low-risk women (with no medical or obstetrical history) during pregnancy and child birth. The zip code of each woman with recurrent miscarriage was used to contact the nearest midwifery practice to take the impact of socio-economic status into account. Women with the same zip code, the same age (difference in birthdate maximal 1 year) and of which the time of first delivery was close to the time of the third miscarriage of the matched exposed woman (maximal 6 months before or 6 months after) were asked to participate.

In both groups pregnant and lactating women (within the last 3 months) were excluded.

Procedures and definitions

After enrolment all women were asked to fill out a web based questionnaire and were invited for risk factor screening including venous blood samples. The questionnaire included general information, medical history, family history of cardiovascular disease, use of medication, intoxications and obstetric history. Information about medical history, use of medication, intoxications and pregnancy outcome was cross checked in obstetrical records to overcome recall bias. Pregnancy outcome in at least one continuing pregnancy was recorded. Gestational diabetes was defined as a glucose intolerance resulting in hyperglycemia with onset during pregnancy[16]. Preeclampsia was defined as systolic blood pressure above 140 mmHg and/or diastolic pressure

above 90 mmHg combined with proteinuria [17], pregnancy induced hypertension as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg or higher measured on two occasions(after 20 weeks' gestation)[18], preterm birth as a delivery between 24 and 37 weeks gestation, intra-uterine growth restriction as birth weight below the 10th percentile for gestational age and sex according to the Perinatal Registry of the Netherlands birth weight percentiles [19]. Recurrent miscarriage was defined as idiopathic when the work-up for causes of recurrent miscarriage showed no abnormalities.

Cardiovascular risk factor assessment

Assessment of cardiovascular risk factors was performed by trained research nurses or physicians at the Leiden University Medical Centre or at the participants' home. Urine was collected for assessment of microalbuminuria immediately after waking up. Venous blood samples were collected after an overnight fast. A panel of novel cardiovascular biomarkers was tested in this study. Biomarkers were classified into; inflammation (HsCRP, Lipoprotein-associated phospholipase A2 (Lp-PLA2)), thrombosis (homocysteine, folate, anti-Cardiolipin antibodies (aCL) and anti-ß-2-Glycoprotein antibodies (aβ2GPI)) IgG and/or IgM, lipid metabolism (Lp(a)), renal function (creatinine, microalbuminuria), myocardial damage (N-terminal pro-brain natriuretic peptide(NT-proBNP), high sensitive Troponine T (hsTroponineT) and multiple mechanisms (albumin, 25-OH-Vitamin D). We describe these biomarkers with their possible association with cardiovascular disease in short in table 1.

The blood samples were centrifuged after coagulation, separated and serum was frozen at -80°C within 2 hours. Routine chemistry analyses were performed on a Roche Modular P800 chemistry analyzer using reagents of Roche Diagnostics (Mannheim, Germany). Analytical variation of all analytes was well below 5%. Homocysteine was analyzed on an Immulite 2000 Xpi immunoanalyzer of Siemens Healthcare Diagnostics (Tarry town, NY, USA). Analytical variation varied between 5% and 8%. NT-proBNP, Folate, hsTroponinT,25-OH-Vitamin D and Lp(a) were analyzed on a Roche Modular E170 Immunoanalyzer. Analytic variation varied between 3% and 6%. Immunological analyses were performed on an Immunocap 250 immunoanalyzer (Thermo Fisher Scientific, Waltham, MA, USA). Analytical variation was up to 5% for aCL and 10% for aβ2GPI antibodies. Lp-PLA2 was measured using an ELISA (Diadexus, San Francisco, CA, USA). All analyses were performed by technicians blinded for obstetrical history.

Table 1. Novel cardiovascular biomarkers, possible associations and mechanisms

Biomarker	Possible association with	Mechanisms
HsCRP	Inflammation	Acute phase protein with hepatic origin [21]
Lp-PLA2	Inflammation	Vascular-specific inflammatory biomarker. (Atherosclerotic plaques in blood vessels) [36]
Homocysteine	Thrombosis	Endothelial cell damage, reduction in the flexibility of vessels, and alters the process of haemostasis. Hyperhomocysteinemia can also arise from nutritional deficiencies of folate, vitamin B6, and vitamin B12. [33]
Folate	Thrombosis	Lowering serum homocysteine.[37]
aCL	Thrombosis	Antiphospholipid antibody: changes in the coagulation cascade, inhibition of protein C, antithrombin and annexin, platelet activation and complement, increased expression of endothelial adhesion molecules. [38] aCL seem to represent the group of autoantibodies with the highest correlation and possible risk outcome with CVD. [39]
aβ2GPI	Thrombosis	Antiphospholipid antibody: changes in the coagulation cascade, inhibition of protein C, antithrombin and annexin, platelet activation and complement, increased expression of endothelial adhesion molecules, [38]. Locally, using cellular models aβ2GPI antibodies seems to have a prominent pathogenic role. [39]
Lp(a)	Lipid metabolism	The physiological function of Lp(a) is unclear, pathogenic role in atherosclerosis and thrombosis formation. [40]
Creatinine	Renal function	May be surrogate marker for generalized vascular damage as well as renal dysfunction. Renal dysfunction may enhance intermediate risk factors such as hypertension, hyperhomocysteinemia and abnormalities of thrombogenic factors. [41]
Microalbuminuria	Renal function	May be a marker of widespread vascular abnormalities, including those of the glomerular capillary wall. Renal dysfunction. [41]
NT-proBNP	Myocardial damage	NT-proBNP is synthesized in response to ventricular stretch and ischemic injury [42]
hsTroponineT	Myocardial damage	Secreted from cardiac (and skeletal) muscles. Myocardial injury and ischemia. [43].
Albumin	Multiple mechanisms	Negative acute-phase protein. Interacts with free fatty acids, and inhibits their promoting effects on platelet aggregation and thrombosis. May act as an indirect and sacrificial antioxidant and inhibits peroxidase, free radical generation. Inhibitor of endothelial apoptosis.[24]
Vitamin D	Multiple mechanisms	Modulation of inflammatory processes. Favourably influence cardiovascular health including downregulation of the renin–angiotensin system, enhancement in insulin secretion and insulin sensitivity, and protection against angiogenesis. [26]

HsCRP: high-sensitivity C-reactive protein. Lp-PLA2: Lipoprotein-associated phospholipase A2, aCL: anti-Cardiolipin, aβ2GPI: anti-ß-2-Glycoprotein, Lp(a):Lipoprotein (a), NT-proBNP : N-terminal pro-brain natriuretic peptide, hsTroponineT: high sensitive Troponine T

Statistics

Matched data were analyzed using SPSS software version 22.0 (Statistical Package for Social Science; SPSS, Chicago, Illinois, USA). Comparisons of normal distributed data were performed using paired T- test. Comparisons of categorical data were performed using the McNemar's test. Skewed variables were log transformed, differences on the logarithmic scale were calculated and then back transformed to the original scale. This results in estimates of the ratio of the (geometric) mean in the recurrent miscarriage group over the mean in the no miscarriage group. For all tests, a p-value < 0.05 is indicated statistical significant. Multivariate analyses were performed using unianova test, to adjust for the following potential confounders (classical cardiovascular risk factors): Model 1: BMI; Model 2: BMI and smoking; and Model 3 BMI, smoking, diabetes mellitus and hypertension. The calculations were repeated in a subgroup analysis including women with idiopathic recurrent miscarriage.

Ethics

Approval from the medical ethics committee of Leiden University Medical Centre (P04-020; October 3, 2012) was obtained and all participants gave informed consent. The study was registered with the Dutch trial registry NTR3408. This study is part of the REMI (REcurrent MIscarriage) studies, studies which investigate consequences and causes of recurrent miscarriage.

Results

A flowchart of the inclusion of the participants is shown in Figure 1. Of the 76 included women (38 women with recurrent miscarriage and 38 women with at least one uncomplicated pregnancy and no miscarriage) 4 women were excluded from analysis, because they did not meet the inclusion criteria for this study as described in Figure 1, leaving 36 matched pairs.

Women with recurrent miscarriage were slightly younger at follow-up and had a significantly higher gravidity and lower parity than those in the no miscarriage group (Table 2). Women with recurrent miscarriage were more often smokers during pregnancy (p=0.05). On all other variables groups were comparable.

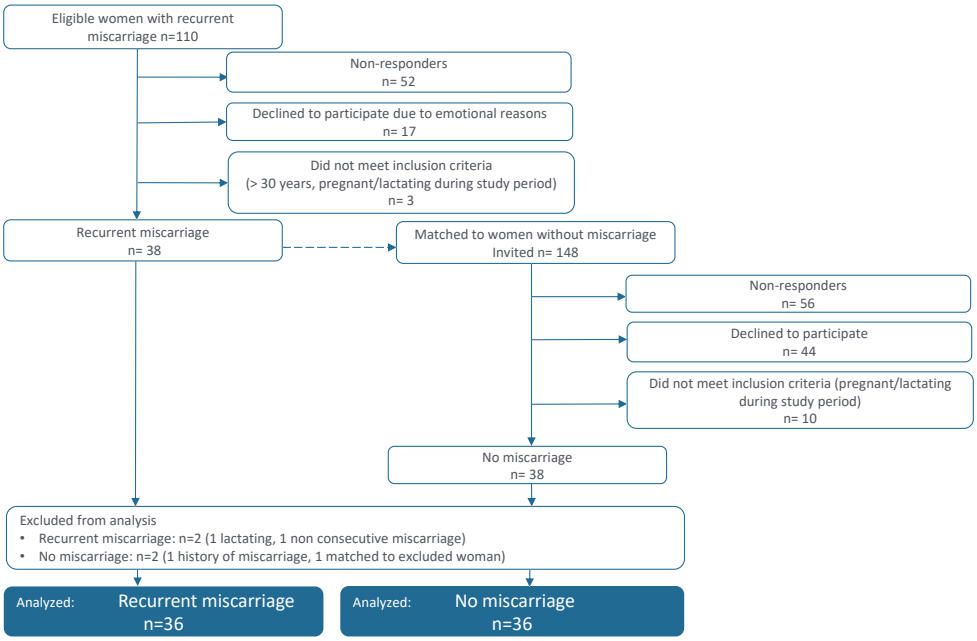

Figure 1. Flow chart Selection of participants

Table 2. Characteristics of participants

	No miscarriage n=36	Recurrent miscarriage n=36	p-value
Maternal age at index pregnancy[a]	26.36 (2.65)	26.47 (2.69)	0.70
Maternal age[b]	34.50 (3.59)	33.28 (3.51)	<0.001
Caucasian (%)	32 (88.9)	30 (83.3)	0.63
University level education (%)	16 (44.4)	8 (22.2)	0.08
BMI[b]	23.78 (3.49)	25.89 (7.08)	0.09
Smoking during pregnancy[d] (%)	5 (13.9)	14 (38.9)	0.05
Smoking[b,c] (%)	5 (13.9)	10 (27.8)	0.23
Diabetes mellitus[b,c] (%)	0 (0)	4 (11.1)	0.04
Antihypertensive medication use[b,c] (%)	0 (0)	3 (8.3)	0.08
Gravidity	2.28 (0.62)	7.11 (2.07)	<0.001
Parity	2.25 (0.60)	1.64 (0.83)	0.001
Primary miscarriages (%)	--	27 (75.0)	--
At least one continuing pregnancy[c] (%)	37 (100)	35 (97.2)	0.31
Gestational diabetes[d] (%)	0 (0)	0 (0)	--
Preeclampsia/Pregnancy induced hypertension[c,d] (%)	0 (0)	3 (8.3)	0.08
Preterm birth[d] (%)	1 (2.8)	4 (11.1)	0.38
Intra uterine growth restriction[d] (%)	4 (11.1)	4 (11.1)	1.00

BMI body mass index
Data are presented as mean (SD)
[a] Age at first pregnancy for unexposed women, age at third consecutive miscarriage for exposed women
[b] At time of follow-up
[c] Chi-squared test. Mc Nemars test not possible (at least one variable in each 2-way table is a constant)
[d] In at least one pregnancy

Table 3. Novel cardiovascular biomarkers in women with recurrent miscarriage

	No miscarriage n=36	Recurrent miscarriage n=36	Mean difference (95% CI)	p-value	Mean difference (95% CI) Model 1	Mean difference (95% CI) Model 2	Mean difference (95% CI) Model 3
Inflammation							
HsCRP mg/L	1.01 (0.32; 1.97)[a]	1.49 (0.65; 5.56)[a]	1.95 (1.17; 3.31)	0.03	1.66 (1.12; 2.40)	1.66 (1.10; 2.51)	1.58 (1.05; 2.45)
Lp-PLA2 ng/ml	360 (75.8)	355 (79.9)	-5.68 (-42.26; 30.90)	0.76	-1.77 (-41.61; 38.07)	-6.12 (-48.14; 35.93)	-12.81 (-56.35; 30.73)
Thrombosis							
Homocysteine µmol/L	9.61 (2.93)	8.17 (2.27)	-1.44 (-2.62; -0.26)	0.02	-1.72 (-2.97; -0.46)	-1.97 (-3.27; -0.67)	-1.96 (-3.34; -0.58)
Folate nmol/L	19.2 (7.05)	20.0 (7.48)	0.79 (-2.41; 4.00)	0.62	1.35 (-2.00; 4.68)	1.54 (-2.01; 5.08)	1.02 (-2.65; 4.69)
aCL[b]				0.51			
weak positive (%)	2 (5.6)	5 (13.9)					
positive (%)	1 (2.8)	1 (2.8)					
aβ2GPI[bc]				0.31			
weak positive (%)	0 (0)	0 (0)					
positive (%)	0 (0)	1 (2.8)					
Lipid metabolism							
Lp(a) nmol/L	20.0 (6.05; 110)[a]	10.6 (6.50; 34.3)[a] *Missing 1*	-1.45 (-2.82; 1.32)	0.25	-1.45 (-2.95; 1.45)	-1.48 (-3.16; 1.48)	-1.23 (-2.63; 1.74)
Renal function							
Creatinine µmol/L	67.4 (7.55)	62.9 (9.58)	-4.50 (-9.20; 0.19)	0.06	-4.81 (-9.96; 0.35)	-5.54 (-10.95; -0.13)	-5.91 (-11.63; -0.19)
Microalbuminuria mg/L	3.00 (3.00; 5.38)[a]	3.00 (3.00; 5.30)[a] *Missing 1*	1.04 (-1.38; 1.55)	0.66	1.05 (-1.38; 1.48)	1.02 (-1.48; 1.41)	1.05 (-1.51; 1.38)
Myocardial damage							
NT-proBNP ng/L	55.9 (53.6)	56.9 (35.0)	1.06 (-21.48; 23.59)	0.93	10.35 (-13.00; 33.70)	7.90 (-16.76; 32.56)	5.67 (-19.35; 30.69)
hsTroponineT ng/L	3.67 (3.66)	3.19 (1.17)	-0.47 (-1.31; 0.37)	0.27	-0.17 (-1.07; 0.72)	0.12 (-0.76; 1.01)	0.10 (-0.77; 0.97)
Multiple mechanisms							
Albumin g/L	47.6 (1.95)	46.0 (2.37)	-1.61 (-2.66; -0.56)	0.004	-1.33 (-2.44; -0.21)	-1.28 (-2.47; -0.09)	-1.19 (-2.43; 0.06)
Vitamin D nmol/L	75.4 (26.8)	55.6 (29.4)	-19.75 (-33.71; -5.79)	0.007	-20.61 (-35.76; -5.45)	-22.09 (-38.11; -6.06)	-24.43 (-40.32; -8.54)
Vitamin D ≤ 60nmol/L N (%)	10 (27.8)	21 (58.3)		0.02			

HsCRP: high-sensitivity C-reactive protein. Lp-PLA2: Lipoprotein-associated phospholipase A2, aCL: anti-Cardiolipin, aβ2GPI: anti-ß-2-Glycoprotein, Lp(a):Lipoprotein (a), NT-proBNP : N-terminal pro-brain natriuretic peptide, hsTroponineT: high sensitive Troponine T.
Model 1: BMI, Model 2: BMI, smoking, Model 3: BMI, smoking, diabetes mellitus, hypertension
Data are presented as mean (SD)
[a] median (25%; 75%)
[b] comparison 'weak positive + positive' vs negative
[c] Chi-squared test. Mc Nemars test not possible (at least one variable in each 2-way table is a constant)

Table 4. Novel cardiovascular biomarkers in women with idiopathic recurrent miscarriage

	No miscarriage n=28	Idiopathic Recurrent miscarriage n=28	Mean difference (95% CI)	p-value	Mean difference (95% CI) Model 1	Mean difference (95% CI) Model 2	Mean difference (95% CI) Model 3
Inflammation							
HsCRP mg/L	0.89 (0.25; 1.56)[a]	1.37 (0.46; 1.38)[a]	1.95 (1.12; 3.47)	0.02	1.51 (-1.01; 2.34)	1.55 (-1.02; 2.40)	1.48 (-1.10; 2.40)
Lp-PLA2 ng/ml	365 (7.14)	354 (80.1)	-11.34 (-51.36; 28.69)	0.57	-10.59 (-55.33; 34.15)	-11.31 (-58.12; 35.50)	-17.77 (-67.35; 31.81)
Thrombosis							
Homocysteine µmol/L	9.55 (2.42)	8.33 (2.32)	-1.22 (-2.54; 0.10)	0.07	-1.45 (-2.93; 0.04)	-1.62 (-3.13; -0.12)	-1.60 (-3.24; 0.04)
Folate nmol/L	19.4 (7.60)	20.7 (6.43)	1.31 (-2.12; 4.74)	0.44	1.78 (-1.91; 5.46)	1.77 (-2.09; 5.63)	1.27 (-2.76; 5.30)
aCL[b]							
weak positive (%)	1 (3.6)	4 (14.3)		0.69			
positive (%)	1 (3.6)	0					
aβ2GPI[bc]							
weak positive (%)	0 (0)	0 (0)					
positive (%)	0 (0)	1 (3.6)					
Lipid metabolism							
Lp(a) nmol/L	20.5 (5.53; 126)[a] *Missing 1*	10.1 *(6.50; 28.5)*[a]	-1.51 (-3.24; 1.38)	0.25	-1.41 (-3.16; 1.62)	-1.41 (-3.31; 1.62)	-1.15 (-2.69; 2.04)
Renal function							
Creatinine µmol/L	66.8 (6.86)	62.9 (10.0)	-3.93 (-9.37; 1.51)	0.15	-4.24 (-10.07; 1.58)	-4.80 (-10.79; 1.19)	-5.13 (-11.45; 1.19)
Microalbuminuria mg/L	3.00 (3.00; 5.23)[a] *Missing 1*	3.00 (3.00; 5.30)[a]	1.07 (-1.45; 1.66)	0.74	1.07 (-1.48; 1.66)	1.01 (-1.55; 1.58)	1.01 (-1.55; 1.51)
Myocardial damage							
NT-proBNP ng/L	53.6 (56.8)	59.4 (32.7)	5.79 (-18.94; 30.51)	0.64	13.80 (-12.79; 40.40)	10.84 (-16.32; 38.00)	8.78 (-19.35; 36.91)
hsTroponineT ng/L	3.86 (4.15)	3.25 (1.32)	-0.61 (-1.71; 0.49)	0.27	-0.30 (-1.46; 0.85)	-0.05 (-1.14; 1.04)	-0.12 (-1.18; 0.94)
Multiple mechanisms							
Albumin g/L	47.5 (2.10)	46.4 (2.27)	-1.11 (-2.27; 0.06)	0.06	-0.75 (-1.89; 0.39)	-0.79 (-1.98; 0.40)	-0.67 (-1.95; 0.61)
Vitamin D nmol/L	77.3 (25.3)	58.1 (30.6)	-19.18 (-35.12; -3.24)	0.02	-21.39 (-38.75; -4.02)	-22.17 (-40.28; -4.07)	-24.76 (-43.76; -5.76)
Vitamin D ≤ 60nmol/L N (%)	8 (28.6)	14 (50.0)		0.18			

HsCRP: high-sensitivity C-reactive protein. Lp-PLA2: Lipoprotein-associated phospholipase A2, aCL: anti-Cardiolipin, aβ2GPI: anti-ß-2-Glycoprotein, Lp(a):Lipoprotein (a), NT-proBNP : N-terminal pro-brain natriuretic peptide, hsTroponineT: high sensitive Troponine T.
Model 1: BMI, Model 2: BMI, smoking, Model 3: BMI, smoking, diabetes mellitus, hypertension
Data are presented as mean (SD)
[a] median (25%; 75%)
[b] comparison 'weak positive + positive' vs negative
[c] Chi-squared test. Mc Nemars test not possible (at least one variable in each 2-way table is a constant)

In 78% of the women with recurrent miscarriage (n=28) no abnormalities were found. Parental chromosomal abnormality was found in one case, antiphospholipid syndrome in one case, hyperhomocysteinemia in three cases and heritable thrombophilia was found in three cases.

Novel cardiovascular biomarkers were presented in Table 3. Women with recurrent miscarriage had a significantly higher HsCRP compared to women with no miscarriage. Significantly lower values of homocysteine, albumin and vitamin D were found in women with recurrent miscarriage compared to women with no miscarriage. These significantly differences remained in the multivariate analyses adjusting for BMI, smoking, diabetes mellitus and hypertension (using three different models), except for albumin adjusted for all four classic cardiovascular risk factors (model 3). A significant lower value of creatinine was found in multivariate analysis, model 2 and 3, in women with recurrent miscarriage compared to women with no miscarriage.

The results for the subgroup analysis including women with idiopathic recurrent miscarriage (table 4) are consistent with the results of the total group. Not all differences were statistically significant.

Discussion

Main findings

Increased levels of HsCRP and decreased levels of albumin and vitamin D were found in women with a history of recurrent miscarriage compared to women with no miscarriage, also after adjustments for classic cardiovascular risk factors (BMI, smoking, diabetes mellitus and hypertension). These cardiovascular biomarkers are involved in mechanisms regarding inflammation.

Interpretation

Our findings indicate a proinflammatory state in women with a history of recurrent miscarriage, which cannot be explained by confounders as BMI, smoking, diabetes mellitus and hypertension. Inflammation plays an important pathogenic role in all stages of atherosclerosis [20]. HsCRP can detect low grade inflammation and adds prognostic information on cardiovascular risk comparable to blood pressure or cholesterol [21]. Values <1, 1 to 3, and >3 mg/l indicate lower, average, or higher relative cardiovascular risk, respectively [21]. Therefore, we can conclude that we found a clinically relevant elevation in the recurrent miscarriage group (unadjusted mean difference HsCRP mg/L 1.95, 95%CI 1.17; 3.31). Lower values of albumin were found in women with recurrent miscarriage. Albumin is inversely associated with cardiovascular disease [22, 23] and several potential biological mechanisms might explain this (Table 1). The most obvious mechanism is inflammation; it is a negative acute-phase protein and concentration falls

during the inflammatory process [24, 25]. Vitamin D (concentrations of 20 to 60 nmol/L) is also inversely associated with cardiovascular disease risk [26]. Vitamin D is involved in many processes of potential relevance to cardiovascular disease (Table 1). A deficiency could lead to increased inflammation, endothelial dysfunction, elevated blood pressure, decreased insulin sensitivity and secretion, arterial stiffness and degradation of atherosclerotic plaque[27]. Vitamin D is produced by the action of UVB light on the skin. Skin pigmentation may reduce capacity to synthesize vitamin D and due to a lower exposure in winter months there are seasonal variations. To a lesser extent, it is also provided in the diet from foods, mostly of animal origin[27]. We found a significantly lower vitamin D concentration in women with recurrent miscarriage, suggesting a decreased intake of vitamin D precursors, whether a decreased exposure to sunlight plays a role is uncertain. As the percentage of Caucasian women was comparable in both groups, we don't expect an impact of ethnicity on our findings. We assessed vitamin D levels only at follow-up. In addition, it is possible that women with recurrent miscarriage already have decreased values of vitamin D during pregnancy. Only one study assessed vitamin D levels and immunological implications, such as presence of autoantibodies and cytokine production, in women with recurrent miscarriage (N=133) and found that 47.4% of these had a vitamin D deficiency (<30 ng/ml) [28]. Over the last decade, the role of vitamin D in human reproduction has been increasingly considered as important. Adverse outcomes linked to vitamin D insufficiency in pregnancy includes pre-eclampsia, gestational diabetes, small-for-gestational age and preterm birth [29-31]. Many potential underlying mechanisms of vitamin D in regulating each of the outcomes are hypothesized, including that vitamin D could act as an immune regulator during implantation [32]. Our findings call for more (observational and intervention) studies to investigate the association between vitamin D and recurrent miscarriage.

Surprisingly, in contrast with the previous described findings, we found that women with recurrent miscarriage had lower levels of homocysteine compared to women with no miscarriage. Hyperhomocysteinemia is associated with recurrent miscarriage and with cardiovascular disease [33, 34]. Our findings could be a confounding effect of the folate and vitamin B supplementation in the recurrent miscarriage group. Homocysteine evaluation is part of the routine work-up in women with recurrent miscarriage. In case of elevated levels, women are advised to use folate and vitamin B supplementation. Women might continue this after their pregnancies and/or at least in between pregnancies. All women are advised to use periconceptional folate supplementation. As women with recurrent miscarriage have a higher gravidity this could also have influenced our results. We found comparable results in the sub analysis including women with idiopathic recurrent miscarriage (although not all significant, probably due to a loss of power). Indicating that our results are independent of a supposed cause for the recurrent miscarriage, such as thrombophilia.

Strengths and limitations

To our knowledge, this is the first study which examined novel cardiovascular biomarkers after recurrent miscarriage. Strengths of our study include a unique well-defined cohort. As recurrent miscarriage is a highly heterogenic condition we strived to more homogeneity and therefore included only women who had their third consecutive miscarriage before the age of 31. A younger age at diagnosis reduces the change of miscarriages due to fetal abnormalities and makes a maternal cause of recurrent miscarriage more plausible [35]. All women had a routine work-up at baseline to identify possible causes for the recurrent miscarriage and therefore we could perform a subgroup analysis including women with idiopathic recurrent miscarriage (Table 3). Because of the matching on zip code, age and time of pregnancy we took several confounding factors into account. Our study has also some limitations. Because women with recurrent miscarriage were included in the study before the matched controls were invited to participate, there was a small difference between the age at time of the follow-up measurements. We don't expect that this will influence the results. Almost 50% of the eligible women did not respond on the invitation and 17 women declined to participate (due to emotional reasons) in this follow-up study (Figure 1), which could possible introduce selection bias. Given the high percentage of women who had at least one continuing pregnancy in the recurrent miscarriage group (97.3%), it is likely that women without live births (women with possibly the most unfavorable profile) more often declined or did not respond. Therefore, our findings in women with recurrent miscarriage could even be an underestimate. Selection bias is also possible in the unexposed group; probably women with a higher education were more likely to participate. Another limitation is the relatively small group of patients, especially in our subgroup analysis. Therefore, this study may partly be underpowered and we should be cautious drawing conclusions. There is a wide range of novel cardiovascular biomarkers which makes it impossible to study all of them. Based on the most recent literature we have selected biomarkers which have the potential to link recurrent miscarriage and cardiovascular disease [11, 12]. Unfortunately, we did not take the accurate blood sample (sodium citrate tube) for the examination of potential interesting biomarkers fibrinogen and lupus anticoagulant.

Perspectives

Our findings suggest a proinflammatory state in women with a history of recurrent miscarriage compared to women with no miscarriage. This suggests an overall poorer health in women with a history of recurrent miscarriage, which could partly explain their increased risk for cardiovascular disease later in life. No differences were found in more specific biomarkers, for example regarding lipid metabolism and myocardial damage. Routine screening of novel cardiovascular biomarkers on patient level seems with the current insight not warranted, although screening for vitamin D status seems plausible. Women with a history of recurrent miscarriage should be given healthy lifestyle advises, such as abstention of smoking, improving dietary habits and sufficient exposure to sunlight.

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5

No increased prevalence of family history of cardiovascular disease in women with recurrent miscarriage

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Abstract

Objectives To assess if a family history of cardiovascular disease is more prevalent in women with recurrent miscarriage. Women with a history of recurrent miscarriage have an increased risk of future cardiovascular disease. This association indicates shared common risk factors, including genetics.

Methods A matched case control study was performed. Women who visited the recurrent miscarriage clinic at Leiden University Medical Centre (between 2000-2014) and had their third consecutive miscarriage ≤ 35 years were included. Controls were women with at least one uncomplicated pregnancy and no miscarriage, matched on zip code, age and date of pregnancy. All women filled out a questionnaire. Family history of premature myocardial infarction and/or stroke was defined as having a first-degree relative with myocardial infarction and/or stroke < 60 years. Family history of cardiovascular disease as having a first-degree relative with myocardial infarction, stroke, hypertension or thrombosis, irrespective of age at event. Odds ratios (OR) were calculated.

Results 103 women with recurrent miscarriage were matched to 143 controls. The prevalence of family history of premature myocardial infarction and/or stroke (OR 0.66 (95% CI 0.31-1.41)) and the prevalence of family history of cardiovascular disease (OR 1.15 (95% CI 0.60-2.21)) was not increased in women with recurrent miscarriage compared to women with no miscarriage. A family history of hypertension seems more prevalent in women with recurrent miscarriage, although not significant (multivariate analysis: OR 1.71 (0.94-3.11)).

Conclusions for practice No increased prevalence of family history of cardiovascular disease was found in women who experienced recurrent miscarriage.

Introduction

Cardiovascular disease(CVD) is the leading cause of death among women in the world (World Health Organization 2013). Women are disproportionally affected by CVD compared to men, it is diagnosed less often and they are less likely to receive appropriate preventive care (Weiss, 2009). It is important to identify women at risk as early as possible so that they can benefit from preventive measures (Daviglus et al. 2006).
In the most recent American Heart Association guidelines for the prevention of CVD in women, pregnancy complications as preeclampsia, gestational diabetes and pregnancy-induced hypertension are indicated as a major risk factor for CVD (Mosca et al. 2011). Recurrent miscarriage is not mentioned in this guideline. There is increasing evidence that (recurrent) miscarriage is a risk factor for CVD as well (Ranthe et al. 2013; Oliver-Williams et al. 2013, Wagner et al. 2015). Miscarriage is defined as the spontaneous loss of pregnancy before 22 weeks' gestation. Three or more consecutive miscarriages are defined as recurrent miscarriage and affects 0.5 to 3% of couples trying to conceive (Stirrat 1990, Jivraj et al. 2001). Recurrent miscarriage is a heterogeneous condition and has many possible etiologic factors: genetic disorders, uterine anomalies, endocrine factors, maternal autoimmune disorders, metabolic disorders, maternal thrombophilia, obesity and toxic factors (de la Rochebrochard et al. 2002), although the percentage of cases without an identifiable cause is about 50% (Porter et al. 2005; Yang et al. 2006). We hypothesize that the association between miscarriages and cardiovascular disease indicates shared common acquired risk factors, such as smoking and antiphospholipid syndrome, and shared heritable (genetic) risk factors.
A Scottish retrospective cohort study found an increased incidence of ischemic heart disease in the parents of women who experienced multiple miscarriages before their first birth, which supports the hypothesis of shared genetic factors (Smith et al. 2011). A family history of (premature) CVD is an independent predictor of myocardial infarction and CVD (Weijmans et al. 2015).
We conducted a matched case-control study to investigate whether a family history of premature CVD was more common in women who experienced recurrent miscarriage compared to women with no miscarriage.

Methods

Study design

Cases

Women who visited the recurrent miscarriage outpatient clinic at Leiden University Medical Centre between 2000 and 2014 and had their third consecutive miscarriage ≤ 35 years of age were invited to participate in this matched case control study. Recurrent

miscarriage was defined as ≥ 3 consecutive miscarriages before 22 weeks of gestation. All women had a routine recurrent miscarriage work-up to identify possible causes for the recurrent miscarriage: a standardized history of the couple was performed, karyotyping of the couple, presence of uterus anomalies by ultrasound or hysteroscopy and presence of acquired and heritable thrombophilia. Acquired thrombophilia: Antiphospholipid syndrome was defined as the presence of IgG anticardiolipin antibodies or lupus anticoagulant in repeated samples taken 3 months apart and at least 10 weeks after a delivery (Visser et al. 2011), after revision of the classification criteria the presence of β2 glycoprotein-I was added to the work-up (Miyakis et al. 2006). Hyperhomocysteinemia was evaluated. Heritable thrombophilia was defined by the presence of a factor V Leiden mutation, factor II (prothrombin) gene mutation, protein C or S deficiency or antithrombin deficiency.

Controls

Women with one or more uncomplicated pregnancy(ies) and no miscarriage were enrolled. In the Netherlands it is common practice that independent community midwives are taking care of low-risk women (with no medical or obstetrical history) during pregnancy and child birth. The zip code of each woman with recurrent miscarriage was used to contact the nearest midwifery practice to take the impact of socio-economic status into account. Women, matched on zip code, age (maximal 1 year younger or 1 year older) and time of delivery (first delivery at the time of the third miscarriage of the matched exposed woman; maximal 6 months before or 6 months after) were asked to participate.
Enrolment took place between 2012 and 2014.

Procedures and definitions

After enrolment all women were asked to fill out a web based questionnaire. The questionnaire included general information, medical history, use of medication, intoxications, obstetric history and family history including cardiovascular disease. The questionnaire was made using ProMISe, an Internet based application for the design, maintenance and use of data management projects according to the security conditions demand by good clinical practice (2016). Information about medical history, use of medication, intoxications and pregnancy outcome was cross checked in obstetrical records to overcome recall bias. Maternal age was defined as age at third consecutive miscarriage for women with recurrent miscarriage or age at first pregnancy for women with no miscarriage. Height and weight was self-reported. Body mass index (BMI) was calculated as weight/length^2. Smoking during pregnancy was self-reported and was found positive if a woman smoked during at least one pregnancy. Primary recurrent miscarriage was defined as three consecutive miscarriages without a prior live birth. Secondary recurrent miscarriage was defined as three consecutive miscarriages with a prior live birth. Ethnicity was based on country of birth of the women. Education was defined as whether

or not university level (college and university education together). Presence of a history of thrombosis, hypertension, diabetes mellitus and hypercholesterolemia was based on the questionnaire, including questions about use of medication. Hypertension at time of questionnaire was found positive if a woman used antihypertensive treatment at that time. Family history was based on the questionnaire which included the following questions: Do you have any relatives with myocardial infarction, stroke, thrombosis and/or hypertension? How many brothers and sisters do you have? How many first-degree relatives had the previous mentioned disease and how many first-degree relatives had the disease before the age of 60?
Family history of premature myocardial infarction(MI) and/or stroke was defined as having at least one first-degree relative with MI and/or stroke before the age of 60. This cut off point of age was used in analyses from the Framingham cohort (Murabito et al. 2004). Family history of cardiovascular disease was defined as having at least one first-degree relative with MI, stroke, hypertension or thrombosis, irrespective of age at event.

Statistics – Sample size

Assumed were the following predictions:
Combination 1: (65%): Cases and controls both don't have family history of CVD.
Combination 2: (17%): Cases have family history of CVD, controls don't have.
Combination 3: (5%): Cases don't have family history of CVD, controls have.
Combination 4 (13%): Cases and controls both have family history for CVD.
The calculation was based on the assumption that we would find a family history of premature CVD in 30% of the women with recurrent miscarriage (cases) and in 18% of the women with no miscarriage (controls). This assumption is based on the Epic-Norfolk study, in which 27% of all healthy participants had a first-degree relative with "outcome" coronary heart disease (Sivapalaratnam et al. 2010). A sample size of 150 women (75 exposed, 75 non-exposed) was sufficient, we took a 10% drop-out in consideration (sided alpha .05. power 80%) We planned to include women in 1:1 ratio, i.e. one women who had recurrent miscarriage compared to at least 1 woman with no miscarriage. PASS, Power Analysis and Sample Size Software (NCSS Statistical Software) was consulted.

Data were analyzed using SPSS software 20.0 (Statistical Package for Social Science; SPSS, Chicago, Illinois, USA). Comparisons of baseline characteristics between cases and controls were performed using independent T-test, Mann-Whitney test, X^2 or Fisher exact test as appropriate. Comparison of family history between cases and matched controls was performed by a conditional matched logistic regression analysis using a stratified Cox regression. Odds ratios (OR) were calculated with 95% confidence intervals (CI). For all tests, a p-value ≤ 0.05 indicated statistical significants. A multivariate analysis was performed including BMI, university level education and smoking during pregnancy as

covariates. A sub analysis was performed, repeating the previous mentioned analyses, in women with primary recurrent miscarriage and their matched controls.

Ethics

Approval from the medical ethics committee of Leiden University Medical Centre (P04-020; October 3 2012) was obtained and all participants gave informed consent. This study is part of the REMI (REcurrent MIscarriages) studies, studies which investigate consequences and causes of recurrent miscarriages.

Results

A flowchart of the inclusion of the participants is shown in Figure 1. For some cases two or three controls agreed to participate. 11 women (five cases, six controls) were excluded from further analysis because they did not meet the matching criteria (maternal age). 103 women with recurrent miscarriage were included and 143 women with no miscarriage.

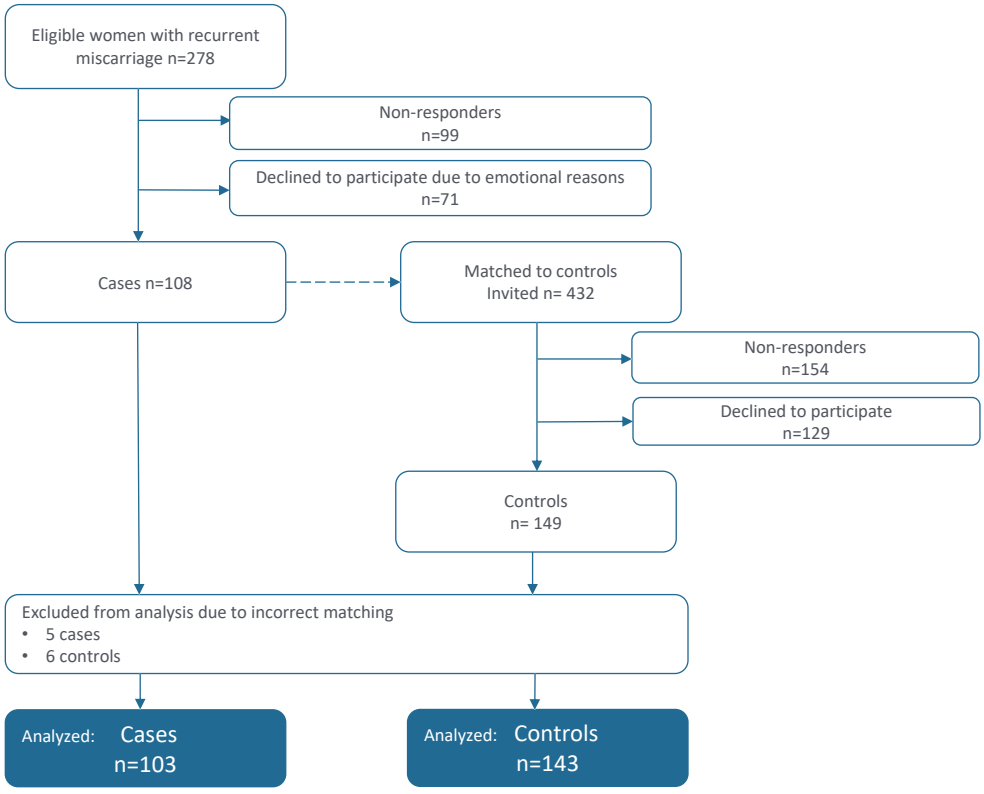

Figure 1. Flow chart of participants

Demographics of the women are presented in the baseline table (Table 1). Mean maternal age at time of questionnaire differed between women with recurrent miscarriage and women with no miscarriage: 34.76 and 35.92 years, respectively (p=0.03). Women with recurrent miscarriage were more often smokers during pregnancy (19.4%) compared to women with no miscarriage (7.0%) (p=<0.001) and had more often hypertension at time of questionnaire (3.9% vs 0%, p=0.03). The number of siblings was comparable between both groups.

Table 1. Baseline table. Characteristics of the participants

	Recurrent miscarriage N=103	No miscarriage N=143	p-value
Maternal age at index pregnancy mean (SD)	29.60 (3.48)	29.28 (3.23)	0.46
Maternal age at questionnaire mean (SD)	34.76 (4.02)	35.92 (4.04)	0.03
Caucasian (%)	94 (92.2) *Missing: 1*	137 (95.9)	0.27
BMI mean (SD)	24.73 (5.18) *Missing: 1*	23.87 (3.32) *Missing: 4*	0.12
University level education (%)	48 (46.6)	83 (58.0)	0.08
Smoking during pregnancy (%)	20 (19.4)	10 (7.0)	< 0.001
Smoking at time of questionnaire (%)	17 (16.5)	18 (12.6)	0.38
Gravidity median (min/max)	6 (3/15)	2 (1/4)	< 0.001
Parity median (min/max)	1(0/4)	2 (1/4)	< 0.001
Primary miscarriage (%)	67 (65)	--	n/a
At least one continuing pregnancy (%)	85 (82.5)	143 (100.0)	< 0.001
Thrombosis (%)*	0 (0.0)	1 (0.7)	1.00
Hypertension (%)*	4 (3.9)	0 (0.0)	0.03
Diabetes Mellitus (1 and 2) (%)*	4 (3.9)	0 (0.0) *Missing: 1*	0.03
Hypercholesterolemia (%)*	0 (0.0)	1 (0.7)	1.00
Number of siblings mean *(SD)*	1.90 (1.47)	1.80 (1.15) *Missing: 1*	0.52

BMI: body mass index
**at time of questionnaire*

Table 2. Analyses of recurrent miscarriage

The existence of	Recurrent miscarriage N=103
Abnormal parental karyotyping (%)	1 (1.0) *Missing: 4*
Uterus anomaly (%)	0 (0.0)
Anti-phospholipid syndrome (%)	4 (3.9) *Missing: 1*
Hyperhomocysteinemia (%)	7 (6.8)
Heritable thrombophilia *(%)*	7 (6.8)

Analyses of recurrent miscarriage are described in table 2. In 81.6 % of the women (N=84) no identifiable cause was found. Seven cases were diagnosed with heritable thrombophilia: factor II (prothrombin) gene mutation (N=3), factor V Leiden (N=5) One case had two clotting defects.
No increase was found in prevalence of a family history of premature MI and/or stroke in women with a history of recurrent miscarriage (15.6%) compared to women with no miscarriage (19.1%) (multivariate analyses: OR 0.65 (95%CI 0.30-1.42)) (Table 3). Eight women with recurrent miscarriage and nine women with no miscarriage did not fill in this part of the questionnaire, indicated as "missing" in the table. No increase was found in prevalence of a family history of any cardiovascular disease, MI, stroke or thrombosis in women with a history of recurrent miscarriage compared to women with no miscarriage. Adjusting for acquired risk factors as BMI, smoking during pregnancy and university level did not change the estimator of interest. Women with recurrent miscarriage had more often a family history of hypertension (54.3%), compared to women with no miscarriage (44.9%), although not significant (multivariate analyses: OR 1.71 (95%CI 0.94-3.11)).

Table 3. Family history of cardiovascular disease in women with recurrent miscarriage

Family history of	Recurrent miscarriage N=103	No miscarriage N=143	Odds unadjusted (95%CI)	Odds adjusted* (95%CI)	p-value**
Premature MI and/or stroke (%)	15 (15.6) *Missing: 7*	26 (19.3) *Missing: 8*	0.66 (0.31-1.41)	0.65 (0.30-1.42)	0.28
Any cardiovascular disease (%)	49 (61.3) *Missing: 23*	69 (57.5) *Missing: 23*	1.15 (0.60-2.21)	1.31 (0.66-2.61)	0.44
MI (%)	15 (16.7) *Missing: 13*	23 (18.3) *Missing:17*	0.90 (0.44-1.83)	0.90 (0.43-1.90)	0.78
Stroke (%)	4 (4.5) *Missing: 15*	9 (6.6) *Missing: 7*	0.51 (0.14- 1.97)	0.56 (0.14-2.28)	0.42
Thrombosis (%)	10 (9.7)	12 (8.4)	1.07 (0.45- 2.50)	0.86 (0.35-2.15)	0.75
Hypertension *(%)*	51 (54.3) *Missing: 9*	61 (44.9) *Missing:7*	1.46 (0.84- 2.56)	1.71 (0.94-3.11)	0.08

MI: myocardial infarction.
CI: confidence interval
**Adjusted for BMI, university level education and smoking during pregnancy*
*** p-value refers to the multivariate analysis.*

Comparable results were found in the subgroup analysis, including 67 women with primary recurrent miscarriage (Table 4).

Table 4. Family history of cardiovascular disease in women with primary recurrent miscarriage

Family history of	Primary recurrent miscarriage N=67	No miscarriage N=94	Odds unadjusted (95%CI)	Odds adjusted* (95%CI)	p-value**
Premature MI and/or stroke (%)*	11 (17.7) *Missing: 5*	18 (20.0) *Missing: 4*	0.77 (0.33-1.81)	0.63 (0.25-1.59)	0.33
Any cardiovascular disease (%)	35 (66.0) *Missing: 14*	51 (61.4) *Missing: 11*	1.26 (0.58-2.76)	1.31 (0.58-2.97)	0.52
MI (%)	10 (16.4) *Missing: 6*	19 (21.6) *Missing: 4*	0.75 (0.33-1.71)	0.69 (0.29-1.67)	0.46
Stroke (%)	3 (5.3) *Missing: 10*	5 (5.6) *Missing: 7*	0.73 (0.13-4.13)	0.65 (0.11-3.78)	0.65
Thrombosis (%)	6 (9.0)	9 (9.6)	0.79 (0.28-2.25)	0.63 (0.19-2.07)	0.45
Hypertension *(%)*	38 (62.3) *Missing: 6*	39 (44.3) *Missing:6*	2.00 (0.97-4.13)	2.11 (0.95-4.72)	0.07

MI: myocardial infarction.
CI: confidence interval
**Adjusted for BMI, university level education and smoking during pregnancy*
*** p-value refers to the multivariate analysis.*

Discussion

Main findings

In this matched case-control study the prevalence of family history of (premature) cardiovascular disease in a first-degree relative did not differ between women with recurrent miscarriage and women with no miscarriage. Therefore, our data does not confirm the assumption that a link exists between familiar cardiovascular disease and recurrent miscarriage when measured by proxy family history.

Strengths and limitations

A strength of the study is that all women visited the recurrent miscarriage outpatient clinic in the past and had a confirmed diagnosis of recurrent miscarriage and therefore eliminating recall bias. We included only women who were ≤ 35 years of age at time of their third consecutive miscarriage to create a more homogeneous group. A younger age at diagnosis makes a maternal cause of recurrent miscarriages more plausible and reduces the chance of miscarriages due to fetal abnormalities (Franssen et al. 2005). We matched the women with recurrent miscarriage (cases) to women with no miscarriage (controls) on zip code (trying to avoid differences in socioeconomic status), age and time of pregnancy to make the groups as comparable as possible. Because we already matched for these factors they were not included as confounders in the multivariate analysis. Another strength of the study is the availability of patient characteristics such as BMI, smoking and education level. Information was crosschecked in obstetrical records

thereby eliminating recall bias. Previous mentioned characteristics are shared acquired risk factors between recurrent miscarriage and cardiovascular disease with a possible familial aggregation and therefore they could be a confounder for the genetic link between the two diseases. Adjusting for BMI, smoking during pregnancy and university level did not change the estimator of interest. It was remarkable that significantly more women in the recurrent miscarriage group were smoking during pregnancy compared to women with no miscarriage. Since we used the definition smoking in at least one pregnancy, exposure to the covariate was not equal in both groups (the number of pregnancies was higher in the recurrent miscarriage group). On the other hand, it is not very likely that women start smoking during their second or next following pregnancy, especially not when they experienced multiple miscarriages and received life style advises. Information about current health status was also available, women with recurrent miscarriage had more often hypertension at time of questionnaire. It is interesting to see this reflected in family history of hypertension as well, which seems more often present in women with recurrent miscarriage: OR 1.71 (95%CI 0.94-3.11), although not significant.

Several limitations of our study should be acknowledged. Selection bias may have been introduced. Possibly women with recurrent miscarriage and no live birth (the 'worst' cases) were more likely to decline to participate due to emotional reasons. A high percentage of 82.5 of the included women with recurrent miscarriage had at least one continuing pregnancy. Family history based on self-report could give recall bias, though this approach of data collection about family history of CVD has been proven reliable (Murabito et al. 2004; Kee et al. 1993). A reason we did not find an increase in the prevalence of a family history of (premature) CVD in women with recurrent miscarriage compared to women with no miscarriage, could be due to lack of power. Our power analysis was based on the assumption that 30% of the women with recurrent miscarriage would have a family history of premature CVD and 18% of the women with no miscarriage. It is possible that there is only a small association between a family history of CVD and recurrent miscarriage (clinically irrelevant), and due to our relatively small group (N=103) we could have missed this association. At time of questionnaire women with recurrent miscarriage were significantly younger compared to women with no miscarriage. This result was to be expected because women with recurrent miscarriage were included in the study before the matched controls were invited to participate. However, the difference in age was only 1.2 years and therefore is unlikely to affect the results in family history of CVD. We did not ask information about current age of the parents of the included women. Some parents will not have reached the age of 60 years at time of questionnaire. Because we matched women on zip code, age and time of pregnancy we do not expect a difference in parents age between both groups and therefore would influence our results. For the composite outcome, any cardiovascular disease the number of missing values was quite high. This is because if a part of the questionnaire was not filled in (regarding MI/ stroke/ thrombosis/ hypertension/ age/ first-degree relative)

and therefore the composite outcome was uncertain 'any cardiovascular disease' was indicated as missing (Table 3 and 4).

Comparison of literature

This is not the first study which compared a family history of CVD between women with recurrent miscarriage and women with no miscarriage. Mahendru et al found a non-significant difference of premature CVD in parents of women (planning to conceive) with a history of unexplained recurrent miscarriage (48%) versus women with previous normal pregnancy (24%), including only 26 women with recurrent miscarriage (Mahendru et al. 2013). Smith et al, performed a large retrospective cohort study and found an increased incidence of ischemic heart disease in the parents of women who experienced three or more miscarriages prior before their first birth (N=224); hazard ratio 1.56 (95%CI 1.14-2.15) (Smith et al. 2011). It is difficult to compare our research with the article by Smith et al because of the different approach; Smith used International Classification of Disease (ICD) codes for family history of CVD in parents compared to self-reports on family history in our study. On the other hand, the incidence of miscarriage was based on self-report in the article by Smith et al in contrast to our study. The included women in Smith's article were very young at time of their recurrent miscarriage: 23 years (median, interquartile range 20-25) at time of first birth, compared to 29.6 years (mean, SD 3.48) at third consecutive miscarriage in our study. Because Smith investigated only women with primary recurrent miscarriage we repeated our analyses for women with primary recurrent miscarriage (Table 4). We found the same results: no increased prevalence of family history of CVD in women with a history of primary recurrent miscarriage.

A recently published large Danish cohort study found an association between non-consecutive miscarriages in women and risk of atherosclerotic disease in their first-degree relatives (Ranthe et al. 2016). The greater the number of daughters with miscarriages or the greater the combined number of miscarriages among daughters, the greater the parental risk of ischaemic outcomes. Persons with ≥3 miscarriages among their daughters had a 15% increased rate of an ischaemic outcome, compared with persons with the same number of children whose daughters had never miscarried. Information about potential confounding factors such as smoking and BMI were not available in this study.

Data from the routine recurrent miscarriage work-up to identify possible causes was described in Table 2. Seven percent of the women in the recurrent miscarriage group were diagnosed with a heritable form of thrombophilia. Heritable forms of thrombophilia, such as a polymorphism in factor V Leiden or prothrombin G20210A gene are associated with recurrent miscarriage and a modest association with coronary disease is described (Kovalevsky et al. 2004; Ye et al. 2006). A stronger association exists between heritable thrombophilia and venous thromboembolism. The prevalence of thrombophilia in women with recurrent miscarriage in current study (6,8%) was lower than what was expected from the literature. Heritable thrombophilia is present in about 15% of the

Western population (Greer 2000). Data about thrombophilia was not available for the controls in this study. The fact that we did not find an increased prevalence of a family history of thrombosis in women with recurrent miscarriage compared to women with no miscarriage is in line with the literature (Smith et al. 2011). Therefore, we do not think that the low prevalence of thrombophilia in our study group can be an explanation for the fact that we did not find an increased prevalence of family history of CVD.

Conclusions

No increase was found in prevalence of family history of (premature) cardiovascular disease in women who experienced recurrent miscarriage compared to women with no miscarriage what contradicts a shared common genetic pathophysiological pathway linking cardiovascular disease and recurrent miscarriage. A possible association was found between family history of hypertension and recurrent miscarriage, which urge more investigation.

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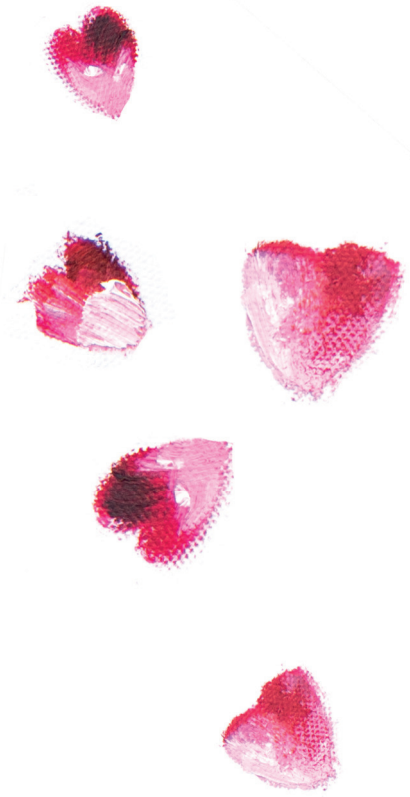

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Genetic polymorphisms in recurrent miscarriage: a meta-analysis

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Abstract

Background The underlying cause of recurrent miscarriage remains often unexplained. A multifactorial etiology, including uterine anomalies, endocrine and metabolic factors, maternal autoimmune disorders, thrombophilia, toxic factors and genetics is assumed. Currently it is still unclear which specific genes are involved. The aim of this study was to investigate systematically which genetic variants are reproducibly associated with recurrent miscarriage.

Methods A systematic review and meta-analysis was performed. PubMed, Embase and Web of Science were searched (till 7 October 2014) for case-control studies that assessed the association between genetic variants and recurrent miscarriage (defined as two or more unexplained miscarriages). The control groups consisted of women with at least one successful pregnancy and no miscarriages. Genetic variants significantly associated with recurrent miscarriages in at least two independent studies were considered as reproduced variants and were included. The association between genes and recurrent miscarriages was assessed at the allele level and a pooled odds ratio was estimated in a random-effects model. A subgroup analysis was performed for the association between genetic variants and three or more consecutive miscarriages. I^2 statistics was reported. Egger test was used to check funnel plot asymmetry.

Results The literature search yielded 4050 articles; a total of 241 studies were included. We identified 25 reproduced genetic variants, of which 16 remained significantly associated with recurrent miscarriage in a random-effects meta-analysis. These variants were in the following genes: F2, FV, FXIIIa, HLA-G, IL10, IL18, MTHFR (two variants), NOS3, PAI1 , STAT3, TNFA (two variants) and VEGFA (three variants). Odds ratios for these 16 variants ranged from 1.21 to 2.64. Eight variants were significantly associated with three or more consecutive miscarriages, in the following genes: FV, IFNG, MTHFR, NOS3, TNFA (two variants) and VEGFA (two variants).

Conclusions This meta-analysis found 16 genetic variants associated with recurrent miscarriage. Major involved pathways seem to be: coagulation and fibrinolysis, immunology and inflammation and angiogenesis. Unravelling mechanisms by which these genetic variants affect the risk of recurrent miscarriage can reveal potential targets in the search for treatment. This meta-analysis also suggests that recurrent miscarriage and cardiovascular disease share genetic risk factors.

Keywords: genetic variants, recurrent miscarriage, risk-factors, cardiovascular disease

Introduction

Spontaneous miscarriage is the most common complication of pregnancy. About 15% of clinically recognized pregnancies end in a miscarriage (Rai and Regan, 2006;Wilcox, Weinberg *et al.*, 1988). The definition of recurrent miscarriage varies between two and three and consecutive versus non-consecutive miscarriages (Kolte, Bernardi *et al.*, 2015). Generally, the definition of three or more consecutive miscarriages prior to the 22nd week of gestation is used (Stirrat, 1990) which affects 0.5-3% of all fertile couples (Jivraj, Anstie *et al.*, 2001). In more than 50% of the cases, the underlying cause remains unexplained after diagnostic tests (Porter and Scott, 2005). Etiologic factors which are assumed to be involved in the pathogenesis of recurrent miscarriage include genetic disorders, uterine anomalies, endocrine factors, maternal autoimmune disorders, metabolic disorders, maternal thrombophilia, obesity and toxic factors, such as smoking and alcohol consumption (Branch, Gibson *et al.*, 2010). Research suggests a multifactorial etiology with a role for genetics (Christiansen, Steffensen *et al.*, 2008;Kolte, Nielsen *et al.*, 2011). An increase in the risk of spontaneous miscarriage was seen in family of women with recurrent miscarriage compared to normal control subjects (Christiansen, Mathiesen *et al.*, 1990;Kolte, Nielsen *et al.*, 2011;Miskovic, Culic *et al.*, 2012;Nybo Andersen, Wohlfahrt *et al.*, 2000). In addition, a case-control study showed that women with two or more unexplained recurrent miscarriages more often had a family history of recurrent miscarriage compared to healthy control subjects, RR 3.2 (95%CI 1.3-8.1) (Parazzini, Bocciolone *et al.*, 1991). However, it is still unclear what specific genes are involved and what individual variants contribute to the development of recurrent miscarriages. Polymorphisms have been investigated in almost 90 different genes (Rull, Nagirnaja *et al.*, 2012).

The aim of this study was to give a systematic overview of the genetic variants associated with recurrent miscarriage. A meta-analysis was performed to assess the pooled effect of the genetic variants that are repeatedly investigated and that are significantly associated with recurrent miscarriage in at least two of the performed studies.

Methods

Literature search

The databases PubMed, Embase and Web of Science were searched in collaboration with a trained librarian (last updated 7 October 2014). Terms used in the search strategy were ‘Recurrent Miscarriage, ‘Habitual Abortion’, ‘Pregnancy Loss’, ‘Polymorphisms, or ‘Genes’. All relevant keyword variants were used, and database specific adaptation of the key terms was performed. In addition, names of specific genes and polymorphisms which are known to be related to preeclampsia (Buurma, Turner *et al.*, 2013) were added

to the search strategy. The only limitation used in the search was language; studies had to be published in English or Dutch. References of related systematic reviews were checked for additional articles.

Eligibility criteria

We searched for case-control studies comparing genetic variants between women with recurrent miscarriage and controls. Inclusion criteria were: 1. Recurrent miscarriage defined as two or more unexplained miscarriages (irrespective whether consecutive or not) in first and/or second trimester; 2. The control group consisted of women who had at least one successful pregnancy and no miscarriages. If a subset of subjects in a study suited the inclusion criteria, only the subset was included. In case of overlapping study populations only the study with the most extensive data was included, to avoid duplication.
All titles and abstracts were reviewed by two researchers (M.W. and A.V. and a subset by M.W. and A.B.), who independently assessed if the study compared at least one genetic variant between women with and without recurrent miscarriage. Genetic variant studies were screened for whether they met the inclusion criteria and whether the investigated genetic variant showed a significant association ($P<0.05$ was defined as significant) at the allelic and/or genotypic level with recurrent miscarriage. When a genetic variant was found to be significantly associated with recurrent miscarriage in at least two studies, that variant was considered a reproduced genetic variant. Finally, only studies that investigated any of these reproduced genetic variants were included in the meta-analysis.

Data extraction

Studies that met the inclusion criteria were entered into separate databases by two researchers (M.W. and A.V.) independently. These two databases were compared, differences were discussed until consensus was reached and allele frequencies were extracted. In case of missing data, at least two attempts were made to contact the corresponding author. When neither the published report nor the corresponding author provided sufficient data to calculate an odds ratio at the allele level or give assurance that the study met the inclusion criteria, the study was excluded.

Statistical analyses

Pooled odds ratio's (OR) with 95% confidence intervals (CI) were calculated at the allele level for the association between reproduced genetic variants and recurrent miscarriage. Data were pooled using a random-effects-model to take into account between-study heterogeneity. I^2 was reported, which reflects the percentage of total variation across studies due to heterogeneity rather than due to change (Higgins, Thompson *et al.*, 2003). Funnel plots were generated for all reproduced genetic variants. Egger test (Egger, Davey *et al.*, 1997) was used to check funnel plot asymmetry (test for small study effect) which

may reflect publication bias. This test was only used when 10 or more studies were included to investigate a genetic variant, because otherwise test power is too low to distinguish chance from real asymmetry (Sterne, Sutton *et al.*, 2011). If funnel plot asymmetry existed and between study heterogeneity was evident a comparison between a random-effects-model and a fixed-effects-model was made. A subgroup analysis was performed for studies which included women with three or more consecutive miscarriages, applying the previously described analyses. All analyses were performed using STATA (StataCorp. 2011. Stata Statistical Software, Release 10, College Station, TX, USA: StataC).

Results

The initial literature search yielded 4050 articles. Finally 129 articles, comprising 241 studies, were included in this meta-analysis (Figure 1). Altogether, 25 reproduced genetic variants in 18 genes were described. Included articles were published between 1997 and 2014. The number of studies per genetic variant ranged from 2 to 38, the number of cases per study ranged from 5 to 1000. A significant association for 16 of the 25 reproduced genetic variants and recurrent miscarriage was found in a random-effects meta-analysis. These variants were in the following genes: F2, FV, FXIIIa, HLA-G, IL10, IL18, MTHFR (two variants), NOS3, PAI1 , STAT3, TNFA (two variants) and VEGFA (three variants) (Figure 2A-B). Odds ratio's ranged from 1.21 to 2.64, no significant protective effects were found (Table 1).
A subgroup analysis was performed for studies which included women with three or more consecutive miscarriages; 19 reproduced genetic variants could be included. A significant association between 8 variants and three or more consecutive miscarriages was found in the random-effects meta-analysis in the following genes: FV, IFNG, MTHFR, NOS3, TNFA (two variants) and VEGFA (two variants) (Table 2) (Figure 2C-D). The references of the included studies per variant are described in Table 3. Characteristics and references of all included studies, forest and funnel plots for all individual genetic variants are provided in the supplementary data.

Genetic variants involved in the renin–angiotensin system
Eleven studies investigating the *angiotensin converting enzyme (ACE) insertion/deletion (I/D)* polymorphism *(rs1799752)* were included in this analysis, resulting in a pooled OR of 1.20 (95% CI 0.95-1.53). In the subgroup analysis of women with three or more consecutive miscarriages, 4 studies were included, resulting in a pooled OR of 0.85 (95% CI 0.72-1.02).

Genetic variants involved in lipid metabolism
Five studies were included in the *apolipoprotein E (APOE) (rs429358)* analysis. E3 versus E4 allele (minor allele) was included in the meta-analysis resulting in a pooled OR of 3.09(95% CI 0.93-10.24).

Genetic variants involved in coagulation and fibrinolysis

Seven reproduced genetic variants in five genes concerning coagulation and fibrinolysis were included in the meta-analysis, six variants remained significantly associated with recurrent miscarriage following meta-analysis. The variant *factor 2 (F2) G20210A (rs1799963)*, also known as prothrombin mutation, was investigated in 26 studies resulting in an OR of 1.94 (95%CI 1.23-3.06). A subgroup analysis was performed for studies defining cases as three or more consecutive miscarriages for *F2 G20210A* resulting in an OR of 1.62 (95%CI 0.82-3.19), including 14 studies. *Factor V Leiden (FVL) (rs6025)* was the most frequently investigated polymorphism regarding recurrent miscarriages. A total of 38 studies were included, resulting in an OR of 1.88 (95%CI 1.47-2.40). For the subgroup three or more consecutive miscarriages 21 studies were included with a pooled OR of 1.79 (95%CI 1.32-2.42) The variant factor XIIIa (*FXIIIa) Val34Leu (rs5985)* was investigated in 8 studies and was associated with recurrent miscarriage; pooled OR of 1.58 (95%CI 1.04-2.41). Three studies were included in the subgroup analysis for three or more consecutive miscarriages, resulting in an OR of 1.36 (95%CI 0.43- 4.28).

Two variants in the methylenetetrahydrofolate reductase (MTHFR) gene were included. The *MTHFR A1298C* polymorphism (rs1801131) was investigated in 14 studies resulting in an OR 1.34 (95%CI 1.05-1.72). The subgroup analysis for three or more consecutive miscarriages resulted in an OR of 1.13 (95%CI 0.91-1.40), including 7 studies. The *MTHFR C677T* variant *(rs1801133)* was investigated in 32 studies and was associated with recurrent miscarriage with an OR of 1.28 (95%CI 1.12-1.47). The subgroup analysis for three or more consecutive miscarriages showed a comparable result. The variant *plasminogen activator inhibitor-1 (PAI1) -675 4G/5G (rs1799889)* was investigated in 14 studies and was associated with recurrent miscarriage with a pooled OR of 1.29 (95%CI 1.05-1.57). The variant *protein Z (PZ) intron F G79A (rs3024718)* was not associated with recurrent miscarriage after meta-analysis, including 4 studies, with a pooled OR of 0.88 (95%CI 0.38-2.04).

Genetic variants involved in inflammation and immunology

Eleven reproduced variants in seven genes involved in inflammation and immunology were included, five remained significantly associated with recurrent miscarriage after meta-analysis. The only reproduced genetic variant concerning human leukocyte antigen (HLA) comprised HLA-G. Eleven studies investigated the *HLA-G 14bp I/D* variant *(rs1704)*. A pooled OR of 1.21 (95%CI 1.01-1.45) was found in the meta-analysis. *Interferon-gamma (IFNG) 874A/T (rs2430561)*, reported in 5 studies, was not associated with recurrent miscarriage after meta-analysis with an OR 1.12 (95%CI 0.82-1.54). However, in the subgroup analysis for three or more consecutive miscarriages an association was found; pooled OR 1.39 (95% CI 1.14- 1.70). The variant *Interleukin (IL)1B -511C/T (rs16944)* was investigated in 4 studies resulting in an OR of 1.17 (95%CI 0.79- 1.73). Four studies investigated the variant *IL6 -174G/C (rs1800795)* resulting in an OR of 1.74 (95%CI 0.96-3.15).

Three variants concerning IL10 were included. The variant *IL10 -592C/A (rs1800872)* was investigated in 7 studies, a pooled OR of 0.84 (95%CI 0.63-1.12) was found. Similar results were found in the subgroup analysis regarding three or more consecutive miscarriages, including 4 studies. *IL10 -819C/T (rs1800871)* was investigated in 4 studies resulting in a pooled OR of 1.22 (95%CI 0.93-1.60). Similar results were found in the subgroup analysis regarding three or more consecutive miscarriages, including 3 studies. *IL10 -1082A/G (rs1800896)* was investigated in 6 studies and was associated with recurrent miscarriage with an OR of 1.25 (95%CI 1.02-1.54). In the subgroup analysis, three or more consecutive miscarriages; a pooled OR of 1.23 (95% CI 0.86-1.76) was found including 3 studies. The variant *IL18 -656C/A (rs1946519)* was investigated in only 2 studies and was associated with recurrent miscarriage with an OR of 1.92 (95%CI 1.61-2.30). Three variants in the tumour necrosis factor –alpha(TNFA) gene were included. Five studies concerned the *-238G/A variant (rs361525)*; a pooled OR of 1.37 (95%CI 0.88-2.14) was found. However, a significant association was found for the subgroup with three consecutive miscarriages (including 3 studies); pooled OR 1.52 (95%CI 1.21-1.92). *TNFA -308G/A (rs1800629)* was investigated in 7 studies, a pooled OR of 1.46 (95% CI 1.17-1.84) was found. A similar result was found in the subgroup analysis for three consecutive miscarriages, including 4 studies. *The TNFA -1031C/T (rs1799964)* variant was investigated in 2 studies and associated with recurrent miscarriage, pooled OR 2.64 (95%CI 1.90-3.66).

Genetic variants involved in oxidative stress

The variant *NOS3 Glu298Asp (rs1799983)* was investigated in 8 studies resulting in a pooled OR of 1.53 (95% CI 1.16-2.01). Seven of these studies included women with three consecutive miscarriage, pooled OR 1.65 (95%CI 1.25-2.17)

Genetic variants involved in oncogenesis

The variant *signal transducer and activator of transcription 3 (STAT3) rs1053004* was investigated in only 2 studies and remained associated with recurrent miscarriage after meta-analysis with a pooled OR of 1.51 (95%CI 1.30-1.75).

Genetic variants involved in angiogenesis

Three reproduced variants in the vascular endothelial growth factor A(VEGFA) were included and all remained associated after meta-analysis. *VEGFA -583T/C (rs3025020)* was investigated in 3 studies and was associated with recurrent miscarriage with a pooled OR of 1.75 (95%CI 1.50-2.03). All the available studies included women with three or more consecutive miscarriages. *VEGFA 936C/T (rs3025039)* was reported in 9 studies resulting in an OR of 1.35 (95%CI 1.09-1.68). A similar result was found in the subgroup analysis for three or more consecutive miscarriages, including 5 studies. The *VEGFA -1154G/A (rs1570360)* variant was reported in 10 studies resulting in an OR of 1.24 (95%CI 1.02-1.50).

Discussion

In this meta-analysis 16 genetic variants were associated with recurrent miscarriage. Meta-analysis of several individual genetic variants has been performed previously in relation to recurrent miscarriage. However, this is the first meta-analysis which gives a complete overview for all genetic variants which were reproducibly significantly associated with recurrent miscarriage. These more robust data could lead to improved understanding of the pathogenesis of recurrent miscarriage and could help to find the right direction for future treatment options.

Strengths/Limitations

This meta-analysis included only reproduced genetic variants, defined as variants that were at least twice independently significantly associated with recurrent miscarriage. This method was described before (Buurma, Turner *et al.*, 2013;Mooyaart, Valk *et al.*, 2011) and aims to minimize the number of false-positive results. However, it is possible that variants which were only described in studies with small sample size and lack of power were missed using this method. Publication bias is a concern in all meta-analyses. Overestimation of effect is possible due to the fact that studies with a non-significant effect are less likely to be published. For this reason Egger tests were performed, which showed no evidence of funnel plot asymmetry. Of note, we only performed this test for analyses including 10 or more studies as recommended by Sterne et al (Sterne, Sutton *et al.*, 2011).

Criteria for study inclusion and exclusion are critical parts of a meta-analysis and can affect results. Recurrent miscarriage is a very heterogenic condition and as described in the introduction the definition of recurrent miscarriage varies in the literature between two and three and consecutive versus non-consecutive miscarriages (Kolte, Bernardi *et al.*, 2015). To reduce the clinical heterogeneity we performed a subgroup analysis for studies which included women with three or more consecutive miscarriages. To further reduce this heterogeneity we included only studies that comprised women with pregnancy loss before the third trimester and the miscarriages had to be unexplained. However, bias is still possible due to the variations in examinations performed to make the diagnosis 'unexplained recurrent miscarriage'. All control women had to be parous women (with no miscarriage), to be sure that all women had been at risk to miscarry. Despite our efforts, statistical heterogeneity was evident for several investigated variants (Table 1 and 2).

Comparisons with literature

Genetic variants in the following genes were associated with recurrent miscarriage in this meta-analysis: F2, FV, FXIIIa, HLA-G, IL10, IL18, MTHFR (two variants), NOS3, PAI1 , STAT3, TNFA (two variants) and VEGFA (three variants). Three main groups or pathways can be distinguished; variants concerning coagulation and fibrinolysis (*F2 G20210A,*

FVL, FXIIIa Val34Leu, MTHFR A1298C, MTHFR C677T, PAI1 -675 4G/5G), variants concerning immunology and inflammation (*HLA-G 14bp I/D, IL10 -1082A/G, IL18 -656C/A, TNFA -308G/A, TNFA -1031C/T*) and variants involved in angiogenesis (*VEGFA -583T/C, VEGFA 936C/T, VEGFA -1154G/A*).

Six genetic variants involved in coagulation and fibrinolysis remained associated with recurrent miscarriage. Inherited thrombophilia is an important topic of research on women with recurrent miscarriage. The major heritable forms of thrombophilia are *F2 G20210A* and *FVL* polymorphisms. Women with inherited thrombophilia have been shown to be at increased risk not only of thromboembolism, but also of complications of pregnancy including preeclampsia and fetal loss (Walker, 2000). *MTHFR A1298C* and *MTHFR C677T* polymorphism remained associated with recurrent miscarriage after meta-analysis. Both polymorphisms reduce the activity of the MTHFR enzyme, causing elevated concentration of homocysteine. Increased homocysteine concentration is associated with neural tube defects and has led to the hypothesis that high concentrations of homocysteine might be embryotoxic (Zetterberg, 2004). Hyperhomocysteinemia is related to recurrent miscarriage (Steegers-Theunissen, Boers *et al.*, 1992;Wouters, Boers *et al.*, 1993). PAI1 regulates fibrinolysis, the 4G deletion allele is associated with high PAI1 plasma levels (Burzotta, Di *et al.*, 1998). An association between the *PAI1 -675 4G* deletion and recurrent miscarriage was found in our meta-analysis with an OR of 1.29, and a 95%CI of 1.05-1.57. The meta-analysis published in 2013 by Su et al (Su, Lin *et al.*, 2013) did not find a significant association between PAI1 and recurrent miscarriage: OR 1.44 (95%CI 0.97-2.14). This is probably due to the number of included studies: we were able to include 14 studies, compared to 11 studies in the meta-analysis performed by Su et al.

It is assumed that HLA-G plays an important role in the maternal-fetal interface (Kovats, Main *et al.*, 1990). HLA-G modulates cytokine secretion to induce immunotolerance, regulates trophoblast invasion for implantation and contributes to vascular remodelling of spiral arteries for pregnancy maintenance (Roussev and Coulam, 2007). Lower circulating levels of soluble(s) HLA-G during the first trimester were found in women with recurrent pregnancy loss versus healthy pregnant controls (Jassem, Shani *et al.*, 2012). We found a pooled OR of 1.21 (95%CI 1.01-1.45) between *HLA-G 14bp* and recurrent miscarriage, although significant heterogeneity was evident across studies (I^2=57.0%). No significant association was found in our subgroup analysis for three or more consecutive miscarriages, likely because of the small number of included studies (4 studies). This result is comparable with the very recently published meta-analysis by Meuleman et al (Meuleman, Lashley *et al.*, 2015).

A significant association between *IFNG 874A/T* and recurrent miscarriage was only found in our meta-analysis including three or more consecutive miscarriages. IFNG is a pro-inflammatory cytokine, produced by type 1 T-helper cells which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective

responses (Romagnani, 1999). Significantly higher levels of IFNG were found in women with recurrent miscarriage compared to women with a normal pregnancy (Raghupathy, Makhseed *et al.*, 1999;Wilson, Jenkins *et al.*, 2004). A meta-analysis performed in 2008 (Bombell and McGuire, 2008) showed a comparable result (OR 1.29), although they did not find a significant association. Our association likely turned out to be significant because we were able to include the large study performed by Parveen et al (Parveen, Shukla *et al.*, 2012) (which found an association: OR 1.49 (1.13-1.95)).

One of the three included polymorphisms in the IL10 gene remained significantly associated with recurrent miscarriage; *IL10 -1082A/G* (OR 1.25 (95%CI 1.02-1.54)). IL10 is known to selectively suppress Th1-mediated cellular responses by inhibiting the production of inflammatory cytokines (IFNG and TNFA). It has been proposed that a decrease in production of IL10 is associated with recurrent miscarriage and increased production is associated with normal pregnancy (Hill, Polgar *et al.*, 1995;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Makhseed, Raghupathy *et al.*, 2000). However this is still controversial; a study found higher IL10 levels at miscarriage than during normal pregnancy (Vassiliadis, Ranella *et al.*, 1998). Our results suggest that women with recurrent miscarriage may have a genetic predisposition to secrete higher levels of IL10. Further research is needed to clarify the role of IL10 in recurrent miscarriages.

A closely related pro-inflammatory cytokine is IL18, also known as IFNG inducing factor (Al-Khateeb, Sater *et al.*, 2011;Boraschi and Dinarello, 2006). We found a significant association between *IL18 -656C/A* with an OR of 1.92, however only two studies were included indicating the need for more research.

Also produced by type 1 T-helper cells is the cytokine TNFA (El-Far, El-Sayed *et al.*, 2009;Liu, Wang *et al.*, 2010). The *TNFA -308G/A* variant remained significantly associated with recurrent miscarriages; pooled OR 1.46 (1.17-1.84), including seven studies. This is in contrast with the meta-analysis performed in 2009 by Medica et al (Medica, Ostojic *et al.*, 2009), which did not find a significant association. Since the publication of the latter meta-analysis, new large studies were published in women with three or more consecutive miscarriages that did find an association between the variant and recurrent miscarriage (Alkhuriji, Alhimaidi *et al.*, 2013;Gupta, Prakash *et al.*, 2012). We were able to include those. The *-1031C/T* variant also remained significantly associated with recurrent miscarriage in our meta-analysis. The variant was investigated in only 2 studies resulting in an OR of 2.64 (95%CI 1.90-3.66). The polymorphisms described above in pro-inflammatory cytokines (in the genes IFNG, IL18 and TNFA) are in line with the hypothesis of a change in the maternal T-helper cell response; in women with recurrent miscarriage towards a persisting Th1- type (Hill, Polgar *et al.*, 1995).

STAT3 belongs to a family of signal transducers and activators of transcription (STAT1-STAT6) of intracellular signalling proteins and plays a role in unrestrained growth of human tumours (Bromberg and Darnell, Jr., 2000). Two studies regarding the *rs1053004* variant could be included in the meta-analysis and the polymorphism remained significantly

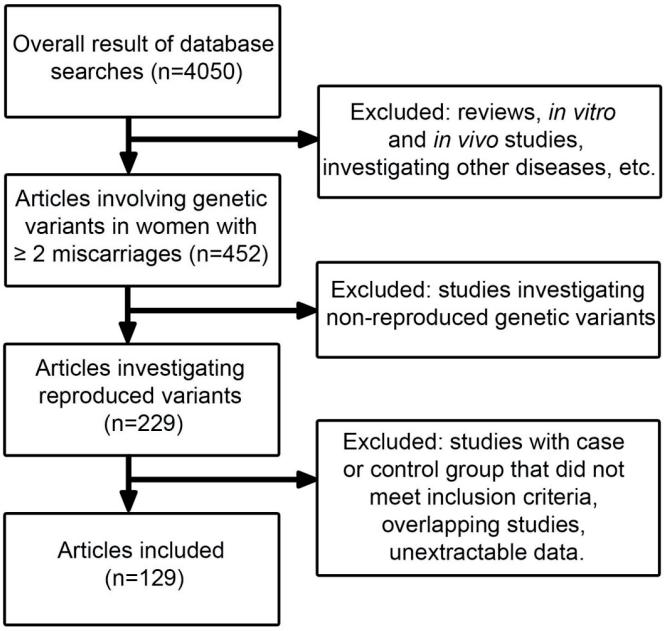


Figure 1. Selection of articles

associated with recurrent miscarriage. Further investigation is needed to clarify the possible mechanisms of the association between the influence of STAT3 on recurrent miscarriage (Garcia, Tirado-Gonzalez *et al.*, 2007). It is hypothesized that altered STAT3 activity leads to a local inflammatory state through recruitment of inflammatory cells and up-regulation of proinflammatory cytokines at the placental membrane, leading to accelerated miscarriage (Messoudi, Al-Sulaiti *et al.*, 2013). VEGFA is an endothelial cell specific protein that acts as a mediator of angiogenesis and vasculogenesis (Almawi, Saldanha *et al.*, 2013). VEGFA is essential in establishing a vascular network during early embryo development (Byrne, Bouchier-Hayes *et al.*, 2005). All three included polymorphisms (*VEGFA -583T/C, 936C/T and -1154G/A*) remained significantly associated with recurrent miscarriage after meta-analysis suggesting an important role in the pathogenesis of recurrent miscarriage.

The use of genetic information is expected to play a major role in future personalized medicine (Chan and Ginsburg, 2011). Personalized medicine is a medical model including each person's unique clinical, genetic, genomic, and environmental information to better classify disease, facilitate the development of new targeted therapies and to more accurately determine disease predisposition which will be helpful in disease prevention.

Cardiovascular disease

Recent epidemiological studies suggest a relationship between recurrent miscarriage and a risk for cardiovascular disease later in life (Oliver-Williams, Heydon *et al.*, 2013;Ranthe,

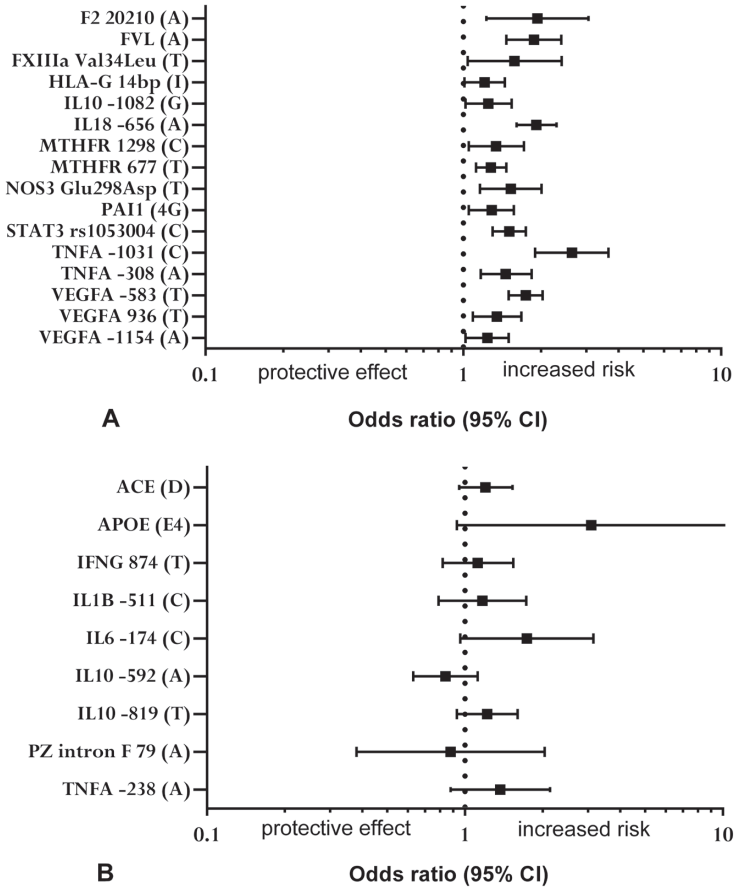
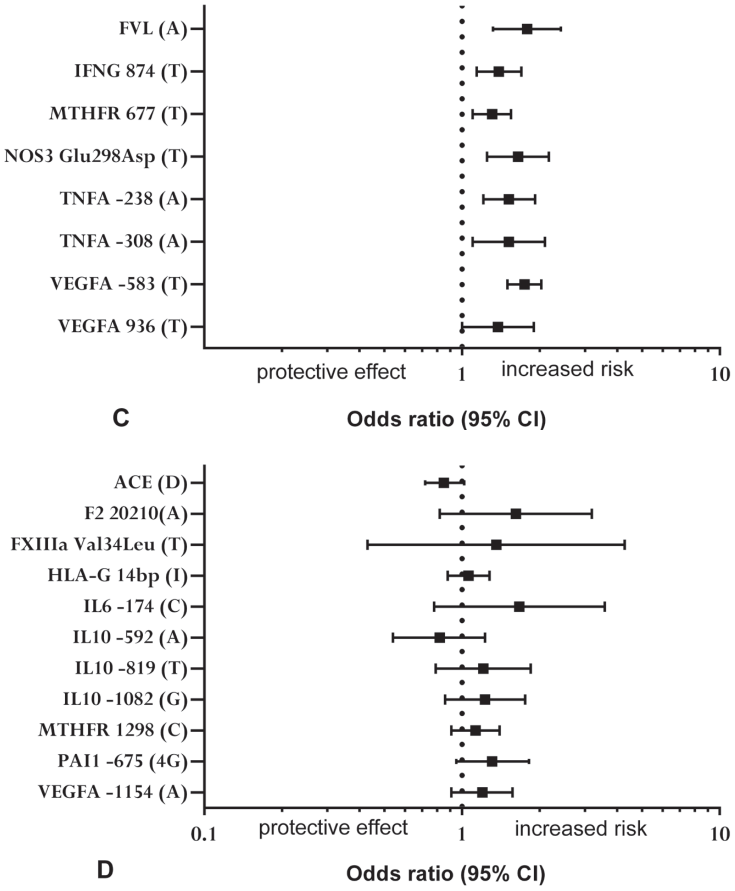


Figure 2. Odds ratio's with 95% confidence intervals for variants that were reproducibly associated with recurrent miscarriage

Andersen *et al.*, 2013). Smith et al found an association between recurrent miscarriage and a family history of ischemic heart disease (Smith, Wood *et al.*, 2011), suggesting a shared genetic predisposition between the two diseases. It is noteworthy to mention that several of the variants that were associated with recurrent miscarriage in this meta-analysis are also identified as risk factors for developing cardiovascular disease. For example *F2 G20210A*, *FVL* and *PAI1 -675 4G/5G* polymorphism were associated with coronary disease in a large meta-analysis (Ye, Liu *et al.*, 2006). In another meta-analysis, *NOS3 Glu298Asp* was found to be significantly associated with ischemic heart disease (Casas, Bautista *et al.*, 2004). *F2 G20210A, FVL, FXIIIa Val34Leu, MTHFR A1298C, MTHFR*



Legend

A. All reproduced genetic variants that were significantly associated with recurrent miscarriage after meta-analysis

B. All reproduced genetic variants that were not significantly associated with recurrent miscarriage after meta-analysis

C. Subgroup analysis: All reproduced genetic variants that were significantly associated with three or more consecutive miscarriages after meta-analysis

D. Subgroup analysis: All reproduced genetic variants that were not significantly associated with three or more consecutive miscarriages after meta-analysis

C677T, NOS3 Glu298/Asp and *PAI1 -675 4G/5G* were associated with ischemic stroke in another meta-analysis (Casas, Hingorani *et al.*, 2004;Kang, Wu *et al.*, 2014).

In summary our study found 16 genetic variants that are associated with recurrent miscarriage. This meta-analysis gives a comprehensive overview of the involved pathways; coagulation and fibrinolysis, immunology and inflammation and angiogenesis. Further studies are needed to investigate the mechanisms of how these genetic variants affect the risk of developing recurrent miscarriages. Additionally, this meta-analysis suggests that recurrent miscarriage and cardiovascular disease share genetic risk factors.

Table 1. Random effect meta-analysis of reproduced genes for recurrent miscarriage

Variant		Minor Allele	Studies(n)	Cases(n)	Controls(n)	OR(95% CI)	I^2 (%)	Egger test P value
ACE I/D	rs1799752	D	11	1928	1170	1.20 (0.95, 1.53)	76.8	0.52
APOE	rs429358	E4	5	969	532	3.09 (0.93,10.24)	84.3	--
F2 G20210A	rs1799963	A	26	3989	3424	1.94 (1.23,3.06)	49.0	0.96
FVL	rs6025	A	38	6025	5246	1.88 (1.47,2.40)	47.2	0.10
FXIIIa Val34Leu	rs5985	T	8	836	568	1.58 (1.04,2.41)	71.3	--
HLA-G 14bp I/D	rs1704	I	11	1449	1362	1.21 (1.01, 1.45)	57.0	0.36
IFNG 874A/T	rs2430561	T	5	596	620	1.12 (0.82, 1.54)	67.6	--
IL1B -511C/T	rs16944	C	4	808	527	1.17 (0.79, 1.73)	81.7	--
IL6 -174G/C	rs1800795	C	4	616	715	1.74 (0.96, 3.15)	87.1	0.34
IL10 -592C/A	rs1800872	A	7	879	1062	0.84 (0.63, 1.12)	73.2	--
IL10 -819C/T	rs1800871	T	4	720	841	1.22 (0.93, 1.60)	67.8	--
IL10 -1082A/G	rs1800896	G	6	1193	1144	1.25 (1.02, 1.54)	52.6	--
IL18 -656C/A	rs1946519	A	2	517	524	1.92 (1.61, 2.30)	0.0	--
MTHFR A1298C	rs1801131	C	14	2289	1758	1.34 (1.05, 1.72)	79.3	0.67
MTHFR C677T	rs1801133	T	32	4146	4573	1.28 (1.12, 1.47)	63.4	0.44
NOS3 Glu298Asp	rs1799983	T	8	1574	1352	1.53 (1.16, 2.01)	72.0	--
PAI1 -675 4G/5G	rs1799889	4G	14	2759	2091	1.29 (1.05, 1.57)	78.8	0.56
PZ intron F G79A	rs3024718	A	4	420	433	0.88 (0.38, 2.04)	87.2	--
STAT3	rs1053004	C	2	670	751	1.51 (1.30, 1.75)	0.0	--
TNFA -238G/A	rs361525	A	5	1058	1201	1.37 (0.88, 2.14)	64.3	--
TNFA -308G/A	rs1800629	A	7	1276	1463	1.46 (1.17, 1.84)	28.5	--
TNFA -1031C/T	rs1799964	C	2	561	484	2.64 (1.90, 3.66)	23.8	--
VEGFA -583T/C	rs3025020	T	3	696	785	1.75 (1.50, 2.03)	0.0	--
VEGFA 936C/T	rs3025039	T	9	1509	1640	1.35 (1.09, 1.68)	50.6	--
VEGFA -1154G/A	rs1570360	A	10	1879	1972	1.24 (1.02, 1.50)	66.1	0.41

Table 2. Subgroup analysis: Random effect meta–analysis of reproduced genes for three or more consecutive miscarriages

Variant	Minor Allele	Studies(n)	Cases(n)	Controls(n)	OR(95% CI)	I^2 (%)	Egger test P value
ACE I/D	D	4	544	593	0.85 (0.72, 1.02)	0.0	--
F2 G20210A	A	14	2314	1911	1.62 (0.82, 3.19)	51.1	0.25
FVL	A	21	3518	3156	1.79 (1.32, 2.42)	34.2	0.10
FXIIIa Val34Leu	T	3	272	268	1.36 (0.43, 4.28)	90.8	--
HLA-G 14bp I/D	I	4	700	405	1.06 (0.88, 1.28)	5.6	--
IFNG 874A/T	T	3	372	485	1.39 (1.14, 1.70)	0.0	--
IL6 -174G/C	C	3	474	537	1.67 (0.78, 3.58)	90.7	--
IL10 -592C/A	A	4	482	601	0.82 (0.54, 1.23)	77.7	--
IL10 -819C/T	T	3	424	536	1.21 (0.79, 1.85)	78.5	--
IL10 -1082A/G	G	3	412	534	1.23 (0.86, 1.76)	68.0	--
MTHFR A1298C	C	7	1019	1106	1.13 (0.91, 1.40)	54.8	--
MTHFR C677T	T	19	1991	2944	1.31 (1.10, 1.55)	60.0	0.31
NOS3 Glu298Asp	T	7	1429	1217	1.65 (1.25, 2.17)	67.3	--
PAI1 -675 4G/5G	4G	7	1042	1377	1.31 (0.95, 1.82)	84.3	--
TNFA -238G/A	A	3	569	813	1.52 (1.21, 1.92)	0.0	--
TNFA -308G/A	A	4	737	1025	1.52 (1.10, 2.10)	53.5	--
VEGFA -583T/C	T	3	696	785	1.75 (1.50, 2.03)	0.0	--
VEGFA 936C/T	T	5	827	901	1.38 (1.00, 1.90)	62.2	--
VEGFA -1154G/A	A	5	1114	1110	1.20 (0.91, 1.57)	71.3	--

Table 3. References of the included articles, per genetic variant

Variant		
ACE I/D		(Al Sallout and Sharif, 2010;Bagheri, Abdi, I *et al.*, 2010;Buchholz, Lohse *et al.*, 2003;Choi, Kwon *et al.*, 2011;Dossenbach-Glaninger, van *et al.*, 2008b;Fatini, Gensini *et al.*, 2000;Kim, Choi *et al.*, 2014;Ozdemir, Yenicesu *et al.*, 2012;Poursadegh, Chaparzadeh *et al.*, 2013;Vettriselvi, Vijayalakshmi *et al.*, 2008;Yenicesu, Cetin *et al.*, 2010)
	Subgroup analysis	(Al Sallout and Sharif, 2010;Bagheri, Abdi, I *et al.*, 2010;Choi, Kwon *et al.*, 2011;Kim, Choi *et al.*, 2014)
APOE		(Agarwal, Parveen *et al.*, 2010;Asgari, Akbari *et al.*, 2013;Korkmazer, Ustunyurt *et al.*, 2013;Ozdemir, Yenicesu *et al.*, 2012;Poursadegh, Farajzadeh *et al.*, 2014)
F2 G20210A		(Abu-Asab, Ayesh *et al.*, 2011;Altintas, Pasa *et al.*, 2007;Bagheri, Rad *et al.*, 2011a;Buchholz, Lohse *et al.*, 2003;Carp, Salomon *et al.*, 2002;Dossenbach-Glaninger, van *et al.*, 2003;Dutra, Fraga *et al.*, 2014;Finan, Tamim *et al.*, 2002;Guerra-Shinohara, Bertinato *et al.*, 2012;Hohlagschwandtner, Unfried *et al.*, 2003;Ivanov, Komsa-Penkova *et al.*, 2009;Karata, Aydin *et al.*, 2012;Lino, Traina *et al.*, 2014;Mitic, Kovac *et al.*, 2010;Mohamed, El Moaty *et al.*, 2010;Mougiou, Androutsopoulos *et al.*, 2008;Mtiraoui, Borgi *et al.*, 2004;Parand, Zolghadri *et al.*, 2013;Parveen, Shukla *et al.*, 2013;Pihusch, Buchholz *et al.*, 2001;Poursadegh, Chaparzadeh *et al.*, 2013;Serrano, Lima *et al.*, 2011;Sotiriadis, Vartholomatos *et al.*, 2007;Sottilotta, Oriana *et al.*, 2006;Yenicesu, Cetin *et al.*, 2010;Yildiz, Yavuzcan *et al.*, 2012

Variant		
	Subgroup analysis	(Altintas, Pasa *et al.*, 2007;Bagheri, Rad *et al.*, 2011a;Guerra-Shinohara, Bertinato *et al.*, 2012;Hohlagschwandtner, Unfried *et al.*, 2003;Karata, Aydin *et al.*, 2012;Lino, Traina *et al.*, 2014;Mitic, Kovac *et al.*, 2010;Mohamed, El Moaty *et al.*, 2010;Mougiou, Androutsopoulos *et al.*, 2008;Mtiraoui, Borgi *et al.*, 2004;Parand, Zolghadri *et al.*, 2013;Parveen, Shukla *et al.*, 2013;Serrano, Lima *et al.*, 2011;Yildiz, Yavuzcan *et al.*, 2012)
FVL		(Abu-Asab, Ayesh *et al.*, 2011;Altintas, Pasa *et al.*, 2007;Bagheri, Rad *et al.*, 2011a;Biswas, Choudhry *et al.*, 2008;Buchholz, Lohse *et al.*, 2003;Carp, Salomon *et al.*, 2002;Dizon-Townson, Kinney *et al.*, 1997;Dossenbach-Glaninger, van *et al.*, 2003;Dutra, Fraga *et al.*, 2014;Eroglu, Yeniel *et al.*, 2006;Finan, Tamim *et al.*, 2002;Guerra-Shinohara, Bertinato *et al.*, 2012;Hashimoto, Shizusawa *et al.*, 1999;Hohlagschwandtner, Unfried *et al.*, 2003;Ivanov, Komsa-Penkova *et al.*, 2009;Karata, Aydin *et al.*, 2012;Kaur, Puri *et al.*, 2013;Kobashi, Kato *et al.*, 2005;Lino, Traina *et al.*, 2014;Mahjoub, Mtiraoui *et al.*, 2005;Mitic, Kovac *et al.*, 2010;Mohamed, El Moaty *et al.*, 2010;Mougiou, Androutsopoulos *et al.*, 2008;Mtiraoui, Borgi *et al.*, 2004;Mukhopadhyay, Saraswathy *et al.*, 2009;Ozdemir, Yenicesu *et al.*, 2012;Parand, Zolghadri *et al.*, 2013;Parveen, Shukla *et al.*, 2013;Pihusch, Buchholz *et al.*, 2001;Poursadegh, Chaparzadeh *et al.*, 2013;Rai, Shlebak *et al.*, 2001;Serrano, Lima *et al.*, 2011;Sotiriadis, Vartholomatos *et al.*, 2007;Sottilotta, Oriana *et al.*, 2006;Yenicesu, Cetin *et al.*, 2010;Yildiz, Yavuzcan *et al.*, 2012;Younis, 2000;Yusoff, Abdullah *et al.*, 2002)
	Subgroup analysis	(Altintas, Pasa *et al.*, 2007;Bagheri, Rad *et al.*, 2011a;Dizon-Townson, Kinney *et al.*, 1997;Eroglu, Yeniel *et al.*, 2006;Guerra-Shinohara, Bertinato *et al.*, 2012;Hashimoto, Shizusawa *et al.*, 1999;Hohlagschwandtner, Unfried *et al.*, 2003;Karata, Aydin *et al.*, 2012;Kaur, Puri *et al.*, 2013;Kobashi, Kato *et al.*, 2005;Lino, Traina *et al.*, 2014;Mahjoub, Mtiraoui *et al.*, 2005;Mitic, Kovac *et al.*, 2010;Mohamed, El Moaty *et al.*, 2010;Mougiou, Androutsopoulos *et al.*, 2008;Mtiraoui, Borgi *et al.*, 2004;Parand, Zolghadri *et al.*, 2013;Parveen, Shukla *et al.*, 2013;Rai, Shlebak *et al.*, 2001;Serrano, Lima *et al.*, 2011;Yildiz, Yavuzcan *et al.*, 2012).
FXIIIa Val34Leu		(Bagheri, Rad *et al.*, 2011b;Dossenbach-Glaninger, van *et al.*, 2003;Elmahgoub, Afify *et al.*, 2014;Jeddi-Tehrani, Torabi *et al.*, 2010;Lino, Traina *et al.*, 2014;Lopez, Vivenes *et al.*, 2006;Poursadegh, Chaparzadeh *et al.*, 2013;Yenicesu, Cetin *et al.*, 2010)
	Subgroup analysis	(Bagheri, Rad *et al.*, 2011b;Elmahgoub, Afify *et al.*, 2014;Lino, Traina *et al.*, 2014;Lopez, Vivenes *et al.*, 2006)
HLA-G 14bp I/D		(Afkhami, Shekari *et al.*, 2014;Aruna, Sirisha *et al.*, 2011;Berger, Hogge *et al.*, 2010;Christiansen, Kolte *et al.*, 2012;Shankarkumar, Shankarkumar *et al.*, 2011;Suryanarayana, Rao *et al.*, 2008;Tripathi, Abbas *et al.*, 2004;Vargas, Sarturi *et al.*, 2011;Xue, Yang *et al.*, 2007;Yan, Lin *et al.*, 2006;Zhu, Huo *et al.*, 2010)
	Subgroup analysis	(Christiansen, Kolte *et al.*, 2012;Suryanarayana, Rao *et al.*, 2008;Tripathi, Abbas *et al.*, 2004;Vargas, Sarturi *et al.*, 2011).
IFNG 874A/T		(Bompeixe, Carvalho Santos *et al.*, 2012;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Parveen, Shukla *et al.*, 2012;Prigoshin, Tambutti *et al.*, 2004;Zastavna, Sosnina *et al.*, 2014)
	Subgroup analysis	(Kamali-Sarvestani, Zolghadri *et al.*, 2005;Parveen, Shukla *et al.*, 2012;Prigoshin, Tambutti *et al.*, 2004)
IL1B -511C/T		(Hefler, Tempfer *et al.*, 2002;Kim, Lee *et al.*, 2014;Ma, Xu *et al.*, 2012;Wang, Yunis *et al.*, 2002)
IL6 -174G/C		(Demirturk, Ates *et al.*, 2014;Parveen, Shukla *et al.*, 2012;Unfried, Bocskor *et al.*, 2003;von, Bompeixe *et al.*, 2005)
	Subgroup analysis	(Demirturk, Ates *et al.*, 2014;Parveen, Shukla *et al.*, 2012;Unfried, Bocskor *et al.*, 2003)

Variant		
IL10 -592C/A		(Alkhuriji, Alhimaidi *et al.*, 2013;Bahadori, Zarei *et al.*, 2014;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Kaur and Kaur, 2011;Parveen, Shukla *et al.*, 2012;Qaddourah, Magdoud *et al.*, 2014;Zastavna, Sosnina *et al.*, 2014)
	Subgroup analysis	(Alkhuriji, Alhimaidi *et al.*, 2013;Bahadori, Zarei *et al.*, 2014;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Parveen, Shukla *et al.*, 2012)
IL10 -819C/T		(Bahadori, Zarei *et al.*, 2014;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Parveen, Shukla *et al.*, 2012;Qaddourah, Magdoud *et al.*, 2014)
	Subgroup analysis	(Bahadori, Zarei *et al.*, 2014;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Parveen, Shukla *et al.*, 2012)
IL10 -1082A/G		(Bahadori, Zarei *et al.*, 2014;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Kim, Lee *et al.*, 2014;Parveen, Shukla *et al.*, 2012;Qaddourah, Magdoud *et al.*, 2014;Zastavna, Sosnina *et al.*, 2014)
	Subgroup analysis	(Bahadori, Zarei *et al.*, 2014;Kamali-Sarvestani, Zolghadri *et al.*, 2005;Parveen, Shukla *et al.*, 2012)
IL18 -656C/A		(Al-Khateeb, Sater *et al.*, 2011;Messaoudi, Dandana *et al.*, 2012)
MTHFR A1298C		(Bae, Choi *et al.*, 2009;Dossenbach-Glaninger, van *et al.*, 2003;Hohlagschwandtner, Unfried *et al.*, 2003;Jeddi-Tehrani, Torabi *et al.*, 2011;Lino, Traina *et al.*, 2014;Mtiraoui, Zammiti *et al.*, 2006;Nair, Khanna *et al.*, 2012;Ozdemir, Yenicesu *et al.*, 2012;Parveen, Tuteja *et al.*, 2013;Poursadegh, Chaparzadeh *et al.*, 2012;Seremak-Mrozikiewicz, Drews *et al.*, 2010;Sotiriadis, Vartholomatos *et al.*, 2007;Yenicesu, Cetin *et al.*, 2010;Yousefian, Kardi *et al.*, 2014)
	Subgroup analysis	(Bae, Choi *et al.*, 2009;Hohlagschwandtner, Unfried *et al.*, 2003;Lino, Traina *et al.*, 2014;Mtiraoui, Zammiti *et al.*, 2006;Parveen, Tuteja *et al.*, 2013;Seremak-Mrozikiewicz, Drews *et al.*, 2010;Yousefian, Kardi *et al.*, 2014)
MTHFR C677T		(Abu-Asab, Ayesh *et al.*, 2011;Bae, Choi *et al.*, 2009;Biswas, Choudhry *et al.*, 2008;Buchholz, Lohse *et al.*, 2003;Creus, Deulofeu *et al.*, 2012;Dossenbach-Glaninger, van *et al.*, 2003;Dutra, Fraga *et al.*, 2014;Eroglu, Yeniel *et al.*, 2006;Hohlagschwandtner, Unfried *et al.*, 2003;Jeddi-Tehrani, Torabi *et al.*, 2011;Karata, Aydin *et al.*, 2012;Kaur, Puri *et al.*, 2013;Kobashi, Kato *et al.*, 2005;Lino, Traina *et al.*, 2014;Mitic, Kovac *et al.*, 2010;Mohamed, El Moaty *et al.*, 2010;Mtiraoui, Zammiti *et al.*, 2006;Nair, Khanna *et al.*, 2012;Ozdemir, Yenicesu *et al.*, 2012;Park, Han *et al.*, 2011;Parveen, Tuteja *et al.*, 2013;Pihusch, Buchholz *et al.*, 2001;Poursadegh, Chaparzadeh *et al.*, 2012;Puri, Kaur *et al.*, 2013;Seremak-Mrozikiewicz, Drews *et al.*, 2010;Sotiriadis, Vartholomatos *et al.*, 2007;Unfried, Griesmacher *et al.*, 2002;Vettriselvi, Vijayalakshmi *et al.*, 2008;Yenicesu, Cetin *et al.*, 2010;Yildiz, Yavuzcan *et al.*, 2012;Yousefian, Kardi *et al.*, 2014)
	Subgroup analysis	(Bae, Choi *et al.*, 2009;Creus, Deulofeu *et al.*, 2012;Eroglu, Yeniel *et al.*, 2006;Hohlagschwandtner, Unfried *et al.*, 2003;Karata, Aydin *et al.*, 2012;Kaur, Puri *et al.*, 2013;Kobashi, Kato *et al.*, 2005;Lino, Traina *et al.*, 2014;Mitic, Kovac *et al.*, 2010;Mohamed, El Moaty *et al.*, 2010;Mtiraoui, Zammiti *et al.*, 2006;Nair, Khanna *et al.*, 2012;Parveen, Tuteja *et al.*, 2013;Poursadegh, Chaparzadeh *et al.*, 2012;Puri, Kaur *et al.*, 2013;Seremak-Mrozikiewicz, Drews *et al.*, 2010;Unfried, Griesmacher *et al.*, 2002;Yildiz, Yavuzcan *et al.*, 2012;Yousefian, Kardi *et al.*, 2014)
NOS3 Glu298Asp		(Almawi, Guarino *et al.*, 2013;Dutra, Fraga *et al.*, 2014;Hefler, Tempfer *et al.*, 2002;Karvela, Papadopoulou *et al.*, 2008;Luo, Li *et al.*, 2013;Parveen, Faridi *et al.*, 2011;Shin, Lee *et al.*, 2010;Suryanarayana, Rao *et al.*, 2006)
	Subgroup analysis	(Almawi, Guarino *et al.*, 2013;Hefler, Tempfer *et al.*, 2002;Karvela, Papadopoulou *et al.*, 2008;Luo, Li *et al.*, 2013;Parveen, Faridi *et al.*, 2011;Shin, Lee *et al.*, 2010;Suryanarayana, Rao *et al.*, 2006)

Variant		
PAI1 -675 4G/5G		(Al Sallout and Sharif, 2010;Buchholz, Lohse *et al.*, 2003;Dossenbach-Glaninger, van *et al.*, 2003;Elmahgoub, Afify *et al.*, 2014;Jeddi-Tehrani, Torabi *et al.*, 2011;Jeon, Kim *et al.*, 2013;Kim, Choi *et al.*, 2014;Lino, Traina *et al.*, 2014;Magdoud, Herbepin *et al.*, 2013;Ozdemir, Yenicesu *et al.*, 2012;Parveen, Tuteja *et al.*, 2013;Poursadegh, Chaparzadeh *et al.*, 2013;Subrt, Ulcova-Gallova *et al.*, 2013;Yenicesu, Cetin *et al.*, 2010)
	Subgroup analysis	(Al Sallout and Sharif, 2010;Elmahgoub, Afify *et al.*, 2014;Kim, Choi *et al.*, 2014;Lino, Traina *et al.*, 2014;Magdoud, Herbepin *et al.*, 2013;Parveen, Tuteja *et al.*, 2013;Subrt, Ulcova-Gallova *et al.*, 2013)
PZ intron F G79A		(Al-Shaikh, Sater *et al.*, 2013;Dossenbach-Glaninger, van *et al.*, 2008a;El-Hamid and El-Khayat, 2011;Topalidou, Effraimidou *et al.*, 2009)
STAT3		(Finan, Mustafa *et al.*, 2010;Messoudi, Al-Sulaiti *et al.*, 2013)
TNFA -238G/A		(Alkhuriji, Alhimaidi *et al.*, 2013;Finan, Al-Irhayim *et al.*, 2010;Gupta, Prakash *et al.*, 2012;Lee, Jeon *et al.*, 2013;Liu, Wang *et al.*, 2010)
	Subgroup analysis	(Alkhuriji, Alhimaidi *et al.*, 2013;Finan, Al-Irhayim *et al.*, 2010;Gupta, Prakash *et al.*, 2012)
TNFA -308G/A		(Alkhuriji, Alhimaidi *et al.*, 2013;Finan, Al-Irhayim *et al.*, 2010;Gupta, Prakash *et al.*, 2012;Kaur and Kaur, 2011;Lee, Jeon *et al.*, 2013;Liu, Wang *et al.*, 2010;Pietrowski, Bettendorf *et al.*, 2004)
	Subgroup analysis	(Alkhuriji, Alhimaidi *et al.*, 2013;Finan, Al-Irhayim *et al.*, 2010;Gupta, Prakash *et al.*, 2012;Pietrowski, Bettendorf *et al.*, 2004)
TNFA -1031C/T		(Finan, Al-Irhayim *et al.*, 2010;Lee, Jeon *et al.*, 2013)
VEGFA -583T/C		(Al-Khateeb, Mustafa *et al.*, 2011;Almawi, Saldanha *et al.*, 2013;Li, Donghong *et al.*, 2013)
	Subgroup analysis	(Al-Khateeb, Mustafa *et al.*, 2011;Almawi, Saldanha *et al.*, 2013;Li, Donghong *et al.*, 2013)
VEGFA 936C/T		(Aggarwal, Parveen *et al.*, 2011;Almawi, Saldanha *et al.*, 2013;Eller, Branch *et al.*, 2011;Lee, Hong *et al.*, 2010;Li, Donghong *et al.*, 2013;Magdoud, Dendana *et al.*, 2012;Papazoglou, Galazios *et al.*, 2005;Samli, Demir *et al.*, 2012;Traina, Daher *et al.*, 2011) (Aggarwal, Parveen *et al.*, 2011;Almawi, Saldanha *et al.*, 2013;Li, Donghong *et al.*, 2013;Magdoud, Dendana *et al.*, 2012;Papazoglou, Galazios *et al.*, 2005;Traina, Daher *et al.*, 2011)
VEGFA -1154G/A		(Aggarwal, Parveen *et al.*, 2011;Almawi, Saldanha *et al.*, 2013;Eller, Branch *et al.*, 2011;Lee, Hong *et al.*, 2010;Li, Donghong *et al.*, 2013;Magdoud, Dendana *et al.*, 2012;Papazoglou, Galazios *et al.*, 2005;Samli, Demir *et al.*, 2012;Su, Lin *et al.*, 2011;Xing, Yan *et al.*, 2011)
	Subgroup analysis	(Aggarwal, Parveen *et al.*, 2011;Almawi, Saldanha *et al.*, 2013;Li, Donghong *et al.*, 2013;Magdoud, Dendana *et al.*, 2012;Papazoglou, Galazios *et al.*, 2005;Xing, Yan *et al.*, 2011)

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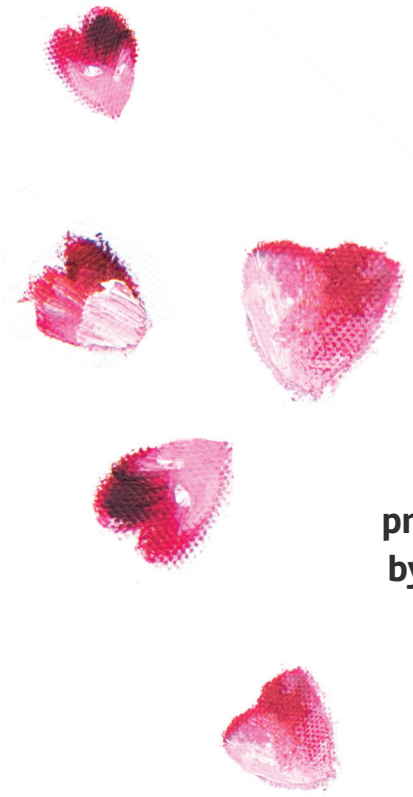

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Pregnancy prior to recurrent pregnancy loss more often complicated by post-term birth and perinatal death

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Abstract

Introduction The cause of recurrent pregnancy loss remains often unknown. Possibly, pathophysiological pathways are shared with other pregnancy complications.

Material and methods All women with secondary recurrent pregnancy loss (SRPL) visiting Leiden University Medical Centre (January 2000-2015) were included in this retrospective cohort to assess whether women with SRPL have a more complicated first pregnancy compared to control women. SRPL was defined as ≥ 3 consecutive pregnancy losses before 22 weeks' gestation, with a previous birth. The control group consisted of all Dutch nullipara delivering a singleton (January 2000-2015). Information was obtained from the Dutch Perinatal Registry. Outcomes were preeclampsia, preterm birth, post-term birth, intrauterine growth restriction, breach position, induction of labor, Caesarean section, congenital abnormalities, perinatal death and severe hemorrhage in the first ongoing pregnancy. Subgroup analyses were performed for women with idiopathic SRPL and for women ≤ 35 years.

Results 172 women with SRPL and 1.196.178 control women were included. Women with SRPL were older and had a higher BMI; 29.7 years versus 28.8 years and 25.1 versus 24.1, respectively. Women with SRPL more often had a post-term birth (OR 1.86 95%CI (1.10-3.17)) and more perinatal deaths occurred in women with SRPL compared to the control group (OR 5.03 95%CI (2.48-10.2)). Similar results were found in both subgroup analyses.

Conclusions The first ongoing pregnancy of women with (idiopathic) SRPL is more often complicated by post-term birth and perinatal death. Revealing possible links between SRPL and these pregnancy complications might lead to a better understanding of underlying pathophysiology.

Introduction

Recurrent pregnancy loss is commonly defined as three or more consecutive pregnancy losses before 22 weeks of gestation(1) and affects 0.5-3% of all fertile couples(2) and includes intrauterine pregnancy demise confirmed by ultrasound or histology and non-visualized pregnancy losses(3). Recurrent pregnancy loss is a heterogeneous condition and has many possible etiologic factors: genetic disorders, uterine anomalies, endocrine factors, maternal autoimmune disorders, metabolic disorders, maternal thrombophilia, obesity and toxic factors, although the percentage of cases without an identifiable cause is about 50%(4, 5). Some of these etiologic factors like autoimmune disorders and thrombophilia might cause a dysregulation of placental vasculature, which can lead to multiple pregnancy complications like miscarriage, pre-eclampsia, fetal growth restriction and fetal death(6).

Approximately 40% of the women with recurrent pregnancy loss have a previous ongoing pregnancy and are diagnosed with secondary recurrent pregnancy loss(SRPL)(2). Few studies described pregnancy outcome prior to recurrent pregnancy loss with different results. One study described that most women did not have obstetric complications(7), a higher rate of fetal death was found in some studies(5,8) and higher rates of preeclampsia(8, 9), low birth weight, hemorrhage and preterm delivery(8) were described. Knowledge of obstetric details regarding the pregnancy prior to the consecutive pregnancy losses may contribute to our understanding of the pathophysiology of recurrent pregnancy loss.

This study was performed to assess whether women with SRPL have a more complicated first pregnancy compared to control women.

Material and methods

Study design

All women with SRPL visiting the recurrent miscarriage clinic at Leiden University Medical Centre, between January 2000 and January 2015 were identified in this retrospective cohort design. SRPL was defined as having ≥ 3 consecutive pregnancy losses before 22 weeks of gestation (including intrauterine pregnancy demise confirmed by ultrasound and non-visualized spontaneous pregnancy losses), with a previous birth ≥ 22 weeks of gestation. Women with a previous multiple pregnancy were excluded from the study. Information was obtained from medical records.

The control group consisted of all Dutch nullipara delivering a singleton ≥ 22 weeks of gestation between January 2000 and January 2015. Data were obtained from the Perinatal Registry of the Netherlands(Perined), a nationwide database that covers around 96% of all maternal, obstetric and neonatal outcomes in the Netherlands(10).

Procedures and definitions

The following maternal characteristics were identified at visiting the recurrent miscarriage clinic: number of consecutive pregnancy losses, parity before recurrent pregnancy loss, presence of late pregnancy loss; defined as having at least one miscarriage after 13 weeks of gestation. All women had a routine recurrent pregnancy loss work-up to identify possible causes for the recurrent pregnancy loss: a standardized history of the couple was performed, karyotyping of the couple (this was offered routinely before 2005 to all couples, after 2005 this was only offered in presence of low maternal age and/or positive family history for recurrent pregnancy loss(11), presence of uterus anomalies by ultrasound or hysteroscopy and presence of acquired and heritable thrombophilia. Acquired thrombophilia: Antiphospholipid syndrome was defined as the presence of anticardiolipin antibodies or lupus anticoagulant in repeated samples taken 3 months apart and at least 10 weeks after a delivery(12), after revision of the classification criteria the presence of anti-β2 glycoprotein-I was added to the work-up(13). Hyperhomocysteinemia was evaluated. Heritable thrombophilia was defined by the presence of a factor V Leiden mutation, factor II (prothrombin) gene mutation, protein C or S deficiency or antithrombin deficiency. Recurrent pregnancy loss was defined as idiopathic when the work-up for causes of recurrent pregnancy loss showed no abnormalities. A sub analysis was performed in women with idiopathic SRPL to clarify if the prevalence of pregnancy complications in women with SRPL solely could be explained by the presence of known causes for recurrent pregnancy loss or that other (unknown) links could be an explanation.

Maternal age was defined as age at first delivery (index pregnancy). Body mass index (BMI) was calculated as weight/ length^2, at time of visiting the recurrent miscarriage clinic. Smoking was self-reported at time of visiting the recurrent miscarriage clinic.

Clinical data from the first ongoing pregnancy (prior to the recurrent pregnancy loss) was obtained. Preeclampsia was defined as systolic blood pressure above 140 mmHg and/ or diastolic pressure above 90 mmHg combined with proteinuria(14), preterm birth as a delivery before 37 weeks of gestation, post-term birth as a delivery ≥ 42 weeks of gestation, intra-uterine growth restriction (IUGR) as birth weight below the 10th percentile for gestational age and sex according to the Perined birth weight percentiles(15). Information about fetal position during labor, induction of labor, delivery by Caesarean section, presence of congenital abnormalities and gender of neonate were collected. Perinatal death was defined as fetal loss after 22 weeks of gestation till 7 days after birth. Severe hemorrhage was defined as ≥ 1000 cc blood loss within 24 hours postpartum.

Statistical analyses

Data were analyzed using SPSS software version 22.0 (Statistical Package for Social Science; SPSS, Chicago, Illinois, USA). Comparisons of normal distributed data were performed using independent T- test. Comparisons of categorical data were performed

using the X^2 test. Odds ratios with 95% confidence intervals were calculated for pregnancy outcome variables. For all tests, a p-value < 0.05 is indicated statistical significant. Sub analyses were performed for women with idiopathic SRPL and for women who were ≤ 35 years at their first delivery.

Ethics

Approval from the medical ethics committee of Leiden University Medical Centre (C15.028; March 2, 2015) was obtained.

Results

A total of 175 women with SRPL were identified. Two women with a multiple pregnancy before their pregnancy losses were excluded from analysis.

Analyzes of recurrent pregnancy loss were described in Table 1. 81.5% of the women had only one ongoing pregnancy before their pregnancy losses. In 105 women (60.7%) recurrent pregnancy loss was defined as idiopathic. 1.196.178 women were included in the control group. Women with SRPL were significantly older and had a higher BMI compared to the control group, 29.7 (4.8) years versus 28.8 (4.9) years and 25.1 (4.9) versus 24.1 (4.4), respectively (Table 2). Information about smoking during pregnancy was not available for the control group.

Comparisons of pregnancy outcome in the first ongoing pregnancy between women with SRPL and the control group were described in Table 3. Women with SRPL more often had a post-term birth compared to the control group (OR 1.85 95%CI (1.09-3.15)). Significantly more perinatal deaths (N=8) occurred in women with SRPL (4.6%) compared to the control group (1.0%), OR 5.00 95%CI (2.46-10.2). Causes of perinatal death were: three intrauterine fetal demises (two had a severe IUGR, in one case no cause was found), one placental abruption, one spontaneous delivery at 23 weeks' gestation, two cases with multiple congenital abnormalities and one severe meconium aspiration.

For all other pregnancy outcomes (preeclampsia, preterm birth, IUGR, breach position, induction of labor, caesarean section, congenital abnormalities, gender and severe hemorrhage) no statistically significant differences were found between both groups.

In the subgroup analysis including only women with idiopathic SRPL (N=105), comparable results were found (Table 3); more often post-term birth in women with idiopathic SRPL (although not significant: OR 1.83 95%CI (0.92-3.62)) and more perinatal deaths OR 7.37 95%CI (3.42-15.9) compared to the control group.

The results of the subgroup analysis including only women who were ≤ 35 years at time of first ongoing pregnancy were similar to the results of the total group (Table 4).

Discussion

In this study women with SRPL had higher rates of post-term birth and perinatal death in their first ongoing pregnancy prior to recurrent pregnancy loss compared to all Dutch nullipara.
A strength of the study is that all women followed a well-documented protocol to identify possible causes for recurrent pregnancy loss as described in Table 1. Therefore, we could perform subgroup analyses including only women with idiopathic SRPL (Table 3) and women ≤ 35 years (Table 4), which in both cases, revealed comparable results to the total group. Therefore, in this study differences in pregnancy outcome cannot be explained by the presence of known causes (such as abnormal karyotyping or acquired/heritable thrombophilia) for recurrent pregnancy loss or a higher age.
Limitations of the study should be acknowledged. For some analyzes our study could be underpowered. For example, for the outcome preterm birth we found a non-significant difference with an OR of 1.2 in the main analysis including 173 women with SRPL. Performing a power analysis learns that, with this number of included women, we could detect an OR of 1.8 (incidence in population 7.8%, using a 2-sided test with 80% power at 5% significance). Therefore, we cannot exclude any possible small differences in pregnancy outcome between SRPL and the control group. Bias could have been introduced because we included only women with SRPL visiting the recurrent miscarriage clinic at Leiden University Medical Centre, which is a tertiary referral center. It is possible that women with adverse pregnancy outcome before their recurrent pregnancy loss were more likely to be referred to this center than women who had a first uncomplicated pregnancy.

Four heterogenous studies with respect to study design and outcome examined pregnancy outcome prior to SRPL before (5, 7-9), with diverse results. In comparison with the other studies we included a relatively large group (Weintraub (matched case-control study, N=58)) (9) and Ooi (cohort study N=85) (7), although not the largest; Nielsen (cohort study) included 358 women with SRPL (8). The study by Yang et al included 675 women, however there was no control group (5). To our knowledge this is the first study investigating the outcome post-term birth. We found that women with SRPL deliver more often post-term in their first pregnancy compared to the control group. In the Netherlands women who reach a gestational age of 41 weeks can choose between two treatment options; induction at or beyond 41 weeks versus expectant management until 42 weeks (only in obstetrical low risk women without contra-indications for expectant management). A possible link between SRPL and post-term birth may be a prostaglandin deficiency. Data from animal studies demonstrate that the primary impulse for the initiation of labor arises from the fetal hypothalamo-pituitary-adrenal (HPA) axis. The HPA axis stimulates steroid synthesis and prostaglandin production and therefore cervical dilation and start of myometrial contractions(16, 17). Relative prostaglandin deficiency

has been suggested to exist in several pregnancy complications; preeclampsia, IUGR and in early pregnancies of women with a history of recurrent pregnancy losses(18). Prostaglandin production increases during full-term labor. No data was found on prostaglandin production in post-term pregnancies(18). In spontaneous preterm labor, inflammation is suggested to be the main underlying mechanism. Several factors have been supposed to start this process; infection, uteroplacental ischemia, and hormonal abnormalities (progesterone or corticotropin-releasing hormone related)(17). In contrast to our results, a study describes a higher risk of preterm birth in women with SRPL(8). If the hypothesis of a relative prostaglandin deficiency is correct, SRPL women with post-term birth will be the ones who did not have an activation of the HPA axis due to other pregnancy complications that causes inflammation and therefore preterm labor.

Remarkable more perinatal deaths occurred in women with SRPL. Our result is in line with the findings by Field et al; women with recurrent miscarriage were more than twice as likely to have previous perinatal deaths compared to women without recurrent miscarriage(19). Yang et al reported a history of perinatal death in 7.8% of women attending a recurrent miscarriage clinic (N=675) (no control group)(5). In both studies, no further details are described. Causes of perinatal death varied from intrauterine fetal demise, IUGR, placental abruption, preterm birth to congenital abnormalities. Independently, preterm birth, IUGR and congenital abnormalities were not elevated in women with SRPL compared to the control group. Although, we could have missed a small difference in outcome between both groups due to a lack of power. Also in the subgroup analyses, including women with idiopathic SRPL and women ≤ 35 years, we found significantly higher values of perinatal death. It is not possible to identify one common cause or pathway to link recurrent pregnancy loss to the observed pregnancy complications. Our findings support the hypothesis that dysregulation of placental vasculature could be an underlying pathophysiologic mechanism linking pregnancy complications such as miscarriage, IUGR and death(6). Preeclampsia is also mentioned in this hypothesis; however we did not find an increased risk of preeclampsia in the first ongoing pregnancy for women with SRPL, in contrast to the (larger) studies of Weintraub and Nielsen(8, 9). Shared maternal risk factors for recurrent pregnancy loss and still birth, the single major determinant of perinatal death, are; maternal age ≥ 40 years, smoking, obesity and diabetes mellitus(20). An association between increasing maternal BMI and post-term birth has also been described(21), although the mechanisms are not fully understood. Mechanical and hormonal interactions between mother, fetus and placenta are involved in the onset of labor. Women with obesity have increased inflammation, circulating leptin concentrations, insulin resistance, lipolysis and dyslipidemia. It has been hypothesized that these metabolic abnormalities influence the onset of labor and uterine contractility(22), possibly via previous mentioned HPA axis(23). In our study women with SRPL had a significantly higher BMI compared to all Dutch women, 25.1(4.9) versus 24.1(4.4) $p<0.01$, respectively, so this could have affected the pregnancy outcome.

Since BMI was not available in the Perinatal Registry we used data on BMI from the Statistics Netherlands(CBS). As only aggregated data was available, it was not possible to perform a subgroup analysis for women with elevated BMI. 9.4% of the women with SRPL reported to smoke at time of visiting the recurrent miscarriage clinic. Smoking was not adequately monitored in the Perinatal Registry for the years 2000-2015. However, a large Dutch study identified approximately 13% of women smoked during their pregnancy in 2001, this prevalence dropped to 6.3% in 2010(24). We cannot specify the impact of smoking behavior on the pregnancy outcome as it is unknown how many of the SRPL women were smoking during their first pregnancy. Possibly women stopped smoking after their adverse pregnancy outcome.

In conclusion, the first ongoing pregnancy of women with (idiopathic) SRPL is more often complicated by post-term birth and perinatal death. More research (epidemiological and basic, including larger groups) is needed to reveal possible links between SRPL and these pregnancy complications as this might lead to a better understanding of the underlying pathophysiology.

Table 1. Analyzes of women with secondary recurrent pregnancy loss

	Secondary recurrent pregnancy loss N=173	
Number of consecutive pregnancy loss *median (min-max)*	4.00 (3-18)	
3 (%)	78 (45.1)	
4 (%)	54 (31.2)	
>4 (%)	41 (23.7)	
Parity before recurrent pregnancy loss *median (min-max)*	1.00 (1-3)	
1 (%)	141 (81.5)	
2 (%)	27 (15.6)	
>2 (%)	5 (2.9)	
Late miscarriage (%)	24 (13.9)	
Abnormal parental karyotyping *(%)*	2 (1.2)	*Missing 21*
Uterus anomaly *(%)*	3 (1.7)	
Anti-phospholipid syndrome *(%)*	10 (5.8)	*Missing 3*
Hyperhomocysteinemia *(%)*	12 (6.9)	*Missing 7*
Heritable thrombophilia *(%)*	27 (15.6)	*Missing 3*

Table 2 Characteristics of participants

		Secondary recurrent pregnancy loss N=173	Idiopathic Secondary recurrent pregnancy loss N=105	Controls N=1196178	p-value
Maternal age[a]	*Mean (SD)*	29.7 (4.8)	29.1 (4.7)	28.8 (4.9)	0.02[b] 0.53[c]
	Missing (%)	15 (8.7)	8 (7.7)	-	
BMI	*Mean (SD)*	25.1 (4.9)	25.4 (5.2)	24.1 (4.4)[d]	<0.01[b] <0.01[c]
	Missing (%)	26 (15.1)	17 (16.2)	n/a	
Smoking (%)		16 (9.2)	8 (7.6)	n/a	n/a
	Missing (%)	1 (0.6)	-		

BMI: body mass index, n/a: not available
[a] at first delivery
[b] comparison between secondary recurrent pregnancy loss and control group
[c] comparison between idiopathic recurrent pregnancy loss and control group
[d] women 20-45 years between 2000-2015 (Statistics Netherlands/ National Institute for Public Health and the Environment 2017)

Table 3. Pregnancy outcome in first ongoing pregnancy

	Secondary recurrent pregnancy loss N=173	Idiopathic Secondary recurrent pregnancy loss N=105	Controls N=1196516
Preeclampsia (%)	5 (2.9)	4 (3.8)	42770 (3.7)
OR(95%CI)	0.79 (0.32-1.92)	1.05 (0.38-2.84)	1
Missing (%)	1 (0.6)	-	25145 (2.1)
Preterm birth (%)	16 (9.2)	12 (11.4)	92589 (7.8)
OR(95%CI)	1.20 (0.72-2.01)	1.52 (0.83-2.78)	1
Missing (%)	-	-	11866 (1.0)
Post-term birth (%)	15 (8.7)	9 (8.6)	57745 (4.8)
OR(95%CI)	1.85 (1.09-3.15)	1.83 (0.92-3.62)	1
Missing (%)	-	-	11866 (1.0)
Intra uterine growth restriction (%)	16 (9.6)	9 (8.8)	107751 (9.1)
OR(95%CI)	1.06 (0.64-1.79)	0.97 (0.49-1.92)	1
Missing (%)	7 (4.1)	3 (2.9)	11780 (1.0)
Breach (%)	11 (6.4)	7 (6.7)	70362 (6.0)
OR(95%CI)	1.07 (0.58-1.96)	1.12 (0.52-2.41)	1
Missing (%)	-	-	21705 (1.8)
Induction of labor (%)	21 (15.2)	16 (19.3)	205683 (17.3)
OR(95%CI)	0.86 (0.54-1.37)	1.15 (0.66-1.98)	1
Missing (%)	35 (20.2)	22 (21.0)	4570 (0.4)
Caesarean section (%)	25 (14.5)	17 (16.2)	207638 (17.4)
OR(95%CI)	0.80 (0.53-1.23)	0.92 (0.55-1.54)	1
Missing (%)	-	-	2741 (0.2)
Congenital abnormalities (%)	6 (3.5)	4 (3.8)	34040 (2.8)
OR(95%CI)	1.23 (0.54-2.77)	1.35 (0.50-3.67)	1
Missing (%)	-	-	-
Neonate gender -male (%)	90 (52.3)	55 (52.4)	614781 (51.4)
OR(95%CI)	1.01 (0.75-1.37)	1.00 (0.68-1.47)	1
Missing (%)	1 (0.6)	-	693 (0.1)
Perinatal death (%)	8 (4.6)	7 (6.7)	11482 (1.0)
OR(95%CI)	5.00 (2.46-10.2)	7.37 (3.42-15.9)	1
Missing (%)	-	-	-
Severe hemorrhage (%)	9 (5.3)	8 (7.8)	67585 (5.8)
OR(95%CI)	0.90 (0.46-1.76)	1.35 (0.66-2.77)	1
Missing (%)	3 (1.7)	2 (1.9)	36009 (3.0)

Table 4. Pregnancy outcome in first ongoing pregnancy if maternal age at delivery ≤ 35 year)

	Secondary recurrent pregnancy loss N=143	Controls N=1094295
Preeclampsia (%)	3 (2.1)	38037 (3.5)
OR(95%CI)	0.59 (0.19-1.84)	1
Missing (%)	1 (0.7)	22455 (2.1)
Preterm birth (%)	15 (10.5)	84025 (7.8)
OR(95%CI)	1.39 (0.82-2.38)	1
Missing (%)	-	10305 (0.9)
Post-term birth (%)	13 (9.1)	51676 (4.8)
OR(95%CI)	1.99 (1.13-3.53)	1
Missing (%)	-	10305 (0.9)
Intra uterine growth restriction (%)	14 (9.8)	96788 (8.9)
OR(95%CI)	1.16 (0.67 -2.02)	1
Missing (%)	6 (4.2)	10211 (0.9)
Breach (%)	10 (7.0)	62964 (5.9)
OR(95%CI)	1.21 (0.64 – 2.30)	1
Missing (%)	-	19077 (1.7)
Induction of labor (%)	14 (12.7)	184184 (16.9)
OR(95%CI)	0.68 (0.39-1.19)	1
Missing (%)	28 (19.6)	4043 (0.4)
Caesarean section (%)	21 (14.7)	179684 (16.5)
OR(95%CI)	0.87 (0.55-1.39)	1
Missing (%)	-	2562 (0.2)
Congenital abnormalities (%)	5 (3.5)	30646 (2.8)
OR(95%CI)	1.26 (0.52-3.07)	1
Missing (%)	-	-
Neonate gender -male (%)	75 (52.4)	562246 (51.4)
OR(95%CI)	1.06 (0.76-1.47)	1
Missing (%)	1 (0.7)	631 (0.1)
Perinatal death (%)	8 (5.6)	10156 (0.9)
OR(95%CI)	6.33 (3.10-12.9)	1
Missing (%)	-	-
Severe hemorrhage (%)	7 (4.9)	60189 (5.7)
OR(95%CI)	0.87 (0.41-1.86)	1
Missing (%)	3 (2.1)	31797 (2.9)

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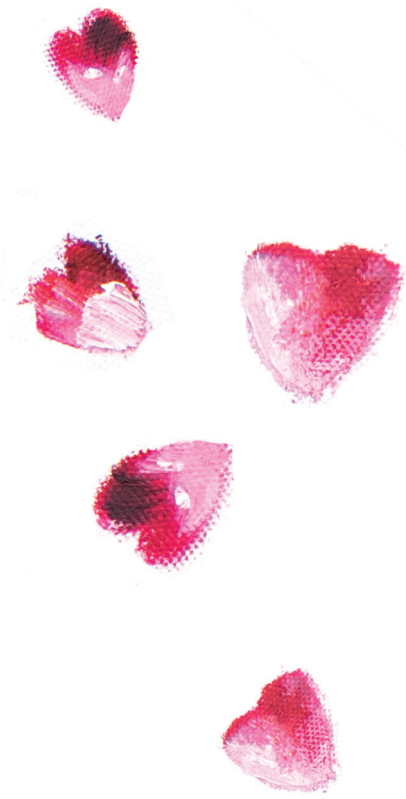

8

General discussion

In this thesis, several studies are presented examining the association between recurrent miscarriage and cardiovascular disease. Main aim of this thesis was to assess whether miscarriages are independently associated with an increased risk of cardiovascular disease later in life. And, if this was true, to identify cardiovascular risk factors and predict long term cardiovascular disease risk in women with a history of recurrent miscarriage.
We found an increased risk of ischemic heart disease in women with a history of two (multivariate analysis HR 1.82) and three or more miscarriages (HR 3.18), irrespective whether consecutive or not (*chapter 2*). Women with a history of recurrent miscarriage have significantly higher 10- and 30-year cardiovascular risk scores compared to women with a history of no miscarriage. These results indicate an opportunity for the early identification of women prone to cardiovascular disease later in life. Women with a history of two or more miscarriages must be made aware of their increased cardiovascular risk and appropriate risk factor modifications will have to be offered, for example life style advises; weight management and smoking control.
In this chapter, I give an interpretation of the findings of the studies described in the previous chapters and propose remaining questions. Suggestions for clinical implementation and future research will be discussed.

(Recurrent) miscarriage; a new cardiovascular risk factor

Studies regarding novel cardiovascular risk factors, including pregnancy complications such as (recurrent) miscarriage are hampered by the nature of the events in question. The events during pregnancy and the development of cardiovascular disease are often separated by decades and observational studies are the best used method to increase our understanding of these novel risk factors. We performed a large cohort study (including 60105 women with a median follow-up of 17 years) and demonstrated an association between miscarriage and ischemic heart disease *(chapter 2)*, independent of classical cardiovascular disease risk factors (maternal age, BMI, social class and smoking). The association between both events was already clinically relevant from 2 miscarriages onwards (multivariate analysis HR 1.82 (95%CI 1.30 to 2.54)). The risk for cardiovascular disease increases with increasing number of miscarriages and was, surprisingly, independent of the consecutive nature of the miscarriages. Our research is in line with a large Danish cohort study of 1 million women, which found a dose-dependent association between miscarriage and future risk of ischemic heart disease and other atherosclerotic endpoints [1]. Overall, at present, several studies have established that (recurrent) miscarriage is associated with ischemic heart disease in the future and possibly other cardiovascular outcomes ([1-4] *chapter 2*).
There are two challenges, which I will discuss in the next paragraphs. Firstly, trying to understand the underlying mechanisms for the association between recurrent miscarriage and cardiovascular disease. This might also contribute to our knowledge of the aetiology of recurrent miscarriage, of which the cause often remains unknown. And

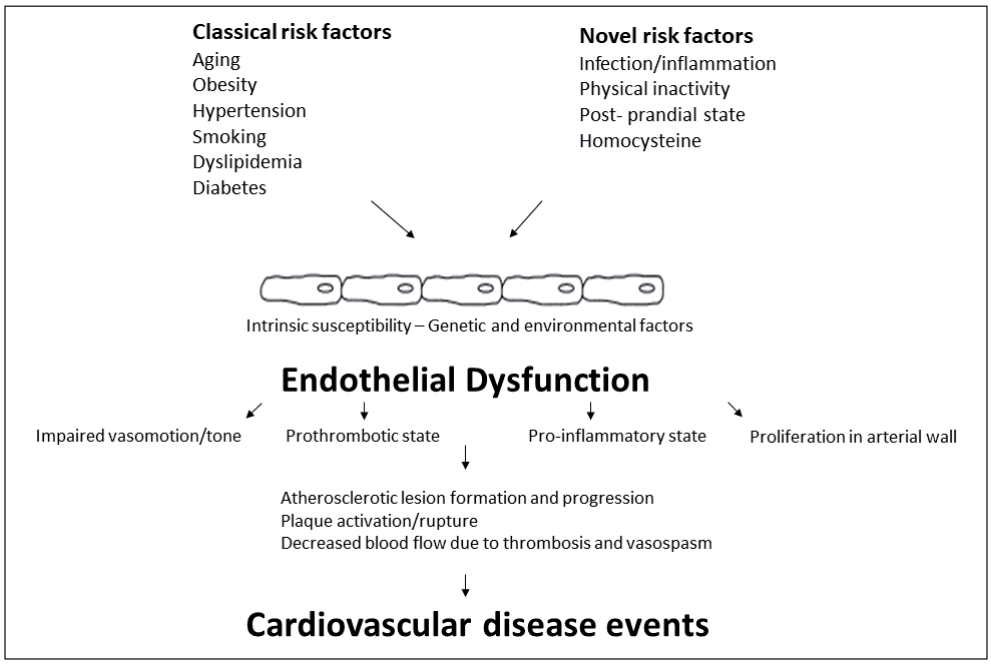

Figure 1. Endothelial dysfunction and Cardiovascular disease. Based on [11]

secondly, how to incorporate (recurrent) miscarriage as a new risk factor in the algorithm for future cardiovascular disease, especially since novel risk factors for cardiovascular disease are rising.

Mechanisms possible involved in the association between recurrent miscarriage and cardiovascular disease

Many underlying mechanisms for the association between recurrent miscarriage and cardiovascular disease are hypothesized. The following mechanisms will be discussed; endothelial dysfunction, shared classic cardiovascular risk factors, antiphospholipid syndrome and immunologic disbalance, genetics and novel cardiovascular biomarkers. Finally, the relation between recurrent miscarriage, other pregnancy complications and cardiovascular disease will be discussed.

Endothelial dysfunction

A unifying mechanism between recurrent miscarriage and cardiovascular disease could be the presence of endothelial dysfunction. Endothelial cells form the inner surface of blood vessels and have many important regulatory functions in the cardiovascular system such as vasoconstriction and dilatation. Healthy endothelium also has anti-thrombotic (through prostacyclin's), anti-inflammatory (through developmental endothelial locus-1) and anti-proliferative (through nitric oxide and prostaglandin I2) functions [5] [6].

In normal pregnancies, maternal uterine arteries are remodelled to create a high-flow, low-resistance uteroplacental vascular system that provides adequate blood flow needed for fetal growth[7]. Placentation starts in the first trimester of pregnancy. The needed invasion of extra villous trophoblasts into the uterine spiral arteries could be impaired by endothelial dysfunction. Dysregulation of the placental vasculature is thought to be the pathophysiologic mechanism leading to multiple pregnancy complications such as miscarriage, preeclampsia, intrauterine growth restriction, and perinatal death[8]. Further, endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis. [9, 10] leading to cardiovascular disease events (Figure 1).
In conclusion endothelial dysfunction is a plausible explanation for the association between miscarriage and cardiovascular disease. It is likely that the endothelium is already damaged before the miscarriages, caused by diverse maternal predisposing factors. Possibly the pregnancy itself leads to endothelium damage (second hit), or it is a combination of both. Underlying maternal factors associated with endothelial dysfunction will be discussed in more detail.

Classic cardiovascular risk factors

Classic cardiovascular risk factors include age, obesity, hypertension, smoking, dyslipidaemia, and diabetes. Cardiovascular disease risk factors adversely affect a diverse range of endothelial homeostatic functions (under influence of environmental and genetic factors) resulting in endothelial dysfunction (Figure 1) which can lead to both recurrent miscarriage and cardiovascular disease. A remarkable higher prevalence of pre-existing hypertension and pre-existing diseases of circulatory system was found in women with miscarriages (dose-dependent) (*chapter 2)*. All women with pre-existing morbidity related to cardiovascular disease (hypertension, type one diabetes mellitus, kidney disease and any disease of circulatory system) were excluded from main analysis. The association between miscarriages and future cardiovascular disease was independent of classical cardiovascular risk factors such as age, BMI, social class and smoking.
In *chapter 3* we determined classical cardiovascular risk factors and calculated cardiovascular disease risk in women after recurrent miscarriage (mean follow-up 7.5 years) and found an increased (extrapolated) 10 and 30-year cardiovascular disease risk compared to women with no miscarriage, calculated by Framingham risk scores. We are not able to answer the question on causal effect as we were only able to look at cardiovascular risk factors in women after they experienced recurrent miscarriage.
The high number of women who smoked in the recurrent miscarriage group was remarkable. Women with recurrent miscarriage were more often smokers during at least one pregnancy (38.9%) compared to women with no miscarriage (13.9%), p=0.05. Active smoking is a known risk factor for recurrent miscarriage; a meta-analysis describes an increased risk of miscarriage (risk ratio 1.23, 95% CI 1.16-1.30) [12]. It is hypothesized that the vasoconstrictive and anti-metabolic properties of some components of cigarette

smoke (nicotine, carbon monoxide, cyanide) could lead to placental insufficiency and embryonic and fetal growth restriction and demise, other studies suggest that smoking results in an increase of mature oocytes leading to miscarriages [13]. Smoking was still more common in women with a history of recurrent miscarriage at time of follow-up (*chapter 3*) although not significant. Also, other values of classical cardiovascular risk factors were higher in women with recurrent miscarriage compared to no miscarriage, although only significant for systolic blood pressure. A lack of power is likely when investigating the individual risk factors as our preliminary sample size analysis was based on the Framingham risk score. It would be interesting to investigate the individual risk factors in a larger study group, and perform multivariate analyses as well, to answer the question which risk factors are contributing the most to the elevated cardiovascular risk score in women with a history of recurrent miscarriage.
In conclusion, women with recurrent miscarriage seem to have a higher prevalence of classical cardiovascular risk factors before, and after their miscarriages. However, this is not the overall explanation for the association between miscarriages and future cardiovascular disease, as the association was found to be independent of classical cardiovascular risk factors such as age, BMI, social class and smoking *(chapter 2)*.

Antiphospholipid syndrome and immunology

Acquired thrombophilia (antiphospholipid syndrome) is clearly related to recurrent miscarriage as well as cardiovascular disease. Antiphospholipid syndrome is an autoimmune disorder characterized by the occurrence of venous and arterial thrombosis and pregnancy complications (such as recurrent miscarriage and early preeclampsia), in the presence of antiphospholipid antibodies. Women with antiphospholipid syndrome are at increased risk for accelerated atherosclerosis and cardiovascular disease events [14]. The exact mechanisms by which the antibodies cause these morbidity is not fully understood. It is suggested that endothelial cells play a central role and represent the common pathway between autoimmunity and inflammation in the pathogenesis of antiphospholipid syndrome. Circulating antibodies and underlying endothelial dysfunction are a necessary 'first hit' for the development of thrombosis, pregnancy complications and cardiovascular disease, an inflammatory 'second hit' by up-regulation of β2-glycoprotein (an apolipoprotein, member of the complement control family, considered to be a natural inhibitor of coagulation) receptors is needed to precipitate the thrombotic event [14]. These inflammatory factors may include infection, immunological and other non-immunological procoagulant factors, such as oestrogen-containing contraceptive pills, surgery and immobility [15].

Acquired thrombophilia is included in the work-up of recurrent miscarriage to identify any causes. Together with: a standardized history of the couple, evaluation of hyperhomocysteinemia, heritable thrombophilia, karyotyping of the couple and

checking the presence of uterus anomalies by ultrasound or hysteroscopy. If no abnormalities are found; a woman is diagnosed with idiopathic recurrent miscarriage. In contrast to the large cohort study described in *chapter 2*, information about causes of recurrent miscarriage was available in our follow-up study in cardiovascular disease risk (*chapter 3*). We performed a subgroup analysis including women with idiopathic recurrent miscarriage which showed comparable results to the results of the total group. The calculated risk scores were even slightly higher in women with idiopathic recurrent miscarriage. Therefore, in our study, the increased cardiovascular disease risk scores in women with recurrent miscarriage cannot be explained by the presence of antiphospholipid syndrome. Recently, the first case control study regarding the association between pregnancy loss and premature arterial thrombosis, which adjusted for both classic cardiovascular risk factors and the presence of antiphospholipid antibodies, was published [16]. An increased risk of arterial thrombosis, independent of the presence of antiphospholipid syndrome, was found in women with ≥ 3 pregnancy losses (miscarriage and stillbirth together), OR 1.95, (95%CI 0.92–4.14). However, it cannot be excluded that other immunological factors will play a role. The maternal immune system plays an important role in the success of pregnancy, optimal regulation is essential to tolerate the allogeneic foetus. As in more than 50% of the cases the cause of recurrent miscarriage remains unknown, it is thought that maladaptation of the maternal immune system could explain part of its pathophysiology. Research shows that in specific cases immunotherapy might benefit pregnancy outcome, although there is no clear evidence yet [17]. On the other hand the activation of the immune system plays a significant role in the pathogenesis of atherosclerosis and other cardiovascular diseases[18]. This possible link between recurrent miscarriage and cardiovascular disease has not been further elaborated in this thesis, but it is an interesting starting point for future research. Vitamin D, that could act as an immune regulator during implantation will be discussed later in this chapter (subheading: novel cardiovascular biomarkers).

Genetic link

A shared genetic background could be a link between recurrent miscarriage and cardiovascular disease. Both diseases have a familial aggregation[19, 20]. The degree of endothelial damage (leading to both conditions) may be in part, related to intrinsic and environmental factors such as genetic polymorphisms (Figure 1 [11]). We were not able to confirm this hypothesis in our matched case-control study concerning family history of premature cardiovascular disease in *chapter 5*; no increased prevalence was found in women with recurrent miscarriage. In *chapter 6* our findings do suggest shared genetic risk factors between recurrent miscarriage and cardiovascular disease. Several of the 16 genetic variants which remained significantly associated with recurrent miscarriage after meta-analysis, are also identified as risk factors for cardiovascular disease.

A higher prevalence of family history of ischemic heart disease and other atherosclerotic

disease (not premature) in women with recurrent miscarriage was found in two other, large cohort, studies [21, 22]. Limitations of our study are the relatively small number of included women with recurrent miscarriage (N=103) and the lack of information about the age of their parents. Some parents will not have reached the age of 60 years at time of questionnaire and are still at risk to develop premature myocardial infarction and/or stroke. In the same study (*chapter 5)* we find that women with recurrent miscarriage had more often hypertension at time of questionnaire. It was interesting to see this reflected in family history of hypertension as well, which seems more often present in women with recurrent miscarriage: OR 1.71 (95%CI 0.94-3.11). Based on our results we cannot rule out a shared genetic background between both conditions, and we conclude that the design of our research probably is not the best to investigate our hypothesis.

In *chapter 6* genetic variants associated with recurrent miscarriage are described of which several, are also identified as risk factors for cardiovascular disease. Most of them are involved in pathways concerning coagulation and fibrinolysis; factor II (prothrombin), factor V Leiden, FXIIIa Val34Leu, MTHFR A1298C, MTHFR C677T and PAI1 -675 4G/5G polymorphism [23-25], and NOS3 Glu298Asp polymorphism [26] is involved in oxidative stress and plays a crucial role in regulating endothelial function [27].

Heritable thrombophilia (defined by the presence of a factor II (prothrombin) gene mutation, factor V Leiden mutation, protein C or S deficiency or antithrombin deficiency) has been an important topic of research in women with recurrent miscarriage for years. It is included in the work-up to identify possible causes of recurrent miscarriage. A strong association exists between heritable thrombophilia and venous thrombosis[28]. Several meta-analyses have reported an increased risk of recurrent miscarriage in women with heritable thrombophilia; however, heterogeneity between studies is significant and the definition of recurrent miscarriage limits firm conclusions[29, 30]. Overall, heritable thrombophilia appears to be only a weak contributor to recurrent miscarriage (and therefore as well to the association between recurrent miscarriage and cardiovascular disease). In line with this is, at first; the lack of association between a family history of venous thrombosis and recurrent miscarriage (*chapter 5*) [21]. And second, the result of a meta-analysis of randomized controlled trials (including eight trials and 483 patients); no increased live birth rate was found with the use of low-molecular-weight heparin in women with recurrent miscarriage and heritable thrombophilia[31]. Suggesting no benefit of low-molecular-weight heparin in preventing recurrent miscarriage in women with inherited thrombophilia. Perhaps, the ongoing study; the Alife2 study, which is evaluating the efficacy of low-molecular-weight heparin on pregnancy outcome, in women (aged 18-42) with heritable thrombophilia and a history of two or more miscarriage and/or intra-uterine fetal death, adds information to this discussion in the future.

MTHFR A1298C and MTHFR C677T polymorphisms reduce the activity of the MTHFR enzyme, causing elevated concentration of homocysteine. Hyperhomocysteinemia is

related to recurrent miscarriage [32] and increases risk of cardiovascular disease. Despite this, we found lower values of homocysteine in women with a history of recurrent miscarriage compared to women with no miscarriage, which could be a confounding effect of the folate and vitamin B supplementation in the recurrent miscarriage group as described in *chapter 4*.

Meanwhile many new studies have been conducted regarding the association between genetic polymorphisms and recurrent miscarriage. Therefore, it could be helpful to repeat meta-analysis with newly added studies to find possible new involved pathways in the aetiology of recurrent miscarriage and their relationship with future cardiovascular disease. And moreover, genetic and more in depth genetic studies such as epigenetics in families with a heavy burden of both miscarriage and cardiovascular disease could help to identify a likely (possibly immunological) link between the two conditions, which could potentially lead to a better understanding of the underlying pathology and possibly new treatment options.

Novel cardiovascular biomarkers

In c*hapter 4* we focused on non- classical cardiovascular risk factors, the so called novel cardiovascular biomarkers, in women with recurrent miscarriage as knowledge about these markers might contribute to a better understanding of the association between recurrent miscarriage and cardiovascular disease. We found that women with recurrent miscarriage had significantly higher values of HsCRP and lower values of albumin and vitamin D compared to women with no miscarriage at time of follow-up. These cardiovascular biomarkers are involved in mechanisms regarding inflammation; indicating a proinflammatory state in women with recurrent miscarriage and will adversely affect a diverse range of endothelial homeostatic functions (Figure 1). No differences were found in more specific cardiovascular biomarkers, for example regarding renal function and myocardial damage.

Especially the decreased concentration of vitamin D is interesting. Vitamin D is inversely associated with cardiovascular disease risk [33] via several mechanisms; increased inflammation, endothelial dysfunction, elevated blood pressure, decreased insulin sensitivity and secretion, arterial stiffness and degradation of atherosclerotic plaque [34]. Over the last decade, the role of vitamin D in human reproduction has been increasingly considered as important. Adverse outcomes linked to vitamin D insufficiency in pregnancy includes pre-eclampsia, gestational diabetes, small-for-gestational age and preterm birth [35-37]. Many potential underlying mechanisms of vitamin D in regulating each of the outcomes are hypothesized, including that vitamin D could act as an immune regulator during implantation [38]. Research showed that trophoblasts produce and response to vitamin D in early pregnancy [39, 40]. Furthermore, it has been established that vitamin D influences local anti-inflammatory responses (via inhibition of TNF-alpha-induced inflammatory cytokines) and induces decidualization for success-

ful pregnancy[41-43]. We assessed vitamin D levels only at follow-up. In addition, it is possible that women with recurrent miscarriage already have decreased values of vitamin D during and/or before pregnancy. Only one study assessed vitamin D levels and immunological implications, such as presence of several autoantibodies and cytokine production, in women with recurrent miscarriage and found that a high proportion of women had a vitamin D deficiency (<30 ng/ml) with immunological implications [44]. With this knowledge, supplementation of vitamin D might be a potential treatment in women with recurrent miscarriage. At first, there is need for more observational studies to investigate the association between vitamin D and recurrent miscarriage (adjusting for ethnicity and seasonal variations). And secondly, if an association is established, intervention studies (randomized controlled trials) are needed to evaluate the effect of vitamin D supplementation on pregnancy outcome in women with recurrent miscarriage. Next to a possible treatment option in women with recurrent miscarriage, vitamin D might be useful in cardiovascular risk estimation. Vitamin D deficiency is a strong risk marker for cardiovascular disease[45]. In the prevention of cardiovascular disease, no significant and consistent protective effect of vitamin D supplementation was found in randomized controlled trials.

Recurrent miscarriage, other pregnancy complications and cardiovascular disease

In *chapter 7* we investigated whether women with secondary recurrent miscarriage had a more complicated first pregnancy compared to all Dutch nullipara. Approximately 40% of the women with recurrent miscarriage have a previous ongoing pregnancy and are as a consequence diagnosed with secondary recurrent miscarriage [46]. We found that women with secondary recurrent miscarriage had higher rates of post-term birth and perinatal death in their first ongoing pregnancy preceding recurrent miscarriage. Causes of perinatal death varied from intrauterine fetal demise, intrauterine growth restriction, placental abruption, preterm birth to congenital abnormalities. The higher rate of perinatal death supports the hypothesis that placental vasculopathy is an underlying pathophysiologic mechanism linking an array of pregnancy complications [8, 47]. Preeclampsia is also mentioned in this hypothesis; however, we did not find an increased risk of preeclampsia in the first ongoing pregnancy for women with secondary recurrent miscarriage, in contrast to the (larger) studies of Weintraub and Nielsen[48, 49]. Shared maternal risk factors for recurrent miscarriage and still birth, the single major determinant of perinatal death, are; maternal age ≥ 40 years, smoking, obesity and diabetes mellitus [50]. An association between increasing maternal BMI and post-term birth has also been described [51], although the mechanisms are not fully understood. These common risk factors are also (classical) risk factors for developing cardiovascular disease[52], contributing to endothelial dysfunction and linking recurrent miscarriage to cardiovascular disease.

In sum, (recurrent) miscarriage and cardiovascular disease are both diseases with a multifactorial aetiology. Previous mentioned mechanisms are involved in the association between both diseases. Although none of them gives a clear explanation on its own, and the contribution of the described mechanisms seems present but not substantial. It is supposed that a unifying mechanism between recurrent miscarriage and cardiovascular disease is the presence of endothelial dysfunction. Described mechanisms of shared classic cardiovascular risk factors, antiphospholipid syndrome, genetic polymorphisms and novel cardiovascular biomarkers involved in inflammation all contribute to this phenomenon. Genetic and immunologic etiology need further elucidation.
It remains unknown if women with recurrent miscarriage have an increased cardiovascular disease risk due to pre-existing common risk factors, due to the complicated pregnancies that leads to permanent (endothelial) damage, or, perhaps the most likely; a combination of these two (second hit) as illustrated in figure 2. For preeclampsia, in which the association with cardiovascular disease is well investigated, this discussion is still ongoing.

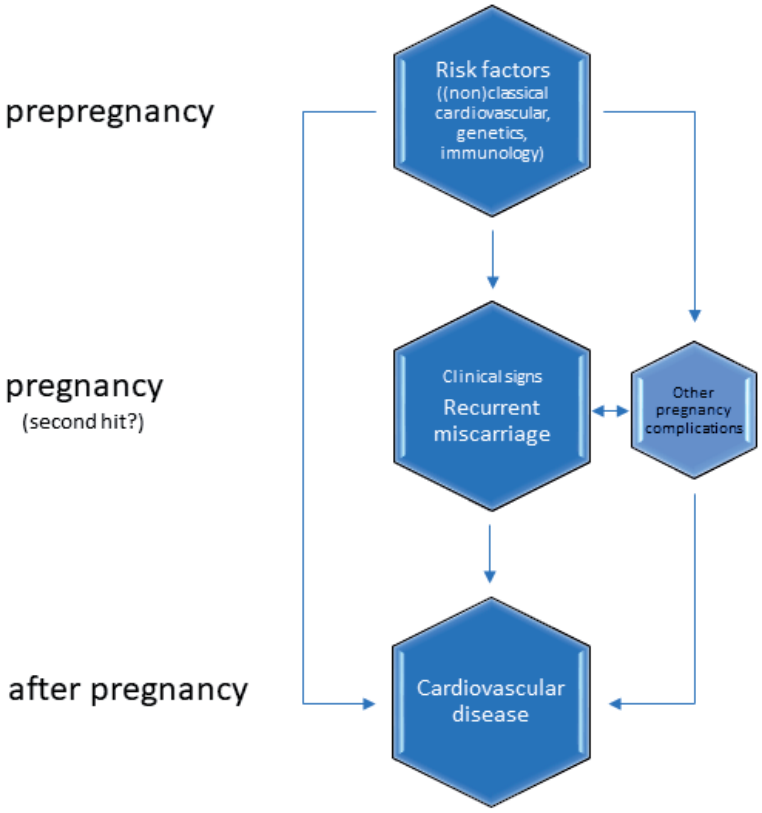

Figure 2. Incorporate (recurrent) miscarriage as a new risk factor in the algorithm for future cardiovascular disease

Definition of recurrent miscarriage

The variation in the definition of recurrent miscarriage, between two and three and consecutive versus non-consecutive miscarriages [53, 54] complicates the incorporation of recurrent miscarriage as a new risk factor for cardiovascular disease. Also, there is disagreement on gestational age at time of miscarriage; early or late, and whether detection of fetal heart activity by ultrasound is obliged or biochemical pregnancies are included as well. Generally, in the international literature, recurrent miscarriage is defined as three or more consecutive pregnancy losses before 22 weeks of gestation[55]. For this reason, we have chosen this definition setting up our research protocols. In the Netherlands, a woman is diagnosed with recurrent miscarriage after two pregnancy losses before 20 weeks of gestation, irrespective whether consecutive or not [53].

As this lack of consensus makes it difficult to compare study results between different centres, the ESHRE special interest group early pregnancy, published a consensus statement in 2015, during the writing of this thesis[54]. They recommend the term recurrent pregnancy loss be used to describe repeated pregnancy demise, and the term recurrent miscarriage be used when all pregnancy losses have been confirmed as intrauterine miscarriages, by ultrasound or histology. For this reason, we changed the term recurrent miscarriage into recurrent pregnancy loss in *chapter 7*, according to the comments of the reviewers, as we included also non-visualized miscarriages. In future research we recommend using this terminology as well [54]. In this thesis, we kept to our predefined definition and terminology of recurrent miscarriage.

Probably we should make a distinction between the most useful definition of recurrent miscarriage regarding aetiology and subsequent pregnancy prognosis on the one hand, and the most useful definition to point out a woman at future risk for cardiovascular disease on the other hand. In this thesis, we focus on the last mentioned. The results in *chapter 2* showed that women who have experienced two or more miscarriages, irrespective of whether consecutive or not, have an increased risk of ischemic heart disease. In subgroup analysis, the risk for ischemic heart disease was lower in women with consecutive miscarriages compared to women with two or more non-consecutive miscarriages. This suggests that the number of events (two or more) is more important than the consecutive nature of events.

Current guidelines

Given the world-wide health problem of cardiovascular disease in women, and its economic implications there is a strong rationale for the prevention of cardiovascular disease in women. More gender specific analyses have been published making it possible to do more definitive recommendations [52], Wilson and Jonner set up ten criteria to determine the suitability for establishing screening programs including for example; the condition should be an important health problem, there should be an accepted treatment recognized for the disease and diagnosis and treatment should be cost-effective [56].

In recent years, pregnancy complications are incorporated in guidelines as novel risk factors for cardiovascular disease in women. The 2011 guidelines for the prevention of cardiovascular disease of The American Heart Association indicate a history of gestational diabetes or hypertensive complications of pregnancy as a major risk factor for developing cardiovascular disease and advises monitoring and control of risk factors in these women postpartum [52]. In 2014, the first Dutch guideline on cardiovascular risk management after reproductive and pregnancy-related disorders was published [57]. This guideline states no increased risk for ischemic heart disease in women with recurrent miscarriage, RR1.99 (95%CI 0.94,4.19). However, only 3 cohort studies were included (up to 2012) and several studies were published afterwards, including a meta-analysis which found a significant increased risk (*chapter 2*) [1-4]. Optimization of modifiable cardiovascular risk factors, by giving lifestyle advices, is recommended to reduce the risk of future cardiovascular disease for all reproductive and pregnancy-related disorders mentioned in this guideline. Follow-up is only recommended for women with a history of preeclampsia, (relative risk CVD; 2.15 (95%CI 1.76–2.61). This includes that at the age of 50 years, women are offered a full cardiovascular risk profile performed according to the Dutch guideline for cardiovascular risk management. The most recent European Guidelines on cardiovascular disease prevention (2016) states that there is no data to suggest that recurrent pregnancy loss is associated with an increased cardiovascular disease risk [58]. They recommend that periodic screening (not further specified) for hypertension and diabetes mellitus should be considered in women with gestational diabetes and hypertensive complications of pregnancy.
Recently published data, including this thesis, suggest it is time to update these mentioned guidelines. As the hazard ratio for ischemic heart disease in women with two or more miscarriages is comparable with the hazard ratio for cardiovascular disease in women with hypertensive disorders of pregnancy [59] an equal approach for women with two or more miscarriages seems justified and I recommend to add a history of two or more miscarriage to the risk factors for cardiovascular disease.

Future perspectives
The important and growing field of the utilization of big data analysis in healthcare is very interesting. In the future, the increased use of statistical machine learning techniques will probably help us to interpret all variables involved in the association between two heterogeneous conditions such as miscarriage and cardiovascular disease. It may be helpful in teasing out subtle information from observational datasets and provide reliable interpretations for individualizing care decisions and personalized medicine.

With current knowledge, women with two or more miscarriage should be made aware of an increased cardiovascular risk and advised lifestyle advises including discontinuing smoking, improving dietary habits, healthy weight and adequate physical exercise

(reducing endothelial damage) to prevent cardiovascular disease events. Appropriate risk factor modifications can lower their risk for future cardiovascular disease. In women with a history of preeclampsia estimates showed that lifestyle interventions after this pregnancy complication have the potential to decrease cardiovascular risk by 4-13%[60]. Individual cardiovascular risk estimation in women with a history of two or more miscarriages should be considered. Evidence on costs and cost-effectiveness of cardiovascular screening in women with miscarriages is lacking. To start, a comparable approach, such as advised in the Dutch guideline on cardiovascular risk management in women with a history of preeclampsia can be applied. At the age of 50 years, performance of a full cardiovascular risk profile according to the Dutch guideline for cardiovascular risk management could be offered. Although this age limit is up for discussion. Evidence is suggesting that screening and prevention should be offered earlier in life to women with a complicated pregnancy. For example, the mean age of stroke onset was about 10 years earlier in women with a history of pregnancy complications (preeclampsia, HELLP syndrome and placental abruption), compared to women without such a history [61]. A suggested new approach is to start screening for cardiovascular risk factors postpartum and repeat it every 10 years. The ideal age of screening should be studied further.
Future research is needed to determine whether women with a history of two or more miscarriages will benefit from screening and preventive interventions. A cohort study with a long follow-up is suggested to evaluate the results of these life style advises and interventions.

Conclusions

In conclusion, given the consistent reporting of an association between miscarriages and later ischemic heart disease (and possibly other cardiovascular disease), it is time to update current guidelines and add a history of two or more miscarriages to the risk factors for cardiovascular disease.
Future studies should aim to determine how the inclusion of information on miscarriages (and other adverse pregnancy events) could improve cardiovascular disease risk evaluation in women.
Women with recurrent miscarriage must be made aware of their increased risk for cardiovascular disease later in life and given lifestyle advises. Individual cardiovascular risk estimation in women with a history of recurrent miscarriage must be implemented.

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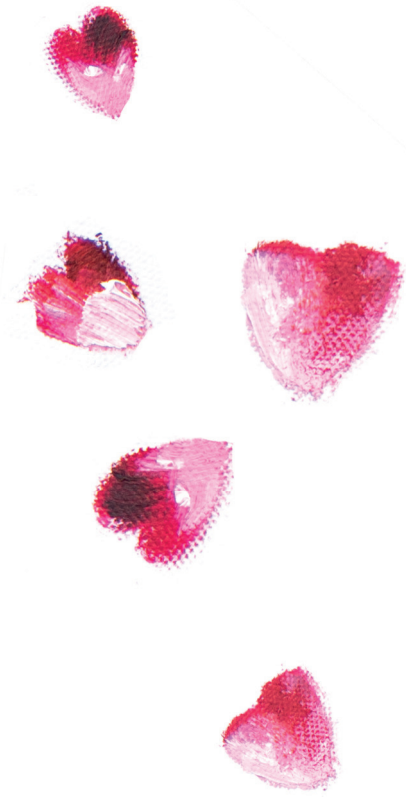

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English summary

Recurrent miscarriage and the subsequent risk of cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death in women in the Western world. There is increasing evidence that women with adverse pregnancy outcomes are at increased risk of CVD. A history of preeclampsia, gestational diabetes or pregnancy-induced hypertension is mentioned as an important factor for cardiovascular disease in women in the American Heart Association guideline. The association between recurrent miscarriage and CVD is less clear. Recurrent miscarriage is affecting 0.5-3% of the fertile couples and is commonly defined as three or more consecutive pregnancy losses before 22 weeks of gestation. It is a highly heterogeneous condition. Possible etiologic factors include genetic disorders, uterine anomalies, endocrine factors, maternal autoimmune disorders, metabolic disorders, maternal thrombophilia, obesity and toxic factors such as smoking and alcohol consumption. In about 50% of the cases the cause remains unknown. Recurrent miscarriage might be a first sign of subsequent CVD in women. Several hypotheses are possible for an association between both diseases; shared common risk factors such as obesity and smoking, endothelial dysfunction and a genetic predisposition is assumed. Early identification of women at increased risk of CVD from their reproductive history may enable them to benefit from screening and preventive interventions.
In this thesis we investigated the possible association between recurrent miscarriage and future cardiovascular disease.

Chapter 1 provides an introduction and outline of this thesis.
Chapter 2 reports a large retrospective cohort study with a long follow-up which assessed whether consecutive miscarriage is associated with an increased risk of cardiovascular disease later in life. Data from the Aberdeen Maternity and Neonatal Databank was used. 60105 women were analysed; 9419 with non-, 940 with two, 167 with three or more consecutive miscarriages and 49579 women with no miscarriage. Women were linked to Scottish Morbidity Records for hospital admissions for cardiovascular conditions, cardiac surgery and death registrations. A sensitivity analysis was performed dividing the women into those who had one, two or ≥ three miscarriages irrespective of these were consecutive or not. In the multivariate analyses (including maternal age, BMI, social class and smoking) a significant association was found between ischemic heart disease and women with two (HR 1.75 (95%CI 1.22-2.52) or ≥ three (HR 3.18 (95%CI 1.49-6.80) consecutive miscarriages. Similar patterns of risk were observed in the sensitivity analysis. The risk for ischemic heart disease was even slightly lower in women with consecutive miscarriages compared to women with 2 or more non-consecutive miscarriages. This suggests that the number of events (2 or more) is more important than the consecutive nature of events. We conclude that women with a history of two or more miscarriages, irrespective of whether consecutive or not, appear to have an increased risk of ischemic heart disease.

Chapter 3 describes a follow-up study which determined cardiovascular risk factors and predicted long term cardiovascular disease risk in women with a history of recurrent miscarriage. Women who visited the recurrent miscarriage clinic at Leiden University Medical Center (between 2000-2010) and had their third consecutive miscarriage before the age of 31 years of age were invited to participate (between 2012-2014). The reference group consisted of women with at least one uncomplicated pregnancy and no miscarriage, matched on zip code, age, and date of pregnancy. All women were invited for risk factor screening, including physical examination and blood collection. Main outcome measures were the (extrapolated) 10- and 30-year cardiovascular risk scores using the Framingham risk score. A sub analysis was performed for women with idiopathic recurrent miscarriage. 36 women were included in both groups, with a mean follow-up of 7.5 years. Women with recurrent miscarriage had a significantly higher extrapolated 10-year cardiovascular risk score (mean 6.24%, SD 5.44) compared to women with no miscarriage (mean 3.56%, SD 1.82, p=0.007) and a significantly higher 30-year cardiovascular risk score (mean 9.86%, SD 9.10) compared to women with no miscarriage (mean 6.39%, SD 4.20, p=0.04). Similar results were found in women with idiopathic recurrent miscarriage (n=28). Therefore, we concluded that women with a history of recurrent miscarriage differ in cardiovascular risk profile at young age compared to women with no miscarriage. The findings support an opportunity to identify women at risk of cardiovascular disease later in life and a possible moment for intervention.

Chapter 4 describes novel cardiovascular biomarkers in women with a history of recurrent miscarriage at time of follow-up. The inclusion criteria of the exposed (women with recurrent miscarriage) and unexposed (women with no miscarriage) women were already described in *chapter 3*. Biomarkers were assessed, regarding the following mechanisms; inflammation (HsCRP, Lp-PLA2), thrombosis (homocysteine, folate, anti-Cardiolipin antibodies and anti-ß-2-Glycoprotein antibodies), lipid metabolism (Lipoprotein (a)), renal function (creatinine, microalbuminuria), myocardial damage (NT-proBNP, hscTroponine T), multiple mechanisms (albumin, 25-OH-Vitamin D). Women with a history of recurrent miscarriage had a significantly higher HsCRP, and significantly lower values of albumin and vitamin D compared to women with no miscarriage which indicates a proinflammatory response in women with recurrent miscarriage. Inflammation plays an important pathogenic role in all stages of atherosclerosis and therefore in the development of cardiovascular disease. No differences were found in more specific biomarkers, for example regarding renal function and myocardial damage. Therefore, we conclude that routine screening of novel cardiovascular biomarkers on patient level is not warranted. Women with a history of recurrent miscarriage should be given healthy lifestyle advises. More research is needed regarding the association between recurrent miscarriage and vitamin D.

Chapter 5 presents a matched case control study to assess if a family history of cardiovascular disease was more prevalent in women with recurrent miscarriage. Is so,

this association indicates shared risk factors, including genetics. 103 women who visited the recurrent miscarriage clinic at Leiden University Medical Centre (between 2000-2014) and had their third consecutive miscarriage ≤ 35 years were included. 143 controls were included, women with at least one uncomplicated pregnancy and no miscarriage, matched on zip code, age and date of pregnancy. All women filled out a questionnaire. Family history of premature myocardial infarction and/or stroke was defined as having a first-degree relative with myocardial infarction and/or stroke < 60 years. Family history of cardiovascular disease as having a first-degree relative with myocardial infarction, stroke, hypertension or thrombosis, irrespective of age at event. We did not find an increased prevalence in women with recurrent miscarriage compared to women with no miscarriage. Therefore, our data does not confirm the assumption that a link exists between familiar cardiovascular disease and recurrent miscarriage when measured by proxy family history. A family history of hypertension seems more prevalent in women with recurrent miscarriage, although not significant (multivariate analysis: OR 1.71 (0.94-3.11)), which urge more investigation.

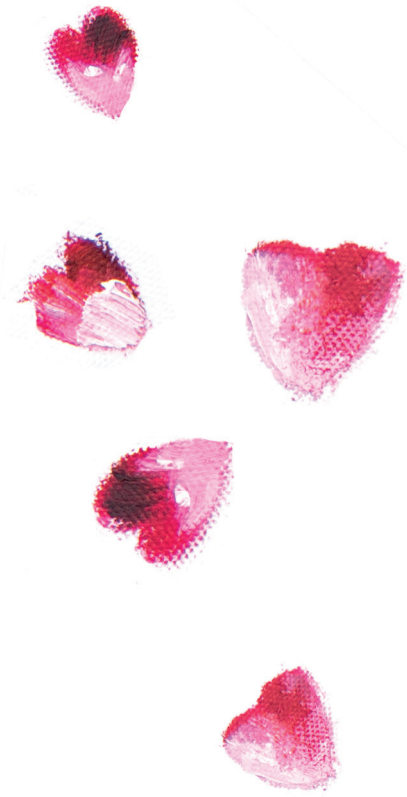
Chapter 6 presents a meta-analysis which describes genetic variants reproducibly associated with recurrent miscarriage. The association between genes and recurrent miscarriages was assessed at the allele level and a pooled odds ratio was estimated in a random-effects model. The literature search yielded 4050 articles; a total of 241 studies were included. We identified 25 reproduced genetic variants, of which 16 remained significantly associated with recurrent miscarriage in a random-effects meta-analysis. Several of these variants are also identified as risk factors for cardiovascular disease; F2 G20210A, FVL, FXIIIa Val34Leu, MTHFR A1298C, MTHFR C677T and PAI1 -675 4G/5G polymorphism, all involved in coagulation and fibrinolysis, and NOS3 Glu298Asp polymorphism, involved in oxidative stress. This meta-analysis suggests that recurrent miscarriage and cardiovascular disease share genetic risk factors.

Chapter 7 describes a retrospective cohort study which assessed whether women with secondary recurrent pregnancy loss (SRPL) (defined as having ≥ 3 consecutive miscarriages before 22 weeks of gestation, with a previous birth ≥ 22 weeks of gestation) (N=172) have a more complicated first pregnancy compared to all Dutch nullipara (N=1.196.178). Some women experience a complication during pregnancy, for instance pregnancy induced hypertension, preeclampsia, intrauterine growth restriction and pre-term delivery, events which may increase the risk of CVD later in life. Recurrent miscarriage might share pathophysiological pathways with other pregnancy complications. It is unclear whether these pregnancy complications are on the causal pathway between miscarriage and cardiovascular disease, they are possibly a confounding factor. Outcomes were the occurrence of preeclampsia, preterm birth, post-term birth, intrauterine growth restriction, breach position, induction of labor, delivery by Caesarean section, congenital abnormalities, perinatal death and severe hemorrhage in the first ongoing pregnancy. Subgroup analyses were performed for women with idiopathic SRPL and for women ≤ 35

years at first pregnancy. Women with SRPL more often had a post-term birth (OR 95%CI 1.86 (1.10-3.17)) and more perinatal deaths occurred in women with SRPL compared to the control group (OR 95%CI 5.03 (2.48-10.2)). Similar results were found in both subgroup analyses. More research is needed to reveal possible links between SRPL and these pregnancy complications as this might lead to a better understanding of the underlying pathophysiology.

Chapter 8 provides a general discussion and discusses remaining questions, suggestions for clinical implementation and future research. We conclude, that given the consistent reporting of an association between miscarriages and later ischemic heart disease (and possibly other CVD), it is time to update current guidelines and add a history of two miscarriages to the risk factors for CVD. Women with recurrent miscarriage should be made aware of their increased risk for cardiovascular disease later in life and given healthy lifestyle advises including discontinuing smoking, improving dietary habits, healthy weight and adequate physical exercise. Individual cardiovascular risk estimation in women with a history of recurrent miscarriage should be considered.

Future research is needed to determine whether women with a history of two or more miscarriages will benefit from screening and preventive interventions.



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Curriculum vitae

Publications

Acknowledgements

Het risico op hart– en vaatziekten na herhaalde miskramen

Hart- en vaatziekten (HVZ) zijn doodsoorzaak nummer 1 bij vrouwen in de westerse wereld. Er is toenemend bewijs dat vrouwen met gecompliceerde zwangerschappen een verhoogd risico hebben op het ontwikkelen van HVZ. In de richtlijn van de American Heart Association worden een voorgeschiedenis van preëclampsie, diabetes gravidarum en zwangerschapshypertensie genoemd als belangrijke risicofactoren voor HVZ. Of er ook een associatie bestaat met herhaalde miskramen is nog niet duidelijk. Herhaalde miskramen komen voor bij 0.5-3% van de fertiele koppels en wordt meestal gedefinieerd als drie of meer achtereenvolgende miskramen tot een amenorroeduur van 22 weken. Het is een erg heterogene aandoening. Mogelijke etiologische factoren zijn genetische afwijkingen, uterus anomalieën, endocriene factoren, maternale auto-immuun afwijkingen, metabole afwijkingen, maternale trombofilie, obesitas en toxische factoren zoals roken en alcohol gebruik. In meer dan 50% van de gevallen blijft de oorzaak onbekend. Herhaalde miskramen zouden een eerste teken van HVZ kunnen zijn. Voor het bestaan van een associatie tussen herhaalde miskramen en HVZ zijn verschillende hypotheses mogelijk; gedeelde risicofactoren zoals obesitas en roken, endotheel dysfunctie en een genetische predispositie worden verondersteld. Als we vrouwen met een verhoogd risico op HVZ op jonge leeftijd kunnen identificeren aan de hand van hun obstetrische voorgeschiedenis kunnen ze wellicht baat hebben bij screening en preventieve interventies. In dit proefschrift wordt de relatie tussen herhaalde miskramen en toekomstige hart- en vaatziekten onderzocht.

Hoofdstuk 1 is de algemene introductie van dit proefschrift.
Hoofdstuk 2 beschrijft een grote retrospectieve cohortstudie met een lange follow-up, waarin onderzocht wordt of er een verband bestaat tussen achtereenvolgende miskramen en een verhoogd risico op HVZ later in het leven. Data van de Aberdeen Maternity and Neonatal Databank werden gebruikt. 60105 vrouwen werden geanalyseerd; 9419 met geen, 940 met twee, 167 met drie of meer achtereenvolgende miskramen en 49579 vrouwen zonder miskraam. Vrouwen werden gelinkt aan de 'Scottish Morbidity Records' aangaande ziekenhuisopnames met betrekking tot cardiovasculaire aandoeningen, hartchirurgie en overlijdensregistraties. Een sensitiviteitsanalyse werd verricht door een onderverdeling te maken van vrouwen met een voorgeschiedenis van één, twee of ≥ drie miskramen, onafhankelijk of deze achtereenvolgend waren of niet. In de multivariate analyses (gecorrigeerd voor maternale leeftijd, BMI, sociale klasse en roken) werd een significante associatie gevonden tussen ischemische hartziekten en vrouwen met twee (HR 1.75 (95%CI 1.22-2.52) en ≥ drie (HR 3.18 (95%CI 1.49-6.80) achtereenvolgende miskramen. Vergelijkbare risicopatronen werden gevonden in de sensitiviteitsanalyse. Het risico op ischemische hartziekten was zelfs iets lager bij vrouwen met achtereenvolgende miskramen in vergelijking met vrouwen met twee of meer niet-achtereenvolgende

miskramen. Wat suggereert dat het aantal events (twee of meer) belangrijker is dan het achtereenvolgende karakter. We concluderen dat vrouwen met een voorgeschiedenis van twee of meer miskramen, onafhankelijk van of deze achtereenvolgend waren of niet, een verhoogd risico hebben op ischemische hartziekten.

Hoofdstuk 3 beschrijft een follow-up studie naar risicofactoren op HVZ bij vrouwen met een voorgeschiedenis van herhaalde miskramen. Tevens wordt aan de hand van deze risicofactoren een voorspelling gedaan op het risico op HVZ op de lange termijn. Vrouwen die de herhaalde miskramen poli van het Leids Universitair Medisch Centrum bezochten tussen 2000-2010 en een derde achtereenvolgende miskraam hadden doorgemaakt voor de leeftijd van 31 jaar werden uitgenodigd om deel te nemen (tussen 2012-2014). De referentiegroep bestond uit vrouwen die tenminste één ongecompliceerde zwangerschap en geen miskraam hadden doorgemaakt, gematcht op postcode, leeftijd en datum van zwangerschap. Alle vrouwen werden uitgenodigd voor een risicofactor screening, inclusief fysiek onderzoek en bloedafname. De belangrijkste uitkomstmaten waren de (geëxtrapoleerde) 10- en 30 jaar risicoscores op HVZ, gebruikmakend van de Framingham risicoscore. Een sub analyse werd verricht naar vrouwen met idiopathische herhaalde miskramen (miskramen waarbij geen oorzaak werd gevonden). In beide groepen werden 36 vrouwen geïncludeerd, met een gemiddelde follow-up van 7.5 jaar. Vrouwen met herhaalde miskramen hadden een significant hogere geëxtrapoleerde 10-jaar risicoscore op HVZ (gemiddeld 6.24%, SD 5.44) vergeleken met vrouwen zonder miskraam (gemiddeld 3.56%, SD 1.82, p=0.007) en een significant hogere 30-jaar risicoscore op HVZ (gemiddeld 9.86%, SD 9.10) vergeleken met vrouwen zonder miskraam (gemiddeld 6.39%, SD 4.20, p=0.04). Vergelijkbare resultaten werden gevonden bij vrouwen met idiopathische miskramen (n=28). Daaruit concluderen we dat vrouwen met een voorgeschiedenis van herhaalde miskramen al op jonge leeftijd een ongunstiger cardiovasculair risicoprofiel hebben in vergelijking met vrouwen zonder miskraam. Deze bevindingen ondersteunen de hypothese dat een voorgeschiedenis van herhaalde miskramen een mogelijkheid biedt om vrouwen met een verhoogd risico op HVZ te identificeren.

Hoofdstuk 4 beschrijft nieuwe cardiovasculaire biomarkers bij vrouwen met een voorgeschiedenis van herhaalde miskramen ten tijde van follow-up. De inclusiecriteria van de vrouwen met en zonder miskramen waren gelijk aan de inclusiecriteria van *hoofdstuk 3*. Er werden biomarkers bepaald met betrekking tot de volgende mechanismes: inflammatie (HsCRP, Lp-PLA2), trombose (homocysteïne, foliumzuur, anti-Cardiolipine antilichamen en anti-ß-2-Glycoproteine antilichamen), lipide metabolisme (Lipoproteïne (a)), nierfunctie (creatinine, microalbuminurie), myocard schade (NT-proBNP, hscTroponine T) en multipele mechanismes (albumine, 25-OH-Vitamine D). Vrouwen met een voorgeschiedenis van herhaalde miskramen hadden een significant hoger HsCRP, en significant lagere waardes van albumine en vitamine D vergeleken met vrouwen zonder miskraam, wat duidt op een pro inflammatie respons in vrouwen met herhaalde miskramen. Inflammatie speelt een belangrijke pathogene rol in alle

stadia van atherosclerose en daarmee de ontwikkeling van hart- en vaatziekten. In de meer specifieke biomarkers werd geen verschil gevonden, zoals bijvoorbeeld nierfunctie en myocard schade. We concluderen dat routine screening van nieuwe cardiovasculaire biomarkers voor de individuele patiënt niet gewenst is. Vrouwen met een voorgeschiedenis van herhaalde miskramen zouden adviezen ten aanzien van een gezonde leefstijl moeten krijgen. Meer onderzoek is nodig om de associatie tussen herhaalde miskramen en vitamine D te onderzoeken.

Hoofdstuk 5 beschrijft een gematchte casecontrole studie om te onderzoeken of HVZ vaker voorkomen in families van vrouwen met herhaalde miskramen, wat zou kunnen wijzen op gedeelde (genetische) risicofactoren. 103 vrouwen die de herhaalde miskramen poli van het Leids Universitair Medisch Centrum (tussen 2000-2014) bezochten en een derde achtereenvolgende miskraam hadden ≤ 35 jaar werden geïncludeerd. 143 controles werden geïncludeerd; dit waren vrouwen met tenminste één ongecompliceerde zwangerschap en geen miskraam, gematcht op postcode, maternale leeftijd en datum van zwangerschap. Alle vrouwen vulden een vragenlijst in. Een familieanamnese van een prematuur myocardinfarct en/ of herseninfarct was gedefinieerd als het hebben van een 1e-graads familielid met een myocardinfarct en/of herseninfarct < 60 jaar. Een familieanamnese van HVZ was gedefinieerd als het hebben van een 1e-graads familielid met myocardinfarct, herseninfarct, hypertensie en/of trombose, onafhankelijk van de leeftijd ten tijde van het event. Er werd geen verhoogde prevalentie gevonden in vrouwen met herhaalde miskramen vergeleken met vrouwen zonder miskraam. Onze data bevestigt de hypothese dat er een link bestaat tussen familiaire HVZ en herhaalde miskramen niet. Een familieanamnese van hypertensie lijkt wel vaker aanwezig in vrouwen met herhaalde miskramen, alhoewel niet significant (multivariate analyse: OR 1.71 (0.94-3.11)), wat meer onderzoek behoeft.

Hoofdstuk 6 beschrijft een meta-analyse naar genetische varianten geassocieerd met herhaalde miskramen. De associatie tussen de genen en herhaalde miskramen werd onderzocht op allel niveau en een samengevoegd odds ratio (OR) werd berekend in een random effects model. Het literatuuronderzoek bestond uit 4050 artikelen, een totaal van 241 studies werd geïncludeerd. Er konden 25 genetische varianten worden geïdentificeerd die ten minste twee keer geassocieerd waren met herhaalde miskramen, waarvan 16 significant geassocieerd bleven met herhaalde miskramen na de random effects meta-analyse. Verschillende van deze varianten zijn ook geïdentificeerd als risicofactoren op HVZ; F2 G20210A, FVL, FXIIIa Val34Leu, MTHFR A1298C, MTHFR C677T and PAI1 -675 4G/5G polymorfisme, allen betrokken bij coagulatie en fibrinolyse, en NOS3 Glu298Asp polymorfisme, betrokken bij oxidatieve stress. Deze meta-analyse suggereert dat herhaalde miskramen en HVZ genetische risicofactoren delen.

Hoofdstuk 7 beschrijft een retrospectieve cohortstudie naar complicaties in de eerste zwangerschap bij vrouwen met secundaire herhaalde miskramen (gedefinieerd als ≥ 3 achtereenvolgende miskramen met een amenorroeduur van < 22 weken, met een

voorafgaande zwangerschap met een amenorroeduur ≥ 22 weken) (N=172) vergeleken met alle Nederlandse nullipara (N=1.196.178). Sommige vrouwen krijgen een zwangerschapscomplicatie die het toekomstige risico op HVZ kan verhogen, zoals zwangerschapshypertensie, preëclampsie, intra-uteriene groeivertraging en/of vroeggeboorte. Mogelijk bestaan er gemeenschappelijke pathofysiologische paden tussen herhaalde miskramen en andere zwangerschapscomplicaties. Het is onduidelijk of deze complicaties zich bevinden op het causale pad tussen herhaalde miskramen en HVZ, of dat ze optreden als confounder. Om deze relatie verder te onderzoeken werden de volgende uitkomsten bekeken: het voorkomen van preëclampsie, vroeggeboorte, serotiniteit, intra-uteriene groeivertraging, stuitligging, inleiding van de bevalling, bevalling door middel van een sectio, congenitale afwijkingen, perinatale sterfte en hemorragie postpartum in de eerste doorgaande zwangerschap. Subgroep analyses werden verricht bij vrouwen met idiopathische secundaire herhaalde miskramen en bij vrouwen die ≤ 35 jaar waren ten tijde van de eerste zwangerschap. Serotiniteit (OR 1.86 (95%CI 1.10-3.17)) en perinatale sterfte (OR 5.03 (95%CI 2.48-10.2)) kwamen vaker voor bij vrouwen met secundaire herhaalde miskramen vergeleken met de controlegroep. Meer onderzoek is nodig om mogelijke verbanden tussen secundaire herhaalde miskramen en deze zwangerschapscomplicaties te ontdekken, omdat dit tot een beter begrip van de onderliggende pathofysiologie kan leiden.

Hoofdstuk 8 bevat een algemene discussie over de bevindingen in dit proefschrift, overblijvende vragen worden bediscussieerd en suggesties voor klinische implementatie en toekomstig onderzoek worden gegeven. Geconcludeerd wordt dat gegeven de consistente bevindingen van een associatie tussen miskramen en ischemische hartziekten (en mogelijk ook andere HVZ), het tijd is de huidige richtlijnen te updaten en een voorgeschiedenis van twee of meer miskramen als risicofactor op het ontwikkelen van HVZ toe te voegen. Vrouwen met herhaalde miskramen zouden geïnformeerd moeten worden over het verhoogde risico op het ontwikkelen van HVZ en leefstijladviezen moeten krijgen, zoals niet roken, verbeteren van dieetgewoontes, gezond gewicht en voldoende beweging. Het bepalen van een individueel cardiovasculair risicoprofiel bij vrouwen met een voorgeschiedenis van herhaalde miskramen zou overwogen moeten worden. Toekomstig onderzoek is nodig om te bepalen of vrouwen met een voorgeschiedenis van twee of meer miskramen baat hebben bij screening en preventieve interventies.

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Marise Wagner was born on January 30th 1986 in Burum. She graduated from secondary school at the Lauwers College Buitenpost in 2004. In the same year, she moved to Leiden to study at the medical faculty of the University of Leiden. During her scientific internship at the division of Fetal medicine (dr. J.M. Middeldorp), department of obstetrics at the Leiden University Medical Center, her enthusiasm for Obstetrics and Gynecology and for research was raised.

In 2010 she graduated (cum laude) from Medical School and after that she worked as a resident (ANIOS) at the Obstetrics and Gynecology department of Medical Center Haaglanden in The Hague (dr. M.J. Kagie). Simultaneously, she started in 2012 with the Recurrent Miscarriage (REMI) research project at the department of obstetrics, Leiden University Medical Center, supervised by prof. dr. K.W.M. Bloemenkamp and prof. dr. J.M.M. van Lith. Between 2013 and 2014 part of her research took place at the Dugald Baird Centre for Research on Women's Health at Aberdeen Maternity Hospital (dr. S. Bhattacharya).

In July 2015 she started her residency training in Obstetrics and Gynecology at the Medical Centre Leeuwarden (dr. L.P. Morssink). Since November 2017 she continued her training at the University Medical Centre Groningen (prof. dr. S. Scherjon and dr. M.H.M. Oonk).

Marise Wagner lives together with Jelmer Remmers, their son Tys (2014) and their daughter Jelka (2016) in Leeuwarden.

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