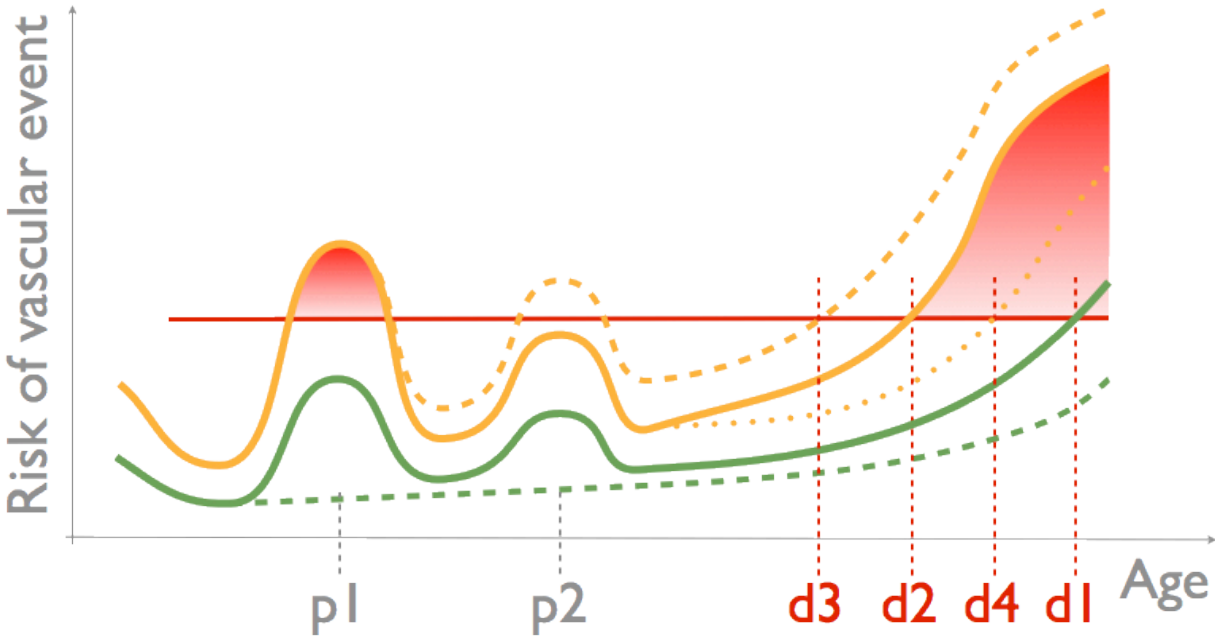
The Vascular Obstetrical Syndrome

– Pregnancy complications and cardiovascular susceptibility.

Doctoral Thesis



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Denne afhandling er i forbindelse med de nedenfor anførte offentliggjorte 9 artikler af Det Sundhedsvidenskabelige Fakultet ved Københavns Universitet antaget til offentligt at forsvares for den medicinske doktorgrad.

København, den 30. august 2013.

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Forsvaret finder sted fredag d. 6. december 2013 kl. 14.00 på Rigshospitalet i auditorium 1.

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The Vascular Obstetrical Syndrome

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1. udgave, 1. oplag, trykt i 50 exemplarer, udgivet oktober 2013.

ISBN: 978-87-996587-0-1

Trykkeri og sted: Hvidovre Hospital, Kettegård Alle 30, 2650 Hvidovre

Layout: Microsoft Word og Keynote for Macintosh.
Skrift: Times New Roman.

1. Contents

2. List of published articles

The present thesis is based on the following 9 articles.

I. Lykke JA, Langhoff-Roos J, Sibai BM, Funai EF, Triche EW, Paidas MJ.
Hypertensive Pregnancy Disorders and Subsequent Cardiovascular Morbidity and Type 2 Diabetes Mellitus in the Mother.
Hypertension 2009;53:944-51.

II. Lykke JA, Paidas MJ, Damm P, Triche EW, Kuczynski E, Langhoff-Roos J.
Preterm delivery and risk of subsequent cardiovascular morbidity and type-II diabetes in the mother.
British Journal of Obstetrics and Gynaecology 2010;117:274-81.

III. Lykke JA, Paidas MJ, Triche EW, Langhoff-Roos J.
Fetal growth and later maternal death, cardiovascular disease and diabetes.
Acta Obstetricia et Gynecologica Scandinavica 2012;91:503-10.

IV. Lykke JA, Langhoff-Roos J, Lockwood CJ, Triche EW, Paidas MJ.
Mortality of mothers from cardiovascular and non-cardiovascular causes following pregnancy complications in first delivery.
Paediatric and Perinatal Epidemiology 2010;24:323-30.

V. Lykke JA, Paidas MJ, Langhoff-Roos J.
Recurring Complications in Second Pregnancy.
Obstetrics & Gynecology 2009;113:1217-24.

VI. Lykke JA, Dideriksen KL, Lidegaard Ø, Langhoff-Roos J.
First-Trimester Vaginal Bleeding and Complications Later in Pregnancy.
Obstetrics & Gynecology 2010;115:935-44.

VII. Lykke JA, Langhoff-Roos J.
First trimester bleeding and maternal cardiovascular morbidity.
European Journal of Obstetrics & Gynecology and Reproductive Biology 2012;164:138-41

VIII. Lykke JA, Bare LA, Olsen J, Lagier R, Arellano A, Tong C, Paidas MJ, Langhoff-Roos J.
Thrombophilias and adverse pregnancy outcomes: results from the Danish National Birth Cohort.
Journal of Thrombosis and Haemostasis 2012;10:1320-5.

IX. Lykke JA, Bare LA, Olsen J, Lagier R, Arellano A, Tong C, Paidas MJ, Langhoff-Roos J.
Vascular associated gene variants in patients with preeclampsia: results from the Danish National Birth Cohort.
Acta Obstetricia et Gynecologica Scandinavica 2012;91:1053-60.

3. Preface

"Pregnancy is the stress test of life". This mantra has been repeated many times during the last decade, and still echoes through scientific circles.[1,2] I became aware of this hypothesis during my early years in residency and it corresponded well to my clinical observations on how pregnancy complications do seem to strike twice and in seemingly susceptible women. These women are now more and more believed to be at an increased risk of health problems during and immediately after pregnancy as well as in later life, where they may encounter manifest diseases of metabolic or cardiovascular origin that ultimately may result in early and untimely death.

The present thesis represents thoughts and reflections on my scientific work carried out from 2007 until 2012.[3-11] During this period, I commenced my residency at Rigshospitalet, the University Hospital in Copenhagen, and was privileged to be recruited to a research group devoted to exploring the link between pregnancy complications and subsequent cardiovascular disease. Later, I was invited to Yale University as a postdoctoral associate to pursue my scientific research and learning.

My one-year stay at Yale allowed me to enhance my understanding of the physiology of pregnancy, pathophysiology of complications as well as research methodology. In the course of this year, epidemiology, statistical science and programming became subjects in themselves, not only mere tools.

I wish to express warm and sincere gratitude to my mentor, Dr. Jens Langhoff-Roos, who supported and encouraged me to explore opportunities and learn from my errors. It has been inspiring, frustrating, and flourishing. And thanks to Prof. Michael J. Paidas for inviting me to Yale University and giving me a unique opportunity to join a dedicated and collaborating scientific group, which has been of fundamental importance to my work. Also thanks to all of my co-authors who have contributed exceptionally to these articles and enriched my scientific life by discussions of the methodology, the interpretation of the results, and teaching me the art of scientific writing.

This thesis is cordially and humbly dedicated to the patience of my wife and children. In the late hours after clinical work, in the neglected weekends, and in the missed family holidays, you witnessed my struggle and you comforted me. I would never have succeeded without you – thank you, Nana!

Copenhagen, December 2012
Jacob

So if you find nothing in the corridors open the doors,
if you find nothing behind these doors there are more floors,
and if you find nothing up there, don't worry, just leap up another flight of stairs.
As long as you don't stop climbing, the stairs won't end,
under your climbing feet they will go on growing upwards.
– Franz Kafka, "Advocates"

4. Summary

4.1. English

Pregnancy poses large demands and challenges the woman's physiology. In some women, the adaptation to these challenges is insufficient and symptoms of pregnancy complications can emerge; these symptoms can be part of complications such as preeclampsia, preterm delivery and fetal growth restriction and may appear in either mild or severe forms. The complications have partly shared pathophysiology associated with poor implantation, which possibly also links early vaginal bleeding to these disorders.

In seven cohort studies and two nested case-cohort studies we investigated the recurrence risk of complications in second pregnancy, the risk of later cardiovascular disease and diabetes mellitus following pregnancy complications, and vascular related genetic variants and thrombophilias, including factor V Leiden G1691A, prothrombin mutation G20210A, and methylhydrofolate reductase C677T, in respect to the pregnancy complications.

For the cohort studies, we used a national census based on the Danish National Patient Registry. We extracted data on n=782,287 women with a first singleton delivery, and n=536,419 women with a second singleton delivery. For the nested case-cohort studies, we used the Danish National Birth Cohort, including a separate national biobank (n=2,032 cases and 1,856 controls).

We found that preeclampsia in first pregnancy increased the risk of recurrence in the second by more than 7-fold corresponding to an overall risk of recurrence of 16%. Furthermore, a strong grading effect was present in that women with preeclampsia at gestational age 28 to 31 weeks had a 32% risk of recurrence. Also, the disorders correlated with each other within and across pregnancies by this grading effect, so preeclampsia at gestational age 28 to 31 weeks increased the risk of fetal growth restriction from 3% to 17%. Fetal growth restriction in first delivery increased the risk of recurrence in second from 1.5% to more than 19%, and also increased the risk of preterm delivery from 2.7% to more than 7%. Early vaginal bleeding operated not only as a marker of complications in same pregnancy, increasing the risk of preterm delivery from 5% to 9% and placental abruption from 1.0% to 1.4%, but also carried risk to the next pregnancy by increasing the risk of preterm delivery from 3.5% to 5.8%.

In the follow-up studies we accrued a median of 14.6 years yielding an average age at censoring of 42 years. Preeclampsia increased the risk of later hypertension by 4-fold from 2% to 8% and for preterm preeclampsia 8-fold to 12%. Fetal growth restriction doubled the risk of later ischaemic heart disease from 1.0% to more than 1.9%. Preterm delivery increased the risk of later diabetes mellitus from 0.8% to 1.5-2.0%. Pre-pregnancy cardiovascular disease doubled the risk of early bleeding (OR 2.28; 95% CI 1.27-4.11), and bleeding increased the risk of later ischaemic heart disease by 60% (HR 1.60; 95% CI 1.41-1.82).

In the genetic studies, we found that factor V Leiden increased the risk of a composite of pregnancy complications by 41% (95% CI 1.13-1.76) and severe preeclampsia by 63% (95% CI 1.10-2.42); other polymorphisms were also associated with severe preeclampsia but high risk of false positive findings necessitates replication.

In conclusion, more than 15% of women with at least one delivery have severe pregnancy complications. These complications tend to recur in a subsequent pregnancy and also increase the risk of other complications. Also, they disclose an increased vascular and metabolic susceptibility, which may lead to subsequent cardiovascular disease and diabetes. We propose to label this phenomenon the "Vascular Obstetrical Syndrome". Future studies should investigate if women with severe pregnancy complications should be examined and counselled as they may benefit from lifestyle changes and possibly also medical prophylaxis – especially if they have additional risk factors such as specific genes or polymorphisms that indicate thrombophilia.

4.2. Danish

En graviditet stiller store krav og udfordringer til kvindens fysiologi. Hos nogle kvinder er tilpasningen til disse udfordringer utilstrækkelig, og symptomer på graviditetskomplikationer kan opstå. Disse symptomer kan være en del af komplikationer såsom svangerskabsforgifting, præmatur fødsel og føtal væksthæmning, og kan forekomme i enten milde eller svære former. Komplikationerne har delvist samme fysiologi forbundet ved dårlig implantation i den tidlige graviditet, der muligvis også forbinder vaginalblødning til disse komplikationer.

I syv kohortestudier og to 'nested' case-cohort studier undersøgte vi recidiv risiko for komplikationer i anden graviditet, risikoen for senere hjerte-kar-sygdomme og sukkersyge efter graviditetskomplikationer, og vaskulære relaterede genetiske varianter og trombofilier, herunder faktor V Leiden G1691A, prothrombin mutation G20210A og methylhydrofolate reduktase C677T, i forhold til graviditetskomplikationer.

I kohortestudierne brugte vi en komplet national kohorte baseret på det danske Landspatientregister. Vi havde data på n = 782287 kvinder med en første singleton fødsel, og n = 536419 kvinder med yderligere en singleton fødsel mere. For de 'nested' case-kohorte studier, brugte vi "Bedre Sundhed for Moder og Barn" kohorten og tilhørende blodprøver lagret i Statens Serum Institut (n = 2032 cases og 1856 kontroller).

Vi fandt, at svangerskabsforgiftning i første graviditet øgede risikoen for recidiv i anden graviditet mere end 7 gange, svarende til en samlet risiko på 16%. Desuden fandt vi en stærk gradueringseffekt således, at kvinder med svangerskabsforgiftning ved 28-31 uger havde en 32% risiko for recidiv. Endvidere var komplikationerne korreleret med hinanden indenfor og på tværs af graviditeterne ved denne gradueringsseffekt: Således øgede svangerskabsforgiftning ved 28-31 uger risiko for føtal væksthæmning fra 3% til 17% i næste graviditet. Fosterets vækstbegrænsning i første fødsel, øget risiko for tilbagefald i anden fra 1.5% til mere end 19%, og også øget risiko for præterm fødsel fra 2.7% til mere end 7%. Tidlig vaginalblødning øgede risikoen for præmatur fødsel i samme graviditet fra 5% til 9% og moderkageløsning fra 1.0% til 1.4%, men samtidig blev risikoen også overført til næste graviditet ved at øge risikoen for præterm fødsel fra 3.5% til 5.8%.

I follow-up studierne fik vi en gennemsnitlig opfølgningstid på 14.6 år og en alder på 42 år ved sygdom eller censurering. Svangerskabsforgiftning omkring terminen øgede risikoen for senere forhøjet blodtryk 4 gange fra 2% til 8%, og ved svangerskabsforgiftning før 37 uger blev risikoen øget 8 gange til 12%. Føtal væksthæmning fordoblede risikoen for senere iskæmisk hjertesygdom fra 1.0% til mere end 1.9%. Præmatur fødsel før 37 uger øgede risikoen for senere sukkersyge fra 0.8% til 1.5%-2.0%, men dette var primært pga. andre komplikationer. Hjertekarsygdom før graviditeten fordoblede risikoen for tidlig vaginal blødning (OR 2.28; 95% CI 1.27-4.11), og tidlig blødning øgede risikoen for senere iskæmisk hjertesygdom med 60% (HR 1.60; 95% CI 1.41-1.82).

I de genetiske studier fandt vi, at faktor V Leiden øgede risikoen for en eller flere komplikationer med 41% (OR 1.41; 95% CI 1.13-1.76) og alvorlig svangerskabsforgiftning med 63% (OR 1.63; 95% CI 1.10-2.42). Andre genetiske variationer var også forbundet med svær svangerskabsforgiftning, men høj risiko for falsk positive fund kræver gentagelse af disse fund i andre populationer.

Samlet set har mere end 15% af kvinder med mindst én fødsel alvorlige graviditetskomplikationer. Disse komplikationer har tendens til at komme igen i en efterfølgende graviditet og også øge risikoen for andre komplikationer, ligesom at de synes at vidne om en øget vaskulær og metabolisk modtagelighed, hvilket kan føre til efterfølgende hjertekarsygdom og sukkersyge. Vi foreslår at navngive dette fænomen det "Vaskulære Obstetriske Syndrom". Fremtidige studier må undersøge om disse kvinder bør rådgives, da de kan drage fordel af livsstilsændringer og muligvis også medicinsk profylakse – især hvis de har yderligere risikofaktorer såsom bestemte gener eller polymorfismer, der viser trombofili.

5. Introduction

Pregnancy is a unique period in every woman's life. It is a period characterized by vast maternal physiological adaptations to increasing demands by the growing fetus and placenta. Successful implantation is a prerequisite for successful pregnancy. Trophoblast invasion and early hemochoroidal placentation have two main goals: to evade the maternal immune system and establish a sufficient and abundant maternal blood supply. Indeed, the placenta can be regarded as a semi-allograft facing the perils of rejection in a host-versus-graft reaction,[12-14] or an evolutionary tug of war between the mother and fetus struggling for resources.[15] This process may result in miscarriage or later pregnancy complications if the implantation and the maternal adaptation are insufficient in extent and degree.[16] The implantation process is so to speak "laying the blue print" of the pregnancy.[17] However, not all of the course of pregnancy can be predicted at this early stage.

Parallel to the development of the placenta and fetus, the maternal physiology comes under considerable and steadily increasing stress. The woman adapts to steadily advancing metabolic and circulatory demands by appropriate changes in physiology and metabolism throughout pregnancy.[18,19] Some women are not able to fully accommodate to this stress and present signs and symptoms of decompensation in specific organ systems. The severity of these symptoms spans a spectrum from modest adaptation challenges with which the body can cope, to severe disorders that endanger the health and even life of both mother and fetus. These symptoms, or complications, may be either maternal or fetal, and they may be mediated by placental changes since the placenta occupies genetically and physiologically a middle-position between mother and fetus. This also means that there can be a strong overlap between different pregnancy complications.

These complications have different, yet also in many ways shared risk factors and common aetiology, which relate to dysfunctional changes in the vascular system. Several concepts that integrate and describe these disorders have been proposed with names such as maternal placental syndrome,[20] ischaemic placental disease,[21] placenta associated syndrome,[7] and great obstetrical syndromes.[22] The core element in all these concepts is a joint metabolic and vascular syndrome emerging in pregnancy and later in life; we propose to merge these concepts into one "vascular obstetrical syndrome".

The consequences of these pregnancy complications for the fetus/offspring can be considerable as exemplified by preterm delivery and risk of cerebral palsy.[23] Other much more long-term consequences are now being elucidated such as those alterations resulting from epigenetic changes conditioned *in utero*: this has been extensively covered in the seminal "Developmental Origin of Adult Health and Disease" (DOHaD) hypothesis,[24] which also frames the evolutionary nature of these pregnancy complications.[15] In this thesis, the focus will be on the association between severe pregnancy complications and subsequent cardiovascular morbidity in the mother.

5.1. Pathophysiology

Several hypotheses have been evolved in the scientific history of pregnancy complications: The focal point, however, is the triad consisting of the development of the placenta, the maternal reaction to pregnancy, and the link between these "two stages" (Figure 1).[25] Depending on the weight of these elements, various forms or presentations of pregnancy complications can arise.

5.1.1. Immune maladaptation

Starting with implantation, the "immune maladaptation hypothesis"[26] proposes that the maternal immune system reacts to fetal/paternal antigens, and that the balance of antigen presentation is critical. Either lack of antigen expression (especially in the HLA-G system) on the surface of the syncytiotrophoblast[27,28] or excessive expression of "unknown" antigens[29] can provoke a maternal

immune response against the invading syncytiotrophoblast. Recently, the emphasis in this hypothesis has shifted to the maternal peripheral regulatory T cells (pTreg), which dampen the immune system; these cells are pivotal in the adaptation process leading to immune tolerance, and in an evolutionary context vital to the class of placental mammals.[30,31]

This hypothesis has been tested in epidemiological settings several times. In general there seems to be a protective effect of pre-pregnancy semen (paternal antigens) exposure, previous full term pregnancy, and short inter-pregnancy interval[32] supporting the concept of an adaptive immune response in pregnancy that induces tolerance after exposure.

5.1.2. Placental development

The lack of immune tolerance, evident in the following placental inflammatory processes[33] and uterine NK-cell activity,[34] can lead to shallow invasion of the extravillous trophoblast, which is associated with impaired re-modelling of the spiral arteries compromising placental perfusion and development.[35] The latter has been associated with pregnancy disorders, such as preeclampsia, inadequate fetal growth,[36,37] fetal demise,[22] and preterm birth[38,39] A strong correlation with each of these disorders has been observed and has been linked to placental bed pathology.[40]

Microthrombi formation in the vascular placental bed[41] together with deposition of fibrin in the villi[36] and acute atherosclerosis[42] can further impair the function of the spiral artery system that feeds the placental intervillous space.[22,43] This hampered development may result in a relative hypoxic state, – or intermediating ischemia and reperfusion –, in the placental tissue, hereby leading to excessive oxidative stress.

5.1.3. "Missing link"

The link or signal between the early implantation deficiency and later pregnancy disorders has been investigated intensively; this may very well be the Grail in preeclampsia. Oxidative stress is likely to be a key signal, and several substances contributing to this are of importance: The "angiogenic imbalance" has attracted considerable attention in recent years,[44-46] and may be detectable as early as in the 1st trimester.[47-49] The angiogenic factors include vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), and the anti-angiogenic factors soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sEng); the latter anti-angiogenic factors are increased in early in pregnancies complicated by later preeclampsia. An imbalance of these substances is likely to be induced by placental chronic hypoxia,[45,46,50] by endothelial inflammation and by tissue damage in the placental villous system,[51-53] which can be observed in the placentas from complicated pregnancies.[51] The primary stimulus, though, is still unresolved.

VEGF is important in maintaining endothelial function, and its soluble receptor, sFlt-1, can obstruct this maintenance, leading to a dysfunctional endothelial state.[54] This includes increased responsiveness towards circulating pressor agents like norepinephrine and angiotensin II, but also decreased secretion of prostaglandin I2, which functions as a potent vasodilator.

5.1.4. Maternal constitution

The maternal genes, phenotypes, and lifestyle preferences can either facilitate or impede a maternal systemic reaction to the adaptations in pregnancy. Family history of cardiovascular disease,[55] personal obesity, sedentary lifestyle, and smoking are some of the factors that modulate the maternal reaction.

Obesity has been implicated in many pregnancy complications,[56] and these associations may reflect processes of an underlying metabolic syndrome.[57] Indeed, many of the individual components of the metabolic syndrome are associated with preeclampsia[58-60] and preterm delivery,[60,61] although not fetal growth restriction.[62]

Smoking has also been linked to poor pregnancy outcome including poor fetal growth, placental abruption, stillbirth,[63] birth defects,[64] and even altered vascular function in the offspring.[65]

However, a protective effect on the risk of developing preeclampsia has also been observed in numerous studies – a finding labelled the "smoking paradox"[66] – which may be due to inhibition of the anti-angiogenic factors.[67]

5.1.5. Haemostatic system

Throughout pregnancy the haemostatic system undergoes considerable changes.[68] From a normal quiescent and balanced state, the activity of the pro- and anti-coagulative systems as well as the fibrinolytic system increase in pregnancy leading to a state of balanced hypercoagulability, which also includes the activity of the platelets.[69,70] Following this, an increased risk of venous thromboembolism in pregnancy is evident.[71]

Locally, the amnion and decidua have distinct homeostatic properties in maintenance of the local uterine haemostasis.[72] This balance may be disrupted by bleeding in the placental bed (clinically recognized by vaginal bleeding), which can provoke and initiate the coagulation cascade leading to thrombin burst; thrombin formation has been linked to later preterm delivery.[73]

5.1.6. "Five paths"

The aetiological pathways to the development of clinical complications are diverse. However, we propose to divide them into "five paths": 1) immunological/inflammatory, 2) placental/throphoblast, 3) constitutional/genetic, 4) endothelial, and 5) haemostatic. These five paths are neither mutually exclusive nor all inclusive, but rather conceptions to ease the understanding of the diversity of aetiologies (Figure 1).

Each path can contribute to the development of pregnancy complications, and the seemingly same clinical complication in two women may have different proportions of these paths illustrating the great heterogeneity in the complications. Also, no element neither is necessary to cause symptoms nor is any one element sufficient to cause symptoms of pregnancy complications.

The interactions and overlap between them are important to recognize: The over-activated haemostatic system interacts with the endothelium, which releases various factors involved in both promoting and inhibiting coagulation,[74] and the maternal constitution will modulate these processes.

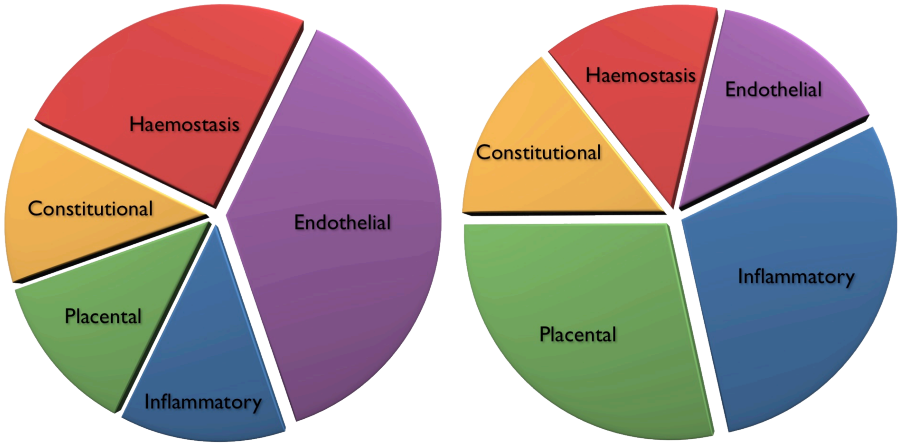

Figure 1. The aggregated five paths. Different proportions of the five paths, that aggregated are sufficient to provoke a pregnancy disorder, will vary from complication to complication. In the left example, the endothelial path is dominating; in the right, the inflammatory and placental paths are dominating.

5.2. Pregnancy complications

The five paths will be more or less pronounced in each pregnancy complication. These complications have shared yet also distinct paths compared to each other.

5.2.1. Preeclampsia

Through the last decades many theories have come in play to explain the complexities in this syndrome, labelling preeclampsia as "the disease of theories". The central feature of the pathophysiology in preeclampsia involves endothelial dysfunction.[75] The stimulus may, however, stem from placental signals and the over-activated coagulation system may enhance the effects of this stimulus further.[76-78]

The symptoms of preeclampsia also compass the endothelium: The hypertension is a result of vasoconstriction,[78] and proteinuria a result of damaged endothelium and podocytes in the glomerular apparatus.[79] Besides the obligate symptoms, other features are now being recognized as part of the syndrome, such as atherosclerosis in the placental vessels[42] and in the maternal systemic arteries,[80] as well as platelet activation and increased risk of thromboembolism in pregnancy.[81] Also, in women with pre-existing hypertension the risk of developing preeclampsia is increased to about 20% and confers a marked risk of poor pregnancy outcome including fetal growth restriction.[82,83]

"Imitators of preeclampsia"[84] and emerging trends of non-classical forms of preeclampsia are perhaps together with the 'upstream' anti-angiogenic markers[76] turning the tide in the definition and concept of preeclampsia.

5.2.2. Fetal growth restriction

In fetal growth restriction the maternal systemic reaction is less pronounced, but the uterine blood flow may be hindered. This is evident in some cases where notch in the uterine artery blood flow pattern assessed by Doppler ultrasound is present. This may be due to microthromboses in the placental bed, local activated platelets,[85] contracted proximate spiral arteries, or maternal vascular dysfunction leading to impaired vasodilatation and uterine flow.[86] Small studies suggest, that this impairment can be reversed by sildenafil (Viagra®), which greatly improves early-onset fetal growth restriction,[87] and normalized the uterine artery notch and Doppler flow profile.[88]

Placental insulin growth factor (IGF) 1 and 2 are important in regulation of fetal growth[89] as well as preeclampsia,[90] and several other hormones act via this IGF-system, such as pregnancy-associated plasma protein-A (PAPP-A).[91] These hormones act as paracrine substances to enhance the placental development. In fetal growth restriction, these actions are hindered.[92]

In this respect, there is much in common with preeclampsia in the placental path, but in fetal growth restriction the endothelial path may be localized to the placenta.[62]

5.2.3. Preterm delivery

Normal parturition is a comprehensive and elaborate event.[93] Preterm birth is not merely the same processes starting prematurely,[94] and many factors and biochemical pathways have been described in the syndrome of preterm birth: infection or inflammation, uteroplacental ischemia or haemorrhage, uterine over-distension, and maternal and fetal stress.[73,95]

Following such ischaemia, the local production of thrombin is increased, which is a potent stimulus of contractility in the myometrium.[96] The thrombin production is further enhanced by tissue factor, which is strongly expressed in the decidual cells.[72] Also, serum tissue factor is elevated in women with preterm rupture of membranes (PPROM)[97] suggesting that systemic endothelial and haematological paths are operative in preterm birth.

In pregnancy, the whole coagulation system is shifted towards an over-activated thrombotic state producing excessive amounts of thrombin and anti-thrombin (TAT) complexes as well as fibrinolytic degradation products.[68] Thrombin has direct degenerative effects on the amnion membrane,[98] and decidual haemostasis is essential for maintaining normal pregnancy.[73] Indeed, first trimester levels of TAT and D-dimer has been associated with spontaneous preterm birth.[99] As such, the haemostatic paths may be pronounced in preterm delivery.

5.2.4. Bleeding in early pregnancy

During implantation and decidualization, local haemostatic factors in the uterus, such as tissue factor expressed in cytotrophoblasts,[100] seem to play distinct orchestrated parts in the establishment of a successful pregnancy; dysfunction of any of these factors could initiate a cascade disrupting this equilibrium with the result of complications later in the pregnancy or miscarriage.[17]

Bleeding in early pregnancy will generate local thrombin formation, and this will stimulate production of the anti-angiogenic factor sFlt-1,[101] which can initiate a maternal endothelial path to later pregnancy as well as perpetuate the defective placentation. Also, early in pregnancy thrombin exposure can change the expression of various regulatory HOX genes in the decidual cells essential for the maintenance of pregnancy hereby linking bleeding to later miscarriage[102] and to later preterm delivery.[103]

The formation of local thrombin and soluble fms-like tyrosine kinase-1 have both been implicated in the development of placental abruption and preeclampsia,[100] and early vaginal bleeding has been linked to systemic thrombin-antithrombin (TAT) generation.[104] In addition, plasma protein Z, an indirect inhibitor of activated factor X, is lower in women experiencing bleeding, and even lower in women experiencing bleeding and a following preterm delivery.[105] Early vaginal bleeding is thus involved in the haemostatic and placental path leading to later complications.

5.3. Genetics

The genetic contribution to cardiovascular disease has been studied for many years starting in 1973 with the discovery of mutations in the low density lipoprotein (LDL) receptor in high-risk families.[106]

In obstetrics, the considerable recurrence risk in women with preeclampsia in their first pregnancy,[7] and the aggregation of preeclampsia in families suggest moderate heritability and penetrance.[107,108] Also, studies using quantitative genetics have estimated that up to 60% of the variation in birth weight can be attributed to fetal genes in contrast to environmental factors.[109] In addition, cardiovascular disease in the woman's family will increase her risk of any pregnancy disorders.[55,110] These findings support genetic elements in pregnancy disorders, which may be shared with cardiovascular diseases.[111,112]

5.3.1. Common or rare variants

The "common disease – rare variant" hypothesis suggests that few variants with low frequency and high penetrance are major disease culprits.[113] Opposed to this is the "common disease – common variant" hypothesis, that suggests that many variants with high frequency but low penetrance, are in an aggregated way major contributors to most of the common complex diseases.[114] These two hypotheses are now driving the search for polymorphisms involved in pregnancy disorders, but with different strategies: candidate gene studies and genome-wide association studies (GWAS).[115] Both of these strategies face different methodological problems but also offer various advantages.[116-118] The candidate gene approach involves a biological *a priori* hypothesis, which is then tested; the GWAS is hypothesis free.[118]

The candidate gene studies are exemplified in the investigation of inherited thrombophilias and genes related to angiotensinogen.[119] The majority of the investigated polymorphisms in obstetrics are related to vascular mechanisms in preeclampsia,[111] and to inflammatory pathways in preterm birth.[120] However, few of the polymorphisms have proven valuable in a clinical setting, but the knowledge gained may provide insights to explore new translational fields and to use proteomics for the mapping of pathophysiologic pathways.

The GWAS are emerging in obstetrics, but because of the demanding number of subjects and statistical processing,[118] only few studies have been published so far.[121,122] The future, undoubtedly, will see more large consortiums collaborating on these huge endeavours.

5.3.2. Inherited thrombophilias

The inherited thrombophilias, including factor V Leiden G1691A (FVL), prothrombin mutation G20210A (PTM), and methylhydrofolate reductase C677T (MTHFR), have in several candidate gene studies been associated with adverse pregnancy outcomes, however, with large inconsistencies regarding the strength and the biological plausibility,[123] and meta-analyses have yielded diverse pooled estimates for these associations.[124-126]

The first reports of the associations between thrombophilias and thrombotic lesions in the placentas from complicated pregnancies were enthusiastic,[127] and the following case-control studies were likewise strongly encouraging spearheaded by the works of Kupferminc et al.[128,129] In the following years, though, several studies were not able to corroborate the previously suggested specificity of the lesions,[41,130] and other case-control studies failed to demonstrate any association to the pregnancy complication.[131] In parallel, the proposed pathophysiology shifted from microthrombotic placental hypo-perfusion to the contemporary concept of an augmentation of the underdevelopment of trophoblast differentiation driven by a local antiangiogenic milieu, which may be further provoked by hypercoagulability.[132-134]

The larger epidemiological studies yield diverse estimates. The main reason for this is methodological differences. In general, case-control studies favour higher estimates as compared to cohort studies, severe phenotypes also favour higher estimates, and differences in population or ethnicity will contribute to the diversity in the estimates. Finally, there seems to be publication bias present favouring higher estimates in the smaller case-control studies.[135-137] All these phenomena will bias away from the null and inflate the estimates.

This heterogeneity in the estimates may, however, not be as unfruitful and confusing as first impression implies: This may simply indicate, that in different populations and in different phenotypes, different proportions of aetiological paths to the complications will vary accordingly. Part of the aetiology of preeclampsia in Scandinavian women will most likely differ from that in African or Asian women.[138,139] Also, an evolutionary concept of the trade-offs for thrombophilias and the diverse frequencies of these in various populations are important for the understanding of the heterogeneity of the estimates.[140] As such, it may be rash to extrapolate finding in one population to a Danish setting.

In the quest for minimizing possible bias some discard all but prospective cohort studies to attain a true estimate,[125] hereby limiting the power to detect a difference. Indeed, this quest for a true unbiased association presents difficulties and the researcher's own choices in judging the quality of the original studies may influence the conclusions.[141] In the end, the interpretation of the vast literature may come down to a matter of pure believe.[133]

5.4. Vascular Obstetrical Syndrome

The elements, the connections and relative balance of the hypotheses – or the "five paths" – of the pregnancy disorders and the link to later maternal cardiovascular disease constitute the Vascular Obstetrical Syndrome.[25]

The centre is the maternal constitution, which will modulate the woman's risk of developing pregnancy complication and also later cardiovascular disease (Figure 2). Included in this constitution is a genetic element, for example thrombophilias that may be involved in placental microthrombi and enhance the underdevelopment of the placenta, or a family predisposition to cardiovascular disease. As such, the maternal constitution will form an accumulated cardiovascular and metabolic susceptibility that can emerge in pregnancy as complications.

The balance of the proportions of the paths will explain the concept of two subtypes of preeclampsia with early-onset and late-onset of symptoms,[142-144] representing two extremes of a continuum. Here, the placental path may be dominating in early-onset preeclampsia, which also is associated with fetal growth restriction, and the maternal constitutional path dominating in late-onset preeclampsia. Also, the higher risk of preeclampsia and fetal growth restriction in first

pregnancy as compared to second pregnancy can be justified by the immune maladaptation hypothesis (inflammatory path) yielding a lower response in second pregnancy due to adaptation.

Depending on the relative proportions of the "five paths", each pregnancy complication will have either a strong or weak association to this cardiovascular susceptibility. As described above, preeclampsia has a strong endothelial component whereas preterm birth may be more related to inflammation and fetal growth restriction to placental development.

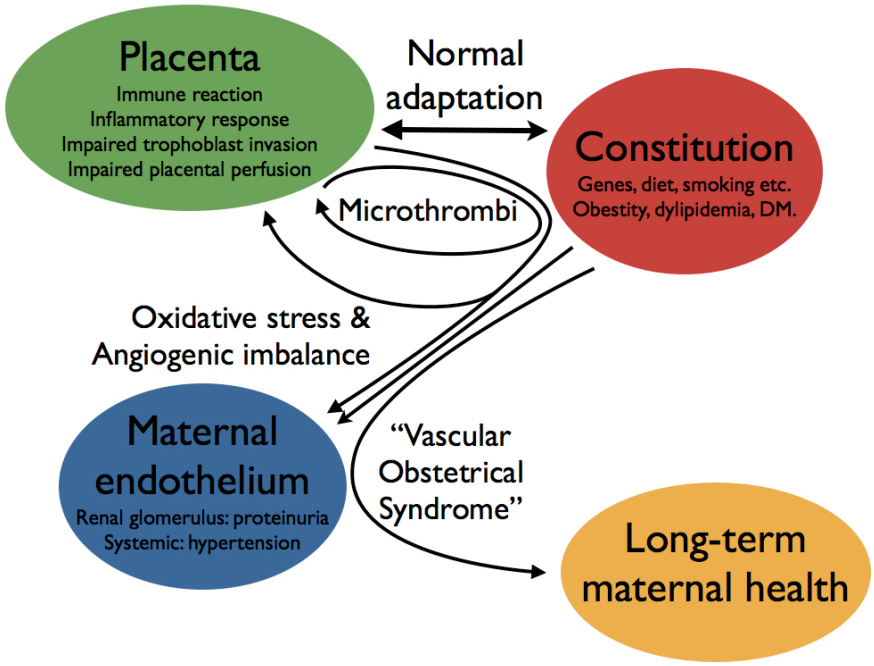

Figure 2. Conceptual model showing patho-physiological processes associated with pregnancy complications and long-term maternal health. The vascular obstetrical syndrome unites the link to later maternal disease.

6. Methodology

Following the pathophysiology of the proposed Vascular Obstetrical Syndrome, we hypothesize that women with cardiovascular and metabolic susceptibility have increased risk of pregnancy complications such as preeclampsia, fetal growth restriction, preterm delivery as well as early vaginal bleeding; these risks are dependent on the proportions of the aetiological paths to the complications.

Based on the aetiological paths of the pregnancy complications, we hypothesize that the complications are associated with later cardiovascular morbidity and diabetes with various strength dependent on the pathophysiology of the cardiovascular disease.

The susceptibility will also affect the recurrence risk of these complications: Based on the shared proportions of aetiological paths, any of the pregnancy complications will strongly tend to recur, but also to a lower degree increase the risk of other complications in next pregnancy. Especially, early vaginal bleeding will increase the risk of complications in the same as well as next pregnancy.

Lastly, we hypothesize that the genetic element of the susceptibility is partly expressed in variations in polymorphisms associated with vascular diseases including the three thrombophilias (FVL, PTM and MTHFR), which will increase the risk of pregnancy complications.

To test these hypotheses, the specific aims of this thesis were to assess:

1. The risk of later cardiovascular disease following pregnancy complications.
    a. The presence of a grading effect by severity of these complications.
    b. Different cardiovascular endpoints as well as diabetes mellitus.

2. The recurrence risk of pregnancy complications across two pregnancies.
    a. The presence of grading effects by severity in first pregnancy.
    b. The transfer of risk to other complications in second pregnancy.
    c. The risk of complications later in pregnancy following early vaginal bleeding.

3. The genetic variation in pregnancy complications.
    a. Inherited thrombophilias (FVL, PTM, and MTHFR) in relation to pregnancy complications (severe preeclampsia, fetal growth restriction, very preterm delivery, placental abruption, and a composite of these and stillbirth).
    b. Specific vascular associated polymorphisms in relation to severe preeclampsia.

In order to design appropriate studies to test these hypotheses, we have considered several issues: Susceptibility, model, population, diseases and their validities, missing values, follow-up time, and choice of statistics. We identified two main sources of data for the studies: 1) the Danish National Patient Registry (NPR), which holds information on diagnoses of all contacts in Danish hospitals including the Medical Birth Registry, and 2) the Danish National Birth Cohort (DNBC) to access blood samples for genotyping. The materials and designs of the studies are described below.

6.1. Vascular susceptibility

The vascular susceptibility is the accumulated risk of vascular event, which will emerge when an arbitrary threshold is reached. In pregnancy and in later life, stress to the vascular system is increased and added to the pre-existing susceptibility. In some women, this may result in pregnancy complications and later premature vascular event (see Figure 3 below).

In the example in Figure 3, two women with different baseline risk of cardiovascular events are shown to go through life including two pregnancies differently: One woman (lower green line) has a low starting point, that is few heritable risk factors, and has not accumulated additional risk factors, such as obesity, before her first pregnancy (p1), and she has also not added enough risk factors in her second pregnancy (p2). Another woman (upper yellow line) has a higher starting point, for example through an inherited thrombophilia, and has accumulated enough risk factors to develop pregnancy disorders (depicted by crossing the red line) in her first (p1); shortly hereafter she will return to a normal state and not present any clinical symptoms, although differences in risk factors may persist and be exaggerated with time.

These two women will, however, eventually accumulate enough risk factors to develop an overt cardiovascular disease or diabetes, but at different ages (d1 and d2, respectively): The difference in time of these diagnoses can easily be assessed.

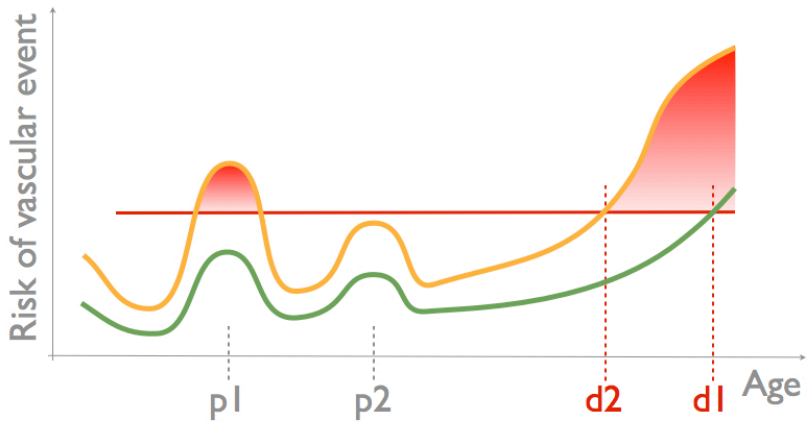

Figure 3. Accumulated risk of vascular event in two women with different risk profiles. Green and yellow lines represent two different women. Crossing the red line represents development of symptoms.
p denotes pregnancy, and d denotes diagnosis of cardiovascular disease.
Adapted from Sattar and Greer, BMJ 2002,[1] with permission.

6.2. Model

Many factors will influence the risk of developing pregnancy disorders as reviewed above (see Figure 4). Genetic or inheritable factors (G) are influencing the risk of developing a metabolic syndrome through various pathways (orange coloured area), and also influencing the fertility directly and indirectly via this metabolic syndrome. The fertility is also influenced by paternal factors as well as the woman's age. The fertility will then affect the woman's final parity (green coloured area). The metabolic syndrome, the parity, the age of the woman, and paternal factors all influence the risk of adverse pregnancy outcomes (APO).

In the association between G and APO, there are no confounders but only intermediate variables. Thus, the crude effect measure of this association is paramount, and adjusting for intermediate variables can be hazardous as unpredicted bias can arise.[145] However, if these variables act as effect modifiers,[146] stratification of the analyses may be necessary.

In the association between APO and CVD, there are many known and presumably many unknown confounders as well as colliders. Adjustment may be necessary but also perilous and misleading as residual and unknown confounding are almost unavoidable in registry studies.

In our data we did not have access to most of these confounders – an innate problem in secondary data analyses. The metabolic syndrome is a strong risk factor for atherosclerosis outside pregnancy,[147] and the same constituting factors have consistently been linked with pregnancy disorders, especially preeclampsia.[148] Inflammatory markers, such as serum C-reactive protein (CRP), are increased before,[59,149] during[150] and after[151,152] pregnancy disorders, preeclampsia in particular.[153] Furthermore, serum CRP is increased in populations with high-risk of cardiovascular disease,[154] as well as intermediate risk.[155] Uric acid, a product in the purine metabolism and involved in oxidative stress, has also been associated with possible direct endothelial damage outside of pregnancy.[156] In some cases of preeclampsia, serum uric acid is rapidly increasing to high levels suggesting accelerated cell damage, although the clinical implication in pregnancy is regarded as controversial.[157]

Smoking has been associated to cardiovascular disease,[158] and also to many pregnancy complications, although a protective effect on preeclampsia has consistently been reported. This will probably render the variable smoking a "negative confounder" in the association preeclampsia to CVD, but a positive confounder in the association fetal growth restriction and later CVD.[159] Socioeconomic status is correlated to all pregnancy outcomes, even in countries where the dissimilarity between social strata is low;[160] however, it seems that other variables, such as smoking, can explain some of this variation,[161] albeit not the full effect.[162,163]

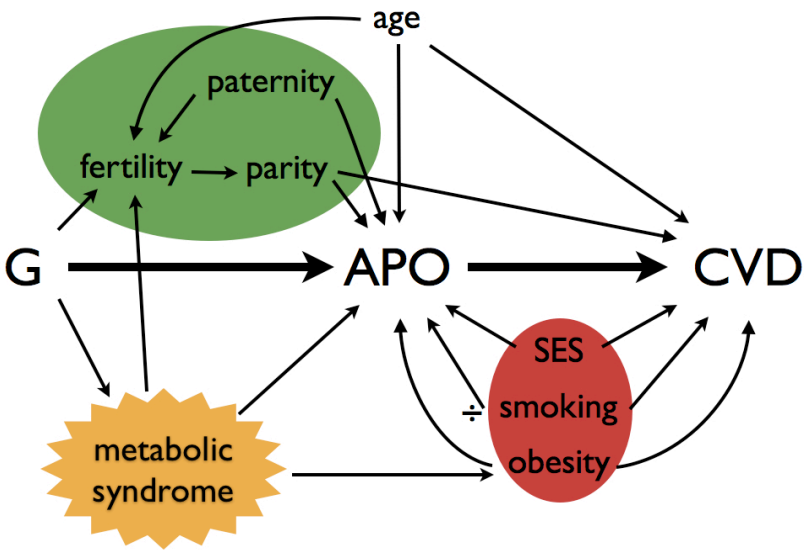

Figure 4. Directed acyclic graph (DAG) illustrating factors influencing adverse pregnancy outcomes and cardiovascular disease. G denotes genomic factors, APO denotes adverse pregnancy outcomes, CVD denotes cardiovascular disease, and SES denotes socioeconomic status.

6.3. Population

We designed nine studies all using the Danish National Patient Registry (NPR).[164] This registry collects information on all deliveries and hospital discharges in Denmark. In the studies on genotypes, we additionally used the Danish National Birth Cohort (DNBC) including stored blood samples.[165]

6.3.1. National census

For the five studies on later risk of cardiovascular disease and diabetes, and two studies on recurrence we performed registry based cohort studies based on a complete national census. In Denmark, all medical diagnoses and procedures in hospitals are reported to the National Patient Registry (NPR). This includes all diagnoses in pregnancy and data on birth, such as birth weight.

From 1978 to 2007, we identified 1,795,806 deliveries to 965,475 women. Of these, 796,915 women had their first delivery between the age of 15 and 50 years. After the exclusion of women with pre-existing cardiovascular diagnosed or diabetes mellitus and women deceased or emigrated within 3 months from the delivery, 782,287 women remained. This constituted cohort one.

Cohort two was a subgroup of cohort one consisting of women aged 15 to 50 years, having a second delivery in the study period and no cardiovascular diagnoses or diabetes, or deceased or emigrated within 3 months from the second delivery. This cohort consisted of 536,419 women.

We assessed status on emigration or death in the National Central Personal Registry in order to censor the women in the survival analyses. In the study period, 2% emigrated and 1% deceased.

6.3.2. Danish National Birth Cohort

For the two studies on genotypes (articles VIII and IX), we accessed the Danish National Birth Cohort (DNBC)[165] for blood samples and performed nested case-cohort studies. In the years 1997 through 2002, half of all pregnant women in Denmark were invited to participate in this prospective cohort, and two thirds of these consented and donated blood samples; these samples were separated into plasma and buffy coat, and stored at the national biobank at "Statens Serum Institut" frozen for later analysis.[165] The 91,661 participating women have been compared to the non-participating women: The authors found no differences in baseline and birth characteristics, hereby making selection bias less likely.[166] Furthermore, when studying genetic associations, no selection bias is expected.

Linking data from the DNBC with the NPR, we identified all women with registered cases of severe preeclampsia, placental abruption, small-for-gestational age offspring, very preterm delivery as well as stillbirth, and a random sample as control cohort; the size of the control cohort was set at the expected largest of the case groups, that is n = 2,000 (see Figure 5). After identification of the registry diagnoses, a trained research midwife validated each medical record of these cases by hand at the local hospitals. In the final study population, only validated cases were used. We identified 315 cases with severe preeclampsia, 743 with very preterm delivery before 34 weeks, 1,415 small-for-gestational-age, 377 with placental abruption, and 2,349 with a composite outcome.

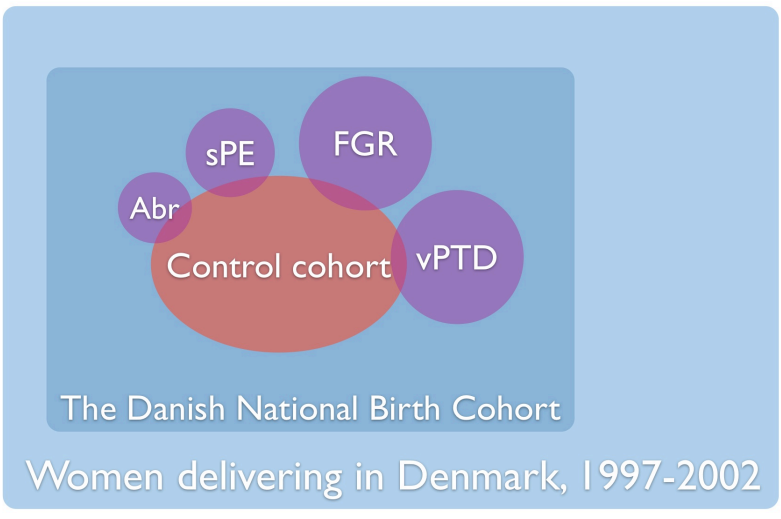

Figure 5. Nested case-cohort studies within the Danish National Birth Cohort. Within the DNBC we identified all registry cases of pregnancy complications and a random control cohort.
Abr denotes placental abruption, sPE severe preeclampsia, FGR fetal growth restriction, and vPTD very preterm delivery.

6.4. Diseases – exposure and outcome

Our primary exposures were early vaginal bleeding, preterm delivery, fetal growth restriction, and hypertensive pregnancy disorders; we also included placental abruption and stillbirth as exposures. The practical handling of these conditions is to categorizing or dichotomizing them into groups by severity; stillbirth is by definition already dichotomized.

The primary outcomes in the follow-up studies were death, hypertension, ischemic heart disease, congestive heart disease, stroke, thromboembolism, and (type 2) diabetes mellitus. These were chosen because of their respectively strong association to the five paths (see above) and general interest. Hypertension will thus represent the vascular path,[167] and thromboembolism the haematological path.[168] However, there are other risk factors for these diseases that will dilute the clarity of these representations. Death was included as the definitive outcome.

We extracted information on the presence of diagnoses of these complications in the NPR. However, because all of the data in the NPR is measured, assessed and reported by the local medical staff, that is midwifes and physicians, errors will eventually occur in these processes.

6.4.1. Pregnancy data

We extracted data on gender, gestational age, birth weight and length of the offspring. We then performed internal validation of these variables (see below), calculated fetal growth and ponderal indices (see below), and categorized the continuous variables into groups.

The NPR does not hold information on the method used for assessment of gestational age. There was a gradual increased in use of ultrasound dating during the time period, which may account for the reduction in mean gestational age at delivery of 2.5 days in the study period from 1978-2007.

The maternal age at delivery was calculated from her personal identification number (PIN) and date of delivery. The parity was listed in the NPR; the validity of this variable has previously been investigated with an almost perfect result.[169]

Presence of vaginal bleeding, hypertensive pregnancy disorders, and placental abruption was extracted using specific codes (International Classification of Death and Diseases [ICD]), and stillbirth was likewise registered separately.

We calculated the timing in pregnancy of the diagnosis vaginal bleeding and only included first trimester cases. This diagnosis has not been validated, but many cases will not be referred to a hospital setting, and the Registry diagnosis is likely biased from this selection, for example maternal anxiety and previous miscarriage. The incidence was 3.2%, which was stable in the study period. Because the diagnosis is registered prospectively, no recall bias is present, which may be a considerable problem in the smaller retrospective case-control studies.

The accuracy of the diagnoses of hypertensive pregnancy disorders is flawed:[170] In a validation study, the positive predictive value (pPV) of severe preeclampsia was 100%, but the sensitivity was 43%. For gestational hypertension, the pPV was 56% and the sensitivity just 10%, and for all types of preeclampsia the pPV was 74% and sensitivity 69%. The reason for these numbers may be a lack of national consensus on diagnostic criteria.[171]

In our studies using the DNBC,[10,11] we manually validated each medical record by hand of the cases only. We were therefore not able assess the true accuracy of the diagnoses as we did not validate the non-cases. Regarding the diagnosis of severe preeclampsia, we followed the ACOG 2002 guideline[172] and found that 60% of the registry cases fulfilled these criteria.[11] The diagnosis of placental abruption could be verified in 69% of the registry cases, 86% in small-for-gestational offspring, and 88% in very preterm delivery.

Because of the low frequencies of these severe complications, any misclassification from underreporting (low sensitivity) will dilute the large reference group only negligibly and the possible bias will hence be minimal. If, however, the misclassification is systematic, for example by including severe cases, then bias away from the null can arise; in gestational hypertension, this may

be the case. Misclassification due to low pPV will dilute the small exposure group and likely bias the estimates towards the null. All of these examples of bias are likely present in our studies.

6.4.2. Cardiovascular diagnoses

It is essential to realize that the NPR is hospital based; the general practitioner in primary care does not report data to the NPR. Therefore, patients only treated in primary care, will not appear in the NPR.

We sought specific ICD codes for the cardiovascular diseases and the date for these in the NPR. Date of decease was assessed in the Central Personal Registry and the primary listed cause in Cause of Death Registry.

Hypertension is in Denmark primarily diagnosed and handled in primary care at the general practitioner. Only when the patient is referred to a hospital setting will the diagnosis be reported to the NPR. This diagnosis is thus likely to represent severe cases or cases with severe co-morbidity demanding hospital treatment. The validity of the diagnosis has not been investigated sufficiently, however, a small study suggested that only 40-60% of true hypertensive patients will have a diagnosis[173] indicating a low sensitivity.

Diabetes mellitus is also handled in primary care; however, many patients will have regular outpatient care at the local hospital or diabetes centre. Again, the severity of the disease will affect the chance for hospital care. Validity studies on the diagnosis in the NPR have yielded a sensitivity of 34% and a pPV 98%.[174]

Stroke is always handled in hospital settings in the acute phase of the disease; later follow-up is partly in primary care. The validity of the diagnosis is generally good with pPV between 81% and 86%, depending on the person reviewing the medical charts.[175] Thromboembolism is also an obligate hospital diagnosis in the acute phase of the disease. The validity is, however, poor with pPV of 57%, which may be related to the diagnostic procedures.[176]

Ischemic heart disease and congestive heart failure are mainly hospital diagnoses, but follow-up is handled in primary care. The validity of ischaemic heart disease has not been evaluated, but the subset of acute myocardial infarction (AMI) has in sample in the NPR a pPV 96%.[177] The validity of congestive heart failure is good with a specificity of 99%, sensitivity of 29%, and pPV of 81%.[178]

The structure of the healthcare system combined with the validity of the diagnosed used will bias the effect measures. Diagnoses in the NPR of hypertension and diabetes probably represent severe cases. Low sensitivity in all of the diagnoses will bias the estimates toward the null, although the low frequencies of the diseases in general will ameliorate this. However, the low sensitivity will increase the chance of a type II error. Low pPV will bias the estimates, most likely toward the null, as the “true” estimate will be diluted with non-cases. Given that most of diagnoses have high pPV, this effect should be minimal.

6.4.3. Genetic polymorphisms

The genetic studies investigated gene polymorphisms and pregnancy complications. The proposed link between the three thrombophilias (FVL, PTM, and MTHFR) and pregnancy complications are still a matter of debate (see later discussion), so we chose to investigate these in respect to each pregnancy complication employing the whole case-cohort design. We also compiled 108 vascular associated polymorphisms[179-188] (see supplemental data for article VIII for full list) and tested these in respect to severe preeclampsia including subtypes based on co-morbidities.

The frozen DNA samples from the DNBC were shipped to Celera (Alameda, CA, USA) where the genotyping of individual DNA samples was done by performing two real-time PCR reactions for each individual sample, using 0.3ng DNA from each sample and allele specific primers.[189] The failure rates of the individual polymorphisms were below 3%.

Blood samples were available and genotyping successful in 2,032 of 2,349 cases (87%) and 1,856 of 2,000 women (93%) in the control cohort (t-test, $p<0.001$). This difference is due to the

use of blood from cases in previous studies in the DNBC; however, this should not affect the internal validity of our studies.

6.5. Missing and implausible values

In the crude data set from the NPR, we performed an "internal validation" of birth weight, gestational age, and crown-heel (body) length. We searched for implausible values: first excluding absurd values of birth weight >7Kg or <200g, gestational age >315d (45 weeks) or <140d (20 weeks), and length = 0cm or ≥90cm. Next, we cross-checked these variables (fetal growth and Ponderal index, see below) and excluded outliers more than 5 standard deviations from the median (both observations, for example the birth weight and the gestational age, or fetal length and birth weight). These observations were reclassified as missing values.

Missing values in total occurred more frequently in the earlier years. As an example, in missing values in gestational age the total number was n=26,889. Of these occurred 53% in the first two years (1978 and 1979), and another 30% in the next two years (1980 and 1981) yielding a total missing values frequency of 22% in the first 4 years. In these early years, just following the implementation of the Registry and the mandatory reporting of trivial data, there seemed to be a culture of neglecting the reporting of the uncomplicated deliveries and of healthy women: In the first 5 years (1978 to 1982), there was a lower than expected frequency of hypertensive disorders, placental abruption, and stillbirth in the group of missing values, and the birth weight in this group was 46g lower and the mothers 0.4 years younger compared to the reference group (t-test, $p<0.001$); the crown-heel length of the offspring was similar in the two groups.

This inequality in years, early vs. late, in the missing values will tend to generate a higher prevalence (%) and rate (events per 10,000 person years) due to the longer follow-up time and thus older subpopulation at censoring. When calculating the Cox models, this false effect will be ameliorated. In some endpoints, the "missing values" group seems be less likely to develop the disease, which can be a consequence of the above mentioned registering.

6.6. Follow-up time

In the studies on later disease, the NPR encompasses an innate restriction in that it was only initiated in 1978 allowing for a maximal follow-up time of 30 years when extracting the data sample in 2007. In our data, the mean maternal age at first delivery in the late 1970'ies was 23 years, which yields a mean age of 42 years at censoring. We also extracted information on emigration and decease for censoring in the survival analyses. During the follow-up period, 15,902 (2.0%) emigrated and 8,876 (1.1%) deceased. In cohort one, the median follow-up time from delivery to time of censoring was 14.6 years (interquartile range 7.6-21.8 years) corresponding to 11,600,945 person-years of observation.

We excluded women with any cardiovascular event in the first 3 months just following the delivery, as these women likely had pre-existing diagnoses or sustaining complications in pregnancy, and as such not representing actual follow-up of later disease.

Previous findings have indicated that the increased risk following pregnancy disorders is most pronounced in the earlier years just following the pregnancy and disappearing after the age of 60-65 years.[190] However, others find a sharp demarcation and increase in cardiovascular risk two decades after the preeclampsia, possibly coinciding with menopause.[191] This may indicate different subtypes of preeclampsia – in first vs. second delivery, immediate vs. prolonged resolution of the hypertension – but also different population being studied with different risk profiles.

6.7. Statistics

We have throughout the studies used logistic regression to assess the associations. We used Cox regression with time from delivery to event as time-dependant variable in the follow-up studies, and

censored women emigrated or deceased. SPSS v16, v18, or v20 for Macintosh (SPSS Inc.; Chicago, Illinois, USA) were used as statistical software throughout the studies, except for the genetic studies (articles VIII and IX). In these studies, we used SAS v9 (SAS Institute Inc., Cary, NC, USA) in the calculation of the associations. Results are presented as numbers (n), percentages (%), and rate per 10,000 years of observation, odds ratios (OR) and hazard ratios (HR) with corresponding 95% confidence intervals (95% CI), where appropriate.

In article VIII we calculated separate effects for genotypic and allelic models and present both in the original article. In the thesis, we present only the allelic model, which compares the additive effect of one risk allele in respect to the wild type.

In article IX we additionally used Benjamini-Hochberg's false discovery rates (FDR) and corresponding q-values[192] to ameliorate the issue of multiple comparison when analysing 108 polymorphisms. Some authorities will advocate for no adjustment, whereas others demand "penalty for peeking" at too much data.[193] In essence, the false positive / false negative ratio is pivotal in this context,[194] and one must carefully evaluate the results of any large study with multiple comparisons.

6.8. Ethics and consents

The Danish National Data Protection Agency (no. 2005–41–5262 and 2007–41–1544), the National Board of Health as well as the Internal Review Board at Yale University approved the protocols. As no direct patient contact was required in the protocols, the approval from the Danish National Ethical Board was not mandatory.

In the genetic studies,[10,11] the board of the DNBC approved the protocols and allowed access to the blood samples as well as validation of the medical records. All women participating in the DNBC had signed full information statements at the time of entry in the cohort.

7. Bleeding in early pregnancy

Bleeding in early pregnancy is perhaps the most common complication in pregnancy affecting 6% to 25% of all pregnancies,[195,196] and followed by miscarriage in about half of these cases; this has labelled early bleeding as "threatened miscarriage".[197]

7.1. Definition

There is no universal definition of this complication.[196] We extracted information in the NPR on the specific diagnosis and related it to first trimester in the ongoing pregnancy; therefore we are unable to collect further information, for example on duration, intensity, or presence of subchorionic haematoma. In our data, early vaginal bleeding complicated 2.3% of all ongoing pregnancies. This frequency is lower than expected from other studies, but the diagnosis is based on a physical examination of women referred to a hospital setting as compared to self-reporting in other studies.

7.2. Consequences in pregnancy

Several studies have identified early vaginal bleeding as a risk factor for preterm delivery (OR 1.74; 95% CI 1.65-1.83),[198,199] placental abruption, fetal growth restriction[200] and preeclampsia[201] later in the pregnancy. In respect to risk of preterm delivery, studies reporting a high incidence of bleeding tend to have low estimates, and low quality studies will tend to have high estimates.[199] Also, previous findings have identified intrauterine haematoma as an aggravating marker of poor obstetrical outcome in women with early bleeding,[202,203] and a grading effect in light or heavy bleeding episodes has also been demonstrated.[199,200] In parallel, actual miscarriage in first pregnancy carries an increased risk into the next pregnancy (first delivery) of early bleeding (OR

1.6; 99% CI 1.4-1.8) and preterm delivery (OR 1.4; 99% CI 1.3-1.6) in comparison to primigravidas.[204]

7.2.1. Own studies

In article VI, we found that a bleeding episode increased the risk of spontaneous preterm delivery, and the risk was more pronounced in the early gestation: Bleeding was associated by OR 1.65 (95% CI 1.55–1.77; n=970 cases with bleeding) for spontaneous preterm delivery at gestational age (GA) 32-36, and OR 2.98 (95% CI 2.50–3.54; n=138 cases) for GA 28-31. This illustrates the different proportions of aetiological paths to preterm delivery in the different parts of pregnancy: In late preterm delivery, other causes will dilute the effect from early bleeding. We also found a small increased risk of prelabour rupture of membranes (OR 1.18, 95% CI 1.11–1.28; n=1,119 cases), both at term (PROM) and prematurely (PPROM), as well as risk of placental abruption (OR 1.48; 95% CI 1.30-1.68; n=250 cases).

Interestingly, when we extended the study to second delivery, bleeding in first pregnancy tended to recur by OR 4.05 (95% CI 3.78–4.34) and carried risk of preterm delivery, PROM, placental abruption, preeclampsia, and stillbirth into the second pregnancy by OR ranging from 1.20 to 1.80.[8] Of note, these associations were independent of bleeding in the second pregnancy; if bleeding did occur in second pregnancy, the risks were further increased. The reverse associations were also present: Preterm delivery, PROM, placental abruption, and stillbirth (but not preeclampsia) in the first delivery were all associated with bleeding in the second delivery by OR ranging from 1.41 to 2.57. Furthermore, bleeding in either pregnancy increased the recurrence risk of these pregnancy disorders. These novel findings suggest that the consequences of the bleeding episode is not confined to the same pregnancy, and reverse causation may be present, the latter supporting a common proclivity to both bleeding episode and later pregnancy complications.

7.3. Long-term follow-up of mothers

No previous studies on the long-term morbidity of mothers experiencing bleeding in pregnancy have been published. In a Scottish cohort study, the authors found a 50% increase (HR 1.51; 95% CI 1.12-2.04) in risk of later ischemic heart disease following one or more miscarriages, rising to more than a double (HR 2.32; 95% CI 0.86-6.27) in risk following three or more miscarriages.[205] This has been confirmed in a Finnish cohort yielding OR 1.4 (95% CI 1.1–1.8) per miscarriage for later myocardial infarction.[206]

7.3.1. Own studies

In article VII, we found that women with pre-pregnancy cardiovascular diseases are at increased the risk of early bleeding in pregnancy (OR 2.28; 95% CI 1.27–4.11), but pre-pregnancy diabetes mellitus was not associated with risk of bleeding in pregnancy (p=0.11 and p=0.10, diabetes type 1 and 2, respectively). This difference indicates that selection bias (referral to hospital) is not likely.

Early bleeding in first pregnancy increased the risk of later ischaemic heart disease (crude HR 1.60; 95% CI 1.41–1.82) and also later diabetes (crude HR 1.60; 95% CI 1.39-1.84). When adjusting for later pregnancy complications, the estimates were attenuated.

These findings parallel the findings on associations between miscarriage and later cardiovascular disease. Also, our findings support the hypothesis of a common vascular and metabolic weakness in the mother that predisposes to both early bleeding and later cardiovascular disease and diabetes.

8. Preterm delivery

A slow rise in preterm delivery (PTD) rate has been observed in most Western countries,[207] including in Scandinavia.[208] Most of the rise in recent years is thought to be due to high-risk pregnancies in general, including medical indications for induction of labour or caesarean section,[209,210] although an increase among low-risk primiparous women is also evident.[208]

8.1. Definition

The gestational age cut-off for preterm delivery (PTD) has been set at 37 full weeks of gestation; the corresponding upper cut-off for defining a post-term delivery is set at 42 full weeks of gestation.[211] Also, other cut-offs in segregating preterm delivery have been used,[94] and recently, even within the major category "at term", some are arguing for dissecting this into finer categories: early term, full term, and late term.[211]

In approximately one-third to half of the cases of preterm delivery, delivery is medically indicated because of either maternal or fetal causes,[94,209] mostly because of preeclampsia or fetal growth restriction.[209] In contrast, spontaneous preterm birth may proceed from either preterm pre-labour rupture of membranes (PPROM) or spontaneous preterm labor.[94] In the NPR, we have for practical reasons defined spontaneous preterm birth as preterm deliveries without any pregnancy disorders.

8.2. Genetics

Previous studies have suggested the presence of a genetic component of preterm delivery and complex modes of inheritance.[212] However, very few polymorphisms have actually been investigated and with large inconsistencies; among these are the inherited thrombophilias.[120,213]

A large Finnish case-control study found an association with factor V Leiden (FVL) by OR 2.6 (95% CI 1.4–5.1; n=324 cases, 19 with FVL),[214] whereas a nested matched case-control study of white women in Boston, USA did not find evidence of an association (OR 0.99; 95% CI 0.43-2.28; n=99 cases, 8 with FVL).[215] Prothrombin mutation (PTM) was also investigated in the two studies from Finland and Boston, again with contrasting findings: The Finnish study found no association (OR 0.3; 95% CI 0.04-2.7; n=324 cases, 1 with PTM) and the Bostonian study found an OR 3.12 (95% CI 1.07-9.13; n=99 cases, 7 with PTM).[214,215] Again, differences in study design may cause this diversity. In the Finnish study, the authors attempted to minimize selection bias by adding cases to the control group; the baseline characteristics were, however, skewed. In the study from Boston, the low incidence of PTD by 2.8% will increase the risk of a type II error, and the choice of performing matching control may introduce bias.

For methylenetetrahydrofolate reductase (MTHFR) polymorphism few studies have investigated the association with PTD. A small Mexican case-control study (n=86) yielded an OR 1.98 (95% CI 0.97–4.08),[216] and a cohort study (n=1,675) from Austria found an OR 1.61 (95% CI 0.71–3.67; n=33 cases) for PTD before 34 full weeks.[217]

8.2.1. Own studies

In article VIII, we investigated these three thrombophilias in respect to overall (not specific spontaneous) very preterm delivery before 34 full weeks of gestation. We identified, validated and genotyped 621 cases.

We found that the association with FVL was borderline significant (OR 1.32; 95% CI 0.98-1.79; 54 with FVL) as compared to PTM (OR 1.36; 95% CI 0.78-2.37; 16 with PTM). MTHFR was not associated with very PTD (OR 1.00; 95% CI 0.86–1.15; 54 homozygote and 254 heterozygote for MTHFR).[10]

Our study is the largest to date investigating these polymorphisms, and together with the existing literature there is at present no strong support for a link to very preterm delivery. However, the studied phenotype is important: We chose to investigate preterm delivery in general as the clinically important endpoint, and as such other complications may increase the strength of the association as compared to spontaneous PTD. Also, the number of cases with the polymorphisms is still low hereby increasing the chance of false negative finding (type II error).

8.3. Recurrence in next pregnancy

One of the strongest risk factors for preterm birth is a history of preterm birth.[218] This applies not only to the woman's personal history, but also to her family.[219,220] The distinction between medical indicated and spontaneous PTD has also been investigated: indicated PTD is linked to a second spontaneous preterm birth, and vice versa, hereby associating the two events by a common aetiology or proclivity.[221]

8.3.1. Own studies

In article V, we investigated the recurrence in second delivery of spontaneous preterm delivery in respect to the gestational age in first delivery. Our estimates on recurrence are comparable to previous findings, and we additionally found a strong grading effect: As compared to delivering at term, delivering at GA 32-36 weeks increased the risk from 3% to 15% corresponding to OR 6.12, and delivering at GA 28-31 weeks further increased the risk to 25% corresponding to OR 12.0 (see Table 1 below).[7] Also, even though these women did not have any pregnancy disorders diagnosed in the first delivery, the grading effect also transferred to moderately increased risk of preeclampsia, fetal growth restriction, and placental abruption in the second delivery.[7] Though, residual confounding and underreporting in the NPR cannot be ruled out as possible bias in these associations.

Table 1. Risk of complications in second delivery by gestation age of spontaneous preterm delivery in first pregnancy.[7]

First delivery	Second delivery					
	Preterm delivery		Small for gestational age		Preeclampsia	
Gest. age	Pct.	OR (95% CI)	Pct.	OR (95% CI)	Pct.	OR (95% CI)
≥ 37 wk	3%	1 (ref.)	2.1%	1 (ref.)	1.1%	1 (ref.)
32 - 36 wk	15%	6.12 (5.84-6.42)	3.2%	1.63 (1.49-1.78)	1.8%	1.60 (1.41-1.81)
28 - 31 wk	25%	12.0 (10.5-13.7)	4.6%	2.17 (1.65-2.85)	2.5%	2.18 (1.52-3.14)
20 - 27 wk	26%	13.1 (10.8-16.0)	4.3%	2.23 (1.47-3.40)	3.2%	2.96 (1.80-4.88)

Spontaneous delivery is defined as a delivery without hypertensive disorders, small-for-gestational-age offspring, placental abruption, or stillbirth. OR denotes odds ratios, and CI confidence interval.

8.4. Long-term follow-up of mothers

Preceding cardiovascular risk factors[60] and overt cardiovascular disease[222] are associated with risk of preterm delivery. Also, previous findings have suggested an increased mortality following preterm delivery,[223] which is predominantly due to cardiovascular disease.[224-227] However, most of these studies are small and the presence of a grading effect has not been investigated.

8.4.1. Own studies

In article II, we found a pronounced grading effect in respect to later hypertension following a preterm delivery in first delivery: Delivering at GA 32-36 weeks as compared to at term imposed an almost 2-fold increased risk (HR 1.74; 95% CI 1.65-1.85), and at GA 28-31 weeks almost 3-fold (HR 2.71; 95% CI 2.40-3.06). However, when controlling for other pregnancy disorders, in order to

yield a proxy for spontaneous PTD, these estimates were severely weakened to 1.30 and 1.50, respectively. This suggests that indicated PTD, from preeclampsia etc., are stronger associated to later hypertension than spontaneous PTD. In respect to later diabetes, the attenuation following the same adjustment was minor, suggesting that spontaneous PTD indeed is associated with later diabetes.

In women having more than two deliveries, we found a grading effect in number of preterm deliveries. Women with two PTD were at higher risk of later diabetes (HR 2.30; 95% CI 1.71-3.10) compared to women with only one PTD and then at term (HR 1.58; 95% CI 1.34-1.86). Notably, women having a PTD in second delivery only were at greater risk of diabetes (HR 1.88; 95% CI 1.59-2.23) compared to PTD in first delivery only indicating different proportions of aetiological paths.

In article IV, we also investigated mortality following PTD in combination with pregnancy disorders. Here, we found that PTD is stronger associated to mortality from cardiovascular causes (HR 1.98, 95% CI 1.64-2.40) than non-cardiovascular causes (HR 1.48; 95% CI 1.34-1.64). These estimates persisted when stratifying for other pregnancy complications.

9. Fetal growth restriction

The concept of fetal growth (FG) is both intuitively simple and scientifically complex. Birth weight (BW) is a simple measurement done with accuracy. BW for GA defines the observed fetal growth (FG) at delivery. A chart of normal values of BW for GA will, however, present an innate problem in preterm deliveries: The BW of delivered offspring might differ from those that continue not to be delivered.[228] This example illustrates the counterfactual problem[229] and extensive selection bias. Following this, ultrasound estimation of fetal weight (EFW) has allowed the evaluation of fetal growth to a certain extent with acceptable accuracy, particularly for epidemiological purposes, despite the limitations imposed by the EFW being based on 2-3 parameters of size *in utero* being measured.

In Scandinavia, the consensus on a normative *in utero* growth curve has for more than a decade now been "the Marsál curve".[230] This was constructed from serial measurements of EFW at various points in gestation in women who eventually had uncomplicated pregnancies. Using this normative chart, a standardized FG score in respect to the median and standard deviation can be calculated. Newer methods have been evolved to construct better, more precise and more valid growth charts to include some inherited paradoxes[231] in these charts.[232-234]

9.1. Definition

Fetal growth restriction (FGR) (also termed intrauterine growth restriction or retardation, IUGR) implies that the pace of growth diminishes and deviates from the growth trajectory hereby resulting in a fetus that does not achieve its predestined BW. In contrast, some fetuses are predestined to be constitutionally small, and these fetuses will follow their trajectory ending at a low BW.[235] This distinction necessitates serial EFW measurements to assess the growth trajectory and the possible deviation.

9.1.1. Small for gestational age

Small-for-gestational-age (SGA) is commonly defined as standardized FG either below the 10th percentile or 2 standard deviations (SD) below the median corresponding to the 2nd percentile; the latter definition is the more severe form. As such, the classification SGA is based on a single point in gestation, usually at delivery.

The distinction between FGR and SGA is crucial. There are presumably great overlap between cases of FGR and SGA, but not universally:[236] However, the more severe the SGA is, the

more specific it is of true FGR. Subsequently, using SGA as a surrogate for FGR can be ambiguous, but in epidemiological studies necessary.

The literature is, however, not precise in this distinction. We have, in order to keep inline with the current literature, used the terminology *fetal growth at delivery* to evaluate degrees of standardized birth weight in respect to median, although the strict definition is SGA (small), AGA (appropriate), and LGA (large).

9.1.2. Ponderal index

The term ponderal index (PI) is defined as BW over the cube of body length (Kg/m^3).[237] This index operates like the body mass index (BMI, Kg/m^2) and measures the build of the offspring (lean, normal, or fat). The PI at birth has been used to differentiate "asymmetric" from "symmetric" FG in preeclampsia,[238] and the PI has been associated with different lipid profiles in the umbilical cord at term suggesting an altered fetal metabolic state.[239] Thus, the PI may relate to the nutritional status at birth of the offspring.[237]

The PI is not independent of GA,[240,241] which also was evident in our data,[6] and therefore we had to construct a standardized PI index (sPI), based on the observed values of BW and body length corrected by GA.

9.2. Genetics

An extensive number of studies have investigated the association between various genetic polymorphisms and SGA. Facco et al. reviewed the 3 inherited thrombophilias in 2009.[135] The majority of the included studies used FG below the 10th percentile as definition of SGA. They found that PTM was weakly associated with SGA (OR 1.52; 95% CI 0.98-2.35; p=0.06) in all case-control studies. MTHFR was not associated to SGA (OR 1.01; 95% CI 0.88-1.17), although case-control studies in general tended to produce higher estimates compared to cohort studies.

FVL was overall associated to SGA (OR 1.23; 95% CI 1.04-1.44), however, this finding may be spurious.[135] The earlier and smaller studies were in general in favour of an association, and the later and larger studies were not. Also, the pooled estimates of case-control studies indicated an association (OR 1.91; 95% CI 1.17-3.12), but the pooled estimate from cohort studies was not as strong (OR 1.16; 95% CI 0.98-1.38). Furthermore, there seemed to be publication bias present in the case-control studies, and amending this by Duval and Tweedie's trim-and-fill method[242] left the pooled estimate attenuated (imputed OR 1.47; 95% CI 0.85–2.52).

9.2.1. Own studies

In article VIII, we investigated the 3 thrombophilias in a nested case-cohort design in respect to severe SGA.[10] We found no evidence of an association to PTM (OR 0.86; 95% CI 0.51-1.45) or MTHFR (OR 1.08; 95% CI 0.97-1.21), but a moderate association to FVL with an OR of 1.43 (95% CI 1.12–1.83). This latter point estimate lies very close to the imputed pooled case-control estimate by Facco et al.[135]

Our definition of SGA is stricter than FG below the 10th percentile used in the inclusion criterion in the meta-analysis by Facco et al., which may increase the effect measurement; the design ranking between the classic case-control and the gold standard of the cohort study hereby limiting the likelihood of selection bias; and the study size larger than all of the included case-controls but one, and also larger than all the cohort studies but one hereby limiting the chance of false negative findings.

9.3. Recurrence in next pregnancy

The risk of recurrence of FGR/SGA offspring has been investigated in several studies, agreeing on an approximately 8-fold risk increase with minor variations based on maternal characteristics.[21,243]

Also, there seems to be an increased risk of stillbirth in a next pregnancy following FGR in a first pregnancy.[244]

9.3.1. Own studies

In article V, we investigated the recurrence risk of FGR/SGA offspring following variations in FG in a first delivery, and found a strong grading effect (see Table 2 below). FG around 2 standard deviations below the median conveyed a 9- to 15-fold increased risk of recurrence in the second delivery, and FG above the median conveyed a lower risk of FGR/SGA in the second delivery. Additionally, the same grading effect carried risk of preeclampsia, preterm delivery, placental abruption and stillbirth in the next delivery, although the estimates were smaller.[7] Also, the grading effect was present only in FG below the median, not above where all the estimates were close to unity.

The finding that these complications are linked across pregnancies by a grading effect supports a shared aetiology or common paths to all these complications. However, underreporting and residual confounding may justify part of the observed grading effect.

Table 2. Variation in fetal growth in first delivery and risk of complications in second delivery.[7]

First delivery	Second delivery			
Fetal growth z-score	SGA OR (95% CI)	Preterm delivery OR (95% CI)	Preeclampsia OR (95% CI)	Placental abruption OR (95% CI)
-3.0 to -2.0	15.1 (14.1-16.3)	2.68 (2.40-2.97)	1.62 (1.34-1.96)	2.05 (1.66-2.52)
-2.0 to -1.5	9.13 (8.59-9.71)	1.76 (1.63-1.91)	1.40 (1.22-1.61)	1.53 (1.31-1.80)
-1.5 to -1.0	4.88 (4.63-5.16)	1.49 (1.40-1.58)	1.18 (1.07-1.30)	1.29 (1.15-1.45)
-1.0 to -0.5	2.40 (2.27-2.53)	1.20 (1.14-1.26)	1.03 (0.95-1.12)	1.18 (1.07-1.30)
-0.5 to +0.5	1 (ref.)	1 (ref.)	1 (ref.)	1 (ref.)
+0.5 to +1.0	0.48 (0.43-0.53)	0.86 (0.81-0.91)	1.05 (0.96-1.14)	0.94 (0.84-1.05)
+1.0 to +1.5	0.34 (0.29-0.39)	0.87 (0.81-0.94)	1.04 (0.94-1.16)	0.85 (0.73-0.98)
+1.5 to +2.0	0.24 (0.19-0.31)	0.87 (0.79-0.97)	1.29 (1.13-1.48)	0.86 (0.70-1.05)
+2.0 to +3.0	0.20 (0.13-0.29)	0.89 (0.78-1.02)	1.15 (0.94-1.39)	0.81 (0.60-1.08)

Women with hypertensive disorders, preterm delivery, placental abruption or stillbirth in first pregnancy were excluded from the analysis. The z-score is the standardized fetal growth by gestational.

9.4. Long-term follow-up of mothers

Follow-up studies of mothers have consistently shown that poor fetal growth is associated to later cardiovascular disease and mortality from this.[225,245,246] Importantly, the effect is larger in mothers compared to fathers suggesting specific maternal factors rather than shared environmental fators.[247] Most of these studies, however, assume a linear effect thus finding an simple inverse association between BW and cardiovascular disease (CVD).[247]

9.4.1. Own studies

In article III, we investigated both variations in the standardized FG and the PI in respect to later maternal CVD and death. We found a bimodal association with U-shaped risk curve of later CVD reflecting increased risk following both excessive and poor FG. The risk profile of overall death was J-shaped with increased risk following poor FG, but not excessive FG. In article IV, we further identified that the primary cause of death was from cardiovascular disease compared to non-cardiovascular disease.

For later diabetes the risk was highest in women delivering offspring with excessive FG, but still increased in women with poor FG. These findings highlight two very different pathways: Gestational diabetes mellitus (GDM) is likely operating as a strong intermediate in excessive FG,

but not poor FG,[248] and as such, other aetiological paths are operating in associating poor FG to later diabetes.

Overall, the findings for standardized PI paralleled the estimates for standardized FG, albeit with lower estimates, suggesting that the nutritional status at delivery – as the PI may be an indicator of[237] – is not as strong a marker of later CVD in the mother as the FG is. This indicates, that inherited maternal constitutional factors, such as genetic growth potential, are more important than the fetal nutritional deprivation to indicate long-term maternal health, in accordance with "the thrifty genotype hypothesis".[249]

10. Preeclampsia

Preeclampsia complicates about 5-8% of pregnancies with a strong preponderance of nulliparous women.[250] The characterization of the disorder has always presented difficulties.[251] Hypertension and proteinuria still remain the hallmarks of the contemporary definition of preeclampsia; edema and weight gain has been omitted from the previous triad. Although there is no universal definition of the *syndrome* of preeclampsia, hypertension and proteinuria are obligate features of the *disorder* preeclampsia.

10.1. Definition

To differentiate the severity of the hypertensive pregnancy disorders including preeclampsia, various classification systems have been proposed: American Collage of Obstetrics and Gynecology (ACOG),[172] National High Blood Pressure Education Program (NHBPEP),[252] Royal Collage of Obstetrics and Gynaecology (RCOG)[253] etc. The majority of these guidelines agrees on the basic definition of preeclampsia, but differs on the classification of the hypertensive disorders in pregnancy including gestational hypertension, mild preeclampsia, severe preeclampsia, syndrome of haemolysis, elevated liver enzymes, low platelets (HELLP), and eclampsia. The classifications are not to be understood as hierarchical systems; neither do they represent a chronologic development of symptoms.[254-256]

We extracted diagnoses from the NPR on gestational hypertension, mild and severe preeclampsia; HELLP syndrome and eclampsia were included in severe preeclampsia. In addition, we used the gestational age at delivery and presence of other pregnancy complications as markers of severity. In the genetic studies, all diagnoses of cases were validated according to the ACOG criteria.[172]

10.2. Genetics

The proposal for a genetic component of preeclampsia is not novel.[257-259] Previous genetic studies have focused on inherited thrombophilias as well as vascular and immunological polymorphisms.[111,260] The inherited thrombophilias (FVL, PTM, and MTHFR) have been extensively investigated with inconsistencies in their association, albeit PTM and MTHFR are most likely not linked to preeclampsia. Differences in study design are in part responsible for these inconsistencies: prospective cohort studies have not supported an association to FVL (OR 1.23; 95% CI 0.89-1.70),[125] whereas cohort studies in general, including also retrospective cohort studies, have a slightly higher estimate (OR 1.49; 95% CI 1.13-1.96).[126]

Contributing to the inconsistency is the heterogeneity of the phenotype studied: The severe form of preeclampsia is associated with FVL by a higher estimate (OR 2.24; 95% CI 1.28-3.94),[124] which may indicate that FVL is not linked to the actual initiation of the disorder, but rather the development into a severe form of it. Various other polymorphisms have been tested in respect to preeclampsia. Polymorphisms in the fat mass and obesity-associated (*FTO*) gene[261] and endothelial nitric oxide synthase gene (*NOS3*),[262,263] are promising, but need further investigation.

10.2.1. Own studies

In article VIII, we tested the association between severe preeclampsia and the 3 inherited thrombophilias (FVL, PTM and MTHFR) in a nested case-cohort design. For FVL, we found an OR 1.63 (95% CI 1.10-2.42), which is lower than expected as our outcome was the severe form of preeclampsia. Of note, in the genotype model, the estimate for heterozygote women was OR 1.94.

We found no association to PTM (OR 1.14; 95% CI 0.50-2.62), but a small association to MTHFR (OR 1.27; 95% CI 1.05-1.55), which was unexpected and most pronounced in the homozygote women (OR 1.74; 95% CI 1.13-2.69).

In article IX, we also tested the association between severe preeclampsia and 108 vascular associated polymorphisms. In this study we calculated Benjamini-Hochberg false discovery rates and q-values to evaluate the multiple comparison.[192] We found overall 17 polymorphisms associated with severe preeclampsia, including polymorphisms in *FTO* (homozygote OR 1.48; 95% CI 1.04-2.12) and *NOS3* (homozygote OR 1.56; 95% CI 1.02-2.38), with p-values ≤0.05, although all polymorphisms with q-values ≥0.43. This means that there is at least a 43% risk of false positive findings within these 17 polymorphisms, or at least 8 of the 17 polymorphisms are false positive. From the alpha-level of 0.05 we had expected 6 false positive findings (0.05 * 108 ≈ 6) in general.

We also stratified the analyses on preterm delivery and small-for-gestational age, which may be indicative of subsets of preeclampsia: Although we did find some polymorphisms with p-values ≤0.05, all of the 108 q-values were ≥0.97 and ≥0.72, respectively, and thus the study not supportive of any significant result in these subtypes.

10.3. Recurrence in next pregnancy

Preeclampsia is a strong risk factor for recurrence in the succeeding pregnancy; previous studies have indicated an overall risk of recurrence around 15%.[7,21,264] The severity of the preeclampsia will affect this risk: The gestational age at delivery functions as a proxy for the severity and confers a grading effect on the recurrence risk ranging up to 40% in preeclampsia remote from term.[7,265] However, the recurrence risk of *severe* preeclampsia is around 7%, corresponding to an OR 19.[266] Also, a trans-generational effect has been demonstrated putting women born of preeclamptic mothers in increased risk of developing preeclampsia themselves.[267]

10.3.1. Own studies

In article V, we showed that the gestational age is important for the recurrence risk: We found the overall risk of recurrence to be 16%, and in preeclamptic women delivering before GA 28 weeks this risk was increased to 38%, corresponding to a 4-fold increase compared to preeclamptic women delivered at term (see Table 3 below). Additionally, we found this grading effect transferred to fetal growth restriction, placental abruption, and stillbirth in the next pregnancy with odds ratios as high as 8, 5, and 6, respectively.[7] This links the disorders across pregnancies and supports a common proclivity to all complications.

Table 3. Preeclampsia in the first pregnancy and risk of complications in the second pregnancy.[7]

First delivery	Second delivery					
	Preeclampsia		Small for gestational age		Placental abruption	
Gest. age	Pct.	OR (95% CI)	Pct.	OR (95% CI)	Pct.	OR (95% CI)
≥ 37 wk	14%	1 (ref.)	3%	1 (ref.)	1.0%	1 (ref.)
32 - 36 wk	25%	2.08 (1.87-2.31)	10%	3.47 (2.95-4.09)	2.2%	2.38 (1.72-3.28)
28 - 31 wk	32%	3.00 (2.45-3.67)	17%	7.21 (5.55-9.36)	3.1%	3.72 (2.13-6.49)
20 - 27 wk	38%	3.89 (2.50-6.05)	17%	8.39 (4.74-14.8)	3.4%	4.77 (1.48-15.3)

Only preeclamptic women in first delivery were included.

10.4. Long-term follow-up of mothers

Half a century ago, suggestions on the long-term morbidity in previously preeclamptic women emerged spearheaded by the works of Leon Chesley.[268,269] Later studies indicated that this was restricted to preeclampsia remote from term,[223] and primarily to mortality from cardiovascular causes.[191,270,271] In the last decade, preeclampsia has also been linked to later hypertension,[270] ischemic heart disease,[270] hyperinsulinemia[272] and overt diabetes.[273]

10.4.1. Own studies

In article I, we investigated several cardiovascular diseases and diabetes in respect to hypertensive pregnancy disorders.[3] We found that severe preeclampsia conveyed an almost 8-fold increase of later hypertension (HR 7.58; 95% CI 7.05-8.14), gestational hypertension 6-fold (HR 5.94; 95% CI 5.55-6.36), and mild preeclampsia 4-fold (HR 3.87; 95% CI 3.70-4.05). Interestingly, we found that there was a marked increase in risk during the first year after a hypertensive pregnancy disorder and this sustained and develop in the years that followed (see Figure 6A below). At the age of 50 years, 20% of women with severe preeclampsia or gestational hypertension had a diagnosis of hypertension as compared to below 5% of women with no hypertensive pregnancy disorders (see Figure 6B below).

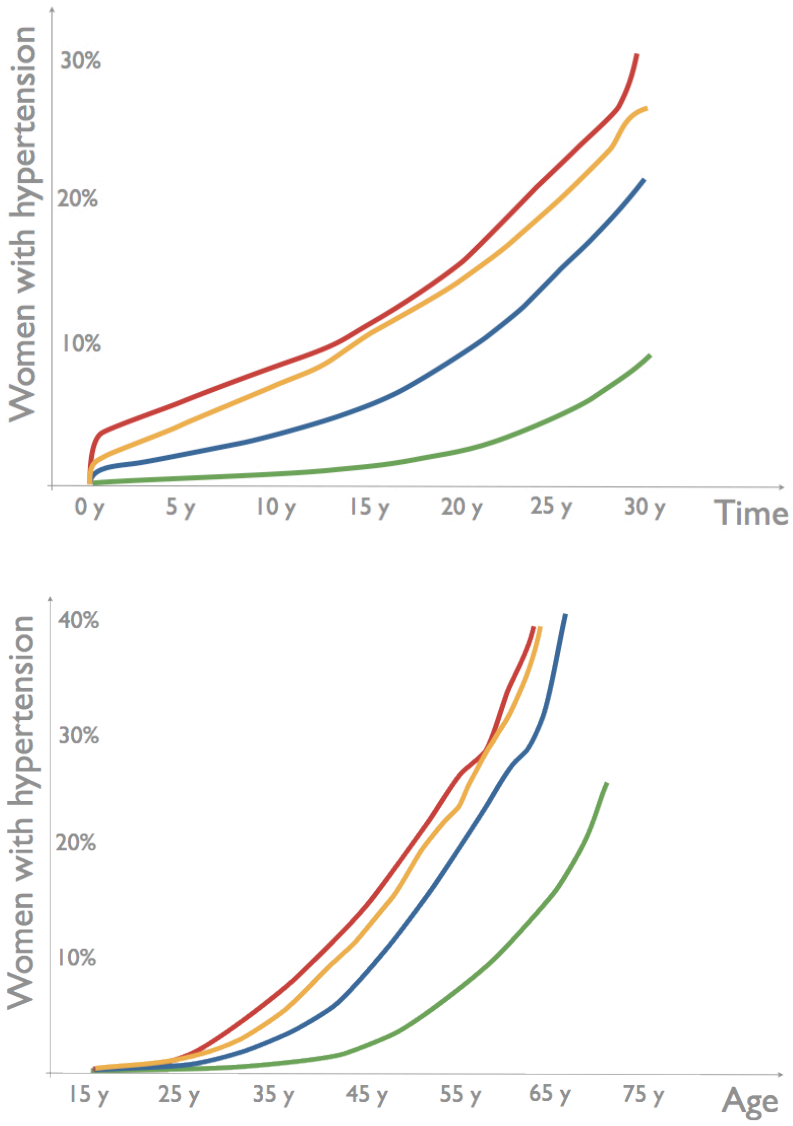

Figure 6. Kaplan-Meier plots of later hypertension following hypertensive pregnancy disorders.
Panel A, upper, shows the proportion of women diagnosed with hypertension in respect to time from first pregnancy.
Panel B, lower, shows the proportion in respect to the age of the women at the time of diagnosis.
Legend: Green line represents no hypertensive pregnancy disorders, blue line represents mild preeclampsia, yellow line represents gestational hypertension, and red line represents severe preeclampsia.

Notably, there was a stronger association following gestational hypertension than following mild preeclampsia: This may be due to underreporting and selection of the grave cases of gestational hypertension in the Registry, and an inclusion of non-cases in the mild preeclampsia diagnosis (low pPV[170]), but also hints differences in the spectrum of preeclampsia. Indeed, previous findings indicate a poorer perinatal prognosis of gestational hypertension compared to mild preeclampsia.[254]

We extended these previous findings and segregated the association in term and preterm preeclampsia, and mild and severe preeclampsia. In relation to later ischemic heart disease, we found an even stronger association in preterm preeclampsia (HR 2.51; 95% CI 1.90-3.33) compared to preeclampsia at term (HR 1.82; 95% CI 1.65-2.00).[3] The same effect of preterm preeclampsia was seen in relation to the risk of later diabetes, which was increased 4- to 7-fold for term and preterm preeclampsia, respectively. The risk of later thromboembolism was highest in preeclamptic pregnancies complicated by fetal growth restriction (HR 2.74; 95% CI 1.93-3.88), compared to preeclampsia at term (HR 1.61; 95% CI 1.39-1.87).[3]

Women having two preeclamptic pregnancies had an even higher risk of later cardiovascular morbidity and risk of diabetes as compared to only preeclampsia in the first pregnancy. Consistently, preeclampsia in the second, but not the first pregnancy conveyed a higher risk as compared to only preeclampsia in the first pregnancy. This latter finding could indicate in preeclampsia in parous women, the maternal constitution is more important than in nulliparous women: The "immune maladaption" hypothesis is more likely to be of importance in preeclampsia in nulliparas as in contrast to the maternal condition in parous women.[265]

11. Discussion

Hypertensive pregnancy disorders, fetal growth restriction, and preterm delivery all convey increased risk of later cardiovascular disease and diabetes. Also, these pregnancy complications tend to cluster in some women, hereby predisposing to recurrence and risk of other complications in a second pregnancy. Bleeding in early pregnancy is also a marker of poor obstetrical outcome: It predisposes to complications later in pregnancy as well as in the following pregnancy, and it is associated with prepregnancy cardiovascular disease and later cardiovascular events. The genetics of pregnancy disorders is still unresolved, and attention has so far been on known polymorphisms, such as FVL, which is linked to thromboembolism outside pregnancy[274] and possibly also severe preeclampsia and fetal growth restriction in pregnancy.

11.1. Strengths and weaknesses

We have endeavoured to unify the perception of a vascular obstetrical syndrome. To do this, we have replicated and expanded previous studies, and also tested novel hypotheses on bleeding in early pregnancy and tested vascular associated polymorphisms in respect to severe preeclampsia.

In our follow-up studies of pregnancy complications, we confirmed previous findings on later mortality, hypertension, stroke, and ischaemic heart disease, and expanded the endpoints to include thromboembolism and diabetes, and stratified the exposures to assess subtypes of the pregnancy complications. Caution must be applied in judging the estimates as no controlling for confounding effects was possible. The possible bias introduced by the imperfect validity of the diagnoses – both exposures and outcomes – will tend to blur the associations and bias toward the null. Due to the relative low follow-up time (median 14.6 years and 42 years of age at censoring), the findings represent a short-term increased risk in a low-risk population; this is also evident in the Kaplan-Meier plots (Figures 6A and 6B).

The recurrence studies expand previous knowledge by linking all complications to each other but stronger to the same complication. Due to the low sensitivity of some of the pregnancy

complications, residual confounding may account for part of the observed associations. However, we were able to demonstrate a pronounced grading-effect, which enhances the biological plausibility of the associations.

The study on early vaginal bleeding corroborates the association to preterm delivery and to complications later in the same pregnancy. We further showed that this risk is carried into the next pregnancy as well and reverse causality may be present.[8] Also, we present evidence for the association to both pre-pregnancy and later cardiovascular disease herby linking early vaginal bleeding to the vascular obstetrical syndrome.[9] Again, bias from the flawed validity of the diagnoses and residual confounding may justify part of the observed estimates.

Our study on thrombophilias is confirmatory, but given the great diversity in previous estimates, we present the first national Danish study. Also, the superior design allowed us to test all pregnancy complications in the same large population at a sensible cost. The case-cohort design diminishes selection bias in the control group, and the ratio between cases and controls were in severe preeclampsia and placental abruption lower than 1:4 indicating that limited additional power could be gain in a full cohort study. A strength was the merging of the data and the validation of the outcome: The phenotypic data was merged with the genotypes just prior to statistical analysis, and the personnel involved in the respective data handling were blinded to the other data; the study may thus be characterized as prospective. The validation of all cases provided accurate exposure groups.

The findings from the study on novel vascular markers in severe preeclampsia[11] were not replicated, as we were unable to find a matching population. The results are as such prone to false positive findings, which is also evident in the high q-values from the FDR-analysis. However, the polymorphisms should be examined further as they may provide insights to new pathways in the aetiology of preeclampsia.

11.2. Vascular Obstetrical Syndrome?

The strengths of the associations are varying from low in the genetic studies over moderate in the later cardiovascular disease follow-up studies to high in the recurrence studies. A high recurrence risk bears evidence of stable risk factors, such as a genetically determined susceptibility or maternal constitution.[275] Moderate risk of later cardiovascular disease suggests that the aetiology is not purely cardiovascular in nature, but multi-factorial, illustrated in the proportions of the "five paths".[276] Low strengths of genetic associations also suggest that no single gene or polymorphism is solely responsible for the disorders.[114]

Aggregated, these findings confirm that pregnancy complications are very heterogenic with diverse aetiologies, even in the same woman experiencing recurrences. Different proportions of the "five paths" will thus dilute the associations with vascular polymorphisms and with later cardiovascular disease, as not all of these are vascular related.

The collected findings of this thesis and previous studies link pregnancy disorders to cardiovascular and metabolic susceptible women; as such, a proclivity for cardiovascular disease and diabetes propels the woman into the Vascular Obstetrical Syndrome. The question arises whether the Vascular Obstetrical Syndrome is merely a pregnancy epiphenomenon of the well-known Metabolic Syndrome: Does the occurrence of this syndrome change the course of a woman's future health, or is it purely a statistical marker of susceptibility, that reiterates already known preceding risk factors?

In contrast, if the syndrome is summarizing or capturing unknown or unmeasured cardiovascular risk factors, the prognostic value of being diagnosed with the Syndrome is novel and relevant for the individual and populations alike. These questions are to date unanswered, and to uncover them we need large population data with ample control mechanisms for confounders and long follow-up times.[277] However, other types of studies and methods have revealed insights.

There seems to be a direct effect related to the number of pregnancies which a woman has, whether complicated or not, on later risk of cardiovascular disease[278] and diabetes.[279] This indicates

a permanent deterioration of the metabolic and endothelial system following pregnancy. Other studies demonstrate accelerated atherosclerosis during the preeclamptic pregnancies[42] and impaired vasodilation of the brachial artery three years after preeclampsia,[280] suggesting ongoing damage of the endothelium.

However, large follow-up cohort studies have indicated that controlling for some baseline confounders relevant to cardiovascular disease diminishes the estimates of later cardiovascular disease, suggesting of a non-causative association.[281] At present, there is, however, no consensus.[282]

11.3. Implications

The actual "susceptibility" of the individual woman is difficult to assess, the weight and interaction of the risk factors hard to judge, the individual threshold for developing symptoms almost impossible to determine, and unknown factors will inevitably be present. As such, counselling of the woman is not easy and ethical considerations are mandatory; the latter is especially important when testing for genetic markers as these may be transferred to the offspring.

Women planning additional pregnancies are faced with upcoming risk of poor obstetrical outcomes, and the testing for genetic markers may discriminate subset of women with even further increased risk of complications. If the recurrence risk by phenotype (e.g. preeclampsia) is independent of the risk by genotype (e.g. FVL), then the post-test probability of recurrence will increase in a FVL heterozygote woman with previous preeclampsia. However, some methodological issues in the latter context have cast doubt on this as the assumptions are flawed: This has been labelled "the thrombophilia paradox".[283]

When stigmatizing the woman with a statistical increased risk of pregnancy complications and/or cardiovascular disease, one is also obligated to offer her guidance to an alternative. This alternative is an effective prophylaxis. The study of pregnancy prophylaxis efficacy is reasonably feasible as the outcome is easily assessed and within a moderate timeframe. This is in contrast to cardiovascular prophylaxis, where future studies should both investigate short-term and long-term impact of the possible benefit as various aetiological factors in pregnancy disorders may influence the risk of cardiovascular disease at different points in time, for example at menopause[191] or just following pregnancy.[190] However, studies that provide solid evidence are not easily performed and documentation of an effect by the use of surrogate markers as well as descriptive studies with geographical or historical control groups may be a realistic foundation for future recommendations.

11.3.1. Pregnancy prophylaxis

Both non-medical interventions based on mostly lifestyle intervention, and medical interventions with various pharmaceuticals have been tested with regard to a next pregnancy.

The aim of lifestyle interventions is to modulate the maternal constitution to a more tranquil state hereby diminishing the overall risk of complications. The studies, which have focused on changes in body weight and dietary habits, have had moderate effects.[284-287]

Supplemental calcium also seems to lower the risk of preeclampsia with no deleterious side effects.[288] In contrast, supplemental fish oils have only a positive effect in women not regularly eating fish,[289] although too much fish may be harmful.[290]

The preventive effect of aspirin with regard to the risk of recurrence of preeclampsia – and probably also FGR – has been established and the treatment seems safe.[291] The proposed prophylactic effect acts via the relative inhibition of production of thromboxane more than of prostacyclin, which will counteract the deleterious effects on the endothelium hereby reversing the vasoconstriction and hypercoagulation.[292-294] The prophylactic effect seems to be restricted to women with severe preeclampsia as compared to mild,[295] and preterm preeclampsia compared to at term.[296] Also, the effect is most pronounced when started early in pregnancy.[297] These differences in effects also hint at the nature of the various forms of preeclampsia: The severe and preterm forms

have a dominating endothelial and haemostatic path as compared to the mild and term preeclampsia; this is also evident from our estimates on later cardiovascular disease.

Low molecular weight heparins (LMWHs) have been evaluated in various studies suggesting a protective effect on many pregnancy complications. Until recently, no proper randomized controlled study (RCT) had yet documented this, and the observational data presented was poor.[298] In the FRUIT-RCT study[299] from 2012, women with previous severe pregnancy disorders and thrombophilias were assigned to either LMWH or LMWH and aspirin: The risk of early hypertensive disorders were lower in women treated with LMWH and aspirin compared to only aspirin. However, some have argued that aspirin resistance[300] is responsible for this observed effect, in that LMWH only conceals the missing effect of aspirin in some predestined women; testing for aspirin resistance will thus identify the women benefitting from additional LMWH or an increase in aspirin dosage.[301] The effects of LMWH are based on the inhibition on factor Xa and IIa in the coagulation cascade, but other unspecific anti-inflammatory effects[302] and direct positive effects on the cytotrophoblast proliferation have been described,[303] herby justifying the rationale for the prophylactic effects in early pregnancy.

11.3.2. Long-term prophylaxis

Regardless of causality in the association between pregnancy disorders and later cardiovascular disease, the knowledge gained can be used to study a possible effect of lifestyle changes in these women who might have a Vascular Obstetrical Syndrome. Probably, we should already encourage lifestyle changes – that seems to be prudent and without side effects – such as smoking cessation, change in dietary habits, promote weight loss, and increase physical activity in daily life.[304]

Whether or not to prescribe medical prophylaxis is a different question. It is difficult to determine the optimal balance of possible benefits, probability of side effects, and use of resources in health care and society in general. No study yet has investigated women with obstetrical complications as possible candidates for intervention, and we are therefore left with other studies on high-risk populations, which are difficult to extrapolate to women with pregnancy complications.

Aspirin should probably be reserved for women with high risk of cardiovascular events as side-effects, such as bleeding episodes, may outweigh the benefit in the general population.[305] However, the protective effect and overall benefit in recurrent venous thromboembolism has been established,[306] but the slight increased risk of thromboembolism following pregnancy complications do probably not justify life-long prophylaxis.

Fish oils are considered harmless but do probably not lower the risk of cardiovascular events,[307] and calcium supplement may increase the risk of cardiovascular events,[308] also with the addition of vitamin D.[309] Vitamin C, a potent anti-oxidant, has been shown to reverse the impaired vasodilation in the years just following preeclampsia.[280] This might be a possible strategy for future research to investigate.

11.4. Revisiting the models

Revisiting the susceptibility model in Figure 5, if the actual experience of pregnancy complications leads to sustained damage to the endothelium, this would contribute to the increased recurrence risk in next pregnancy and the risk of premature cardiovascular disease; this is illustrated by the upper yellow dotted line and in the left-shift from d2 to d3 in Figure 7 below. Also, if prophylaxis – medical or non-medical – were effective, this would postpone the development of overt cardiovascular disease; this is illustrated by the lower yellow dotted line and the right-shift from d2 to d4 in Figure 7 below. Of note, even women having uncomplicated pregnancies may have alterations in cardiovascular and metabolic state in comparison to women not having any pregnancies, although these nulliparous women might represent a very different kind of women; this is illustrated in the lower green dotted line in Figure 7.

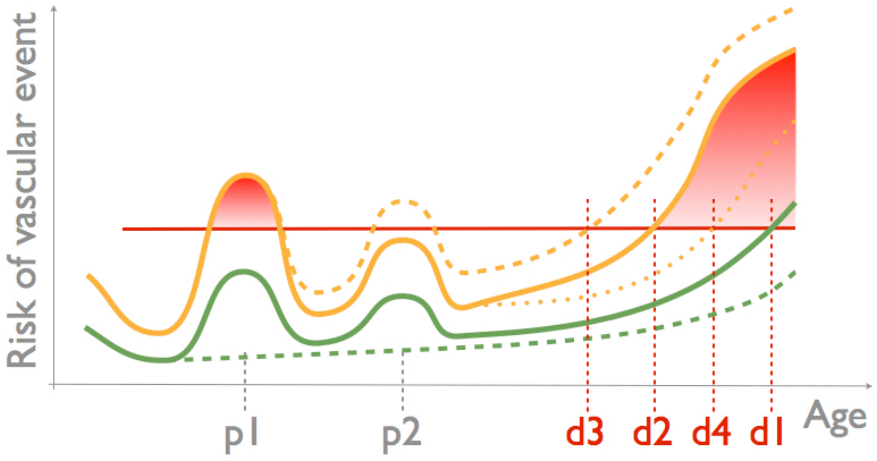

Figure 7. Accumulated risk of vascular event in two women with different risk profiles and three hypothetical scenarios (dotted lines). See text for further explanation. p denotes pregnancy, and d denotes diagnosis of cardiovascular disease. Adapted from Sattar and Greer, BMJ 2002,[1] with permission.

11.5. Conclusion

Complications in pregnancy identify a group of women consisting of more than 15%. These women are liable to be diagnosed with later cardiovascular disease and diabetes, which supports the hypothesis of a common origin and aetiology. Genetic factors may be present in this association, such as factor V Leiden, and may also define subsets of women especially prone to prophylactic measures. These prophylactic measures come from inspiration in cardiovascular medicine and have already proven effective in preventing the recurrence of severe preeclampsia.

The pregnancy complications differ in their strength of association to this common vascular obstetrical syndrome, and these associations are dependent on the proportions of aetiological paths in each pregnancy complication. The association to severe preeclampsia is the stronger one, and spontaneous preterm delivery the weaker one.

Whether these associations represent causal links or the pregnancy complications are only intermediary factors in the associations are still unresolved. Also, future studies are needed to investigate the effectiveness of secondary life-long prophylaxis – medical or non-medical – in women with the vascular obstetrical syndrome.

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