Linda Tømmerdal Roten

# Genetic predisposition for development of preeclampsia

Candidate gene studies in the HUNT (Nord-Trøndelag Health Study) population

Thesis for the degree of Philosophiae Doctor

Trondheim, August 2009

Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine

**NTNU**
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**NORGES TEKNISK-NATURVITENSKAPELIGE UNIVERSITET**
**DET MEDISINSKE FAKULTET**

**Linda Tømmerdal Roten**

# Genetisk disposisjon for utvikling av svangerskapsforgiftning. Kandidatgenstudier i HUNT (Helseundersøkelsen i Nord-Trøndelag) populasjonen.

Svangerskapsforgiftning (preeklampsi) er en av de viktigste årsakene til sykdom og død hos gravide kvinner i den vestlige verden, og på verdensbasis fører tilstanden til at omkring 63,000 kvinner dør per år. I Norge får omtrent 3% av alle gravide svangerskapsforgiftning. Syndromet består av blodtrykksstigning og protein (eggehvite) i urinen, eventuelt med væskeansamling (ødem) i kroppen. Blodtrykkstigningen og proteinutskillelsen utvikler seg gjerne i siste halvdel av svangerskapet, det vil si etter svangerskapsuke 20. Alvorligheten av utslagene varierer, de kan være alt fra bagatellmessige med lett blodtrykksstigning og litt protein i urinen til en tilstand som truer liv. Svangerskapsforgiftning er en uforutsigbar sykdom, og i enkelte tilfeller kan situasjonen forverre seg meget raskt. Som oftest er det bare kvinnen som utvikler tegn på preeklampsi, men noen ganger vil også fosteret involveres. En dårlig utviklet morkake (placenta) kan føre til sviktende oksygen- og næringstilførsel, noe som kan resultere i veksthemning hos fosteret. Per i dag eksisterer det ingen effektiv behandling, og hvis de preeklamptiske manifestasjonene blir så alvorlige at de truer kvinnens liv, må svangerskapet avbrytes og fosteret forløses eller fødes uansett hvor kort svangerskapet har kommet. Dette innebærer at preeklampsi er en viktig årsak til prematur fødsel. Omtrent 15% av alle premature fødsler er assosiert med svangerskapsforgiftning.

Svangerskapsforgiftning kan være arvelig, og enkelte familier viser økt forekomst av sykdommen. Ved å studere slike familier fant man ut at arvestoffet/genene påvirker om man utvikler svangerskapsforgiftning eller ikke. Det er åpenbart at sykdomsutviklingen inkluderer genetiske mekanismer og risiko faktorer, men det er fortsatt uklart hvilke, hvordan de virker inn og hvordan de virker sammen med andre. Den genetiske komponenten ble først tilskrevet maternell arv, men det har siden vist seg at både mor og foster (via fars gener) bidrar til den genetiske risikoen for svangerskapsforgiftning. Men genetikken i svangerskapsforgiftning er kompleks og har de samme kjennetegnene som vanlige komplekse humane sykdommer som for eksempel hjerte-kar sykdommer og diabetes. Disse sykdommene kjennetegnes ved at det ikke er noe entydig og klart arvemønster, og at det er effekten av mange gener, i kombinasjon med livsstil og miljøfaktorer, som bidrar til risiko.

Hel-genom skanning (koblingsanalyser) har blitt brukt til å kartlegge genetiske områder som predisponerer for sykdom i familier med økt forekomst av svangerskapsforgiftning. Slike familier har mest sannsynlig en sterk genetisk predisposisjon. I tillegg er slektninger veldig like i det genetiske materialet (DNA), dette gjør det enklere å lete etter områder som er koblet

til sykdom når man sammenligner syke og friske individer. Men selv om slike familiestudier har vært en suksess når det gjelder å finne kromosomområder som er koblet til sykdom, har det vist seg at å identifisere gener som predisponerer for svangerskapsforgiftning er vanskelig. Dette er derfor fortsatt en av de mest fundamentale og viktige utfordringene innen fødselsmedisin (obstetrikk).

Arbeidet som presenteres i denne avhandlingen har hatt som mål å identifisere gener hos mor som predisponerer for svangerskapsforgiftning innenfor kromosomområder som har blitt vist å være koblet til sykdommen (2q, 5q, 13q). Grunnlaget for at det har vært mulig å gjennomføre dette prosjektet er den andre store helseundersøkelsen i Nord-Trøndelag (HUNT2), der det ble samlet inn blodprøver fra alle deltagerne. Ved å sammenligne arvestoffet (DNA) fra kvinner som har hatt svangerskapsforgiftning (n=1.139) med kvinner som ikke har hatt svangerskapsforgiftning (2.269) har vi klart å finne gener (*SEPS1, ACVR2A, ERAP1, ERAP2, CRHBP, TNFSF13B*) som sannsynligvis er viktige for utvikling av sykdommen. Felles for disse genene er at de er viktige for at forholdet mellom mor og foster skal være bra under svangerskapet. Disse genene er viktige for at morkaken og blodårene mellom mor og morkaken skal utvikle seg normalt, eller for at mors immunsystem skal akseptere fosteret (som for mor oppleves som delvis ukjent på grunn av at halve arveanlegget er fra far). Noen av disse genene (*SEPS1, ACVR2A*) er også kjent å være involvert i andre sykdommer, som også er assosiert med svangerskapsforgiftning. Hjerte-kar sykdom er for eksempel mer hyppig blant kvinner som har hatt svangerskapsforgiftning, og noen av risiko faktorene og mekanismene som er involvert i sykdomsutviklingen er felles for disse to sykdommene. Også såkalte metabolske tilstander, med forstyrrelser i karendotel, sukker- og fett-omsetning, er mer vanlig blant kvinner som har hatt svangerskapsforgiftning. Det er derfor sannsynlig at kunnskapen om gener/genetiske mekanismer som er viktige i svangerskapsforgiftning også vil kunne gi relevant innsikt når det gjelder å forstå utvikling av hjerte-kar sykdom.

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# ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| ACVR2A | Activin receptor type 2A |
| AGT | Angiotensinogen |
| AGTR | Angiotensin receptor |
| ApoE | Apolipoprotein E |
| ASO | Allele specific oligo |
| Aust/NZ | Australia/New Zealand |
| BMI | Body mass index |
| BP | Blood pressure |
| CHD | Coronary heart disease |
| COL4A | Type IV collagen alpha |
| CRH | Corticotrophin releasing hormone |
| CRHBP | Corticotrophin releasing hormone binding protein |
| CS | Cesarean section |
| CTL | Cytotoxic T lymphocyte |
| CVD | Cardiovascular disease |
| DBP | Diastolic blood pressure |
| DNA | Deoxyribonucleic acid |
| eNOS | Endothelial nitric oxide synthase |
| EVT | Extravillous trophoblast |
| ER | Endoplasmic reticulum |
| ERAP | Endoplasmic reticulum aminopeptidase |
| FDR | False discovery rate |

| | |
|---|---|
| FGR | Fetal growth restriction |
| FRET | Förster resonance energy transfer |
| FVL | Factor V Leiden |
| GWAS | Genome-wide association study |
| HDL | High density lipoprotein |
| HIF | Hypoxia inducible factor |
| HLA | Human leukocyte antigen |
| H/R | Hypoxia re-oxygenation |
| HUNT | Health study of Nord-Trøndelag |
| HWE | Hardy-Weinberg expectation |
| ICD | International classification of disease |
| IGF | Insulin-like growth factor |
| IGFBP | Insulin-like growth factor binding protein |
| IL | Interleukin |
| IS | Ischemic stroke |
| IUGR | Intrauterine growth restriction |
| KIR | Killer cell immunoglobulin-like receptor |
| LD | Linkage disequilibrium |
| LDL | Low density lipoprotein |
| LNPEP | Leucyl-cystinal aminopeptidase |
| LOD | Logarithm of the odds ratio |
| LSO | Locus-specific oligo |
| MAD | Malondialdehyd |
| MBRN | Medical Birth Registry of Norway |
| MMP | Matrix metalloproteinase |

| | |
|---|---|
| MoBa | The Norwegian Mother and Child Cohort Study |
| MTHFR | Methylentetrahydrofolate reductase |
| NCBI | National Center for Biotechnology Information |
| NFQ | Non-fluorescent quencher |
| NGF | Norsk Gynecologisk Forening (The Norwegian Society of Gynaecology and obstetrics) |
| NK cell | Natural killer cell |
| OMIM | Online Mendelian Inheritance in Man |
| PCR | Polymerase chain reaction |
| PIGF | Placental growth factor |
| PIH | Pregnancy induced hypertension |
| QTDT | Quantitative transmission disequilibrium test |
| QTL | Quantitative trait locus |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SBP | Systolic blood pressure |
| sENG | Soluble endoglin |
| SEPS1 | Selenoprotein S |
| sFlt | Soluble fms-like tyrosine kinase |
| SGA | Small for gestational age |
| SNP | Single nucleotide polymorphism |
| SOD | Superoxide dismutase |
| STBM | Syncytiotrophoblast microparticles |
| STOX | Storkhead box |

| | |
|---|---|
| STR | Short tandem repeat |
| TDT | Transmission disequilibrium test |
| TGF | Transforming growth factor |
| TNF | Tumor necrosis factor |
| TNFR | Tumor necrosis factor receptor |
| uNK cell | Uterine natural killer cell |
| UPR | Unfolded protein response |
| UTR | Untranslated region |
| VEGF | Vascular endothelial growth factor |
| 2-ME | 2-methoxyestradiol |

## LIST OF PAPERS

I Moses, EK, Johnson, MP, Tømmerdal, L, Forsmo, S, Curran, JE, Abraham, LJ, Charlesworth, JC, Brennecke, SP, Blangero, J, Austgulen, R. **Genetic association of preeclampsia to the inflammatory response gene *SEPS1*** (2009). *American Journal of Obstetrics and Gynecology* **198,** 336 e1-5.

II Roten, LT, Johnson, MP, Forsmo, S, Fitzpatrick, E, Dyer, TD, Brennecke, SP, Blangero, J, Moses, EK, Austgulen, R. **Association between the candidate susceptibility gene *ACVR2A* on chromosome 2q22 and pre-eclampsia in a large Norwegian population-based study (the HUNT study)** (2009). *European Journal of Human Genetics* **17**(2), 250-7.

III Roten LT, Johnson MP, Løset M, Mundal SB, Forsmo S, Fenstad MH, Skorpen F, Dyer TD, Blangero J, Moses EK, Austgulen R. **Evaluation of *COMT* as a maternal preeclampsia candidate susceptibility gene, assessed by genotyping of the Val158Met polymorphism and by transcriptional profiling of decidual tissue** (2009). Submitted to *Molecular Human Reproduction.*

IV Roten LT, Johnson MP, Dyer TD, Forsmo S, East CE, Brennecke SP, Blangero J, Moses EK, Austgulen R. The ***ERAP2* gene is associated with preeclampsia in Australian and Norwegian populations**. Published online in *Human Genetics,* 2009, DOI 10.1007/s00439-009-0714-x..

V Roten LT, Johnson MP, Dyer TD, Forsmo S, East CE, Brennecke SP, Blangero J, Austgulen R, Moses EK. **Identification of *TNFSF13B* as a genetic risk factor for preeclampsia: replication of association in Australian and Norwegian populations** (2009). Submitted to *European Journal of Human Genetics.*

## SUMMARY

Preeclampsia is a major cause of maternal morbidity and mortality in the western world, and approximately 63,000 women dye yearly of the disease worldwide. This disorder is characterized by increased maternal blood pressure (hypertension) and proteinuria, sometimes also with edema. The clinical manifestations develop during the latter half of the pregnancy. However, preeclampsia is a condition which may aggravate very quickly, from minor (mild hypertension and traces of protein in the urine) to life-threatening manifestations. Generally, preeclampsia manifestations are maternal, but sometimes the fetus is also involved. The most important fetal sign is growth restriction, due to an insufficiently developed placenta and reduced supply of oxygen and nutrition. Unfortunately, women who develop preeclampsia have an increased risk of loosing their baby prior to, during or immediately after birth. Preeclampsia lacks an effective prevention strategy or curative treatment, and if the preeclamptic manifestations threaten maternal or fetal life, the only alternative is to deliver the baby and placenta, irrespective of gestational age. This implies preeclampsia as one of the most important causes of premature delivery.

Preeclampsia may be heritable, and some families show an increased occurrence of the disease. By studying such families, it was discovered that development of preeclampsia is influenced by the genetic material/genes. Genetic mechanisms and risk factors are obviously included in development of this disease, however it is still unclear which, what their influence is and how they work together with other mechanisms or risk factors. The genetic component was initially ascribed to maternal inheritance, but it has later turned out that both mother and fetus (via paternal genes) contribute to the genetic risk of developing preeclampsia. But the genetics of preeclampsia are complex and shares characteristics of common complex diseases

such as cardiovascular diseases and diabetes. These diseases are characterized by no clear-cut and distinct pattern of inheritance, and it is the effect of many genes in combination with lifestyle and environmental factors that contribute to the risk.

Whole genome scans (linkage analyses) have been used to map genetic loci that predispose to disease in families with increased occurrence of preeclampsia. These families are likely to have a strong genetic predisposition. In addition, it is easier to search for regions in linkage with disease by comparing affected and unaffected individuals that are related since relatives are very much alike genetically (in their DNA). Although these family studies have been successful in identifying chromosomal regions in linkage with disease, it has proven difficult to identify the actual genes predisposing to preeclampsia. Thus, this is still one of the fundamental and important challenges in obstetrics.

# 1 INTRODUCTION

## *1.1 Preeclampsia – maternal characteristics*

Preeclampsia is a pregnancy specific disorder affecting approximately 3-5% of all pregnant women in the western world,[1] exhibiting substantial maternal and fetal morbidity and mortality.[2] The world-wide prevalence of preeclampsia is estimated to be as high as 8%, although this rate is higher in developing countries[3] and amongst selected ethnic ancestry[4]. Approximately 63,000 women dye yearly of preeclampsia worldwide.[5] Preeclampsia typically develops in the latter half of pregnancy (after 20 weeks of gestational age), and is particularly prominent at the end of the pregnancy. The maternal preeclamptic syndrome is characterized by increased blood pressure (BP) (development of hypertension) and protein in the urine (proteinuria), and edema of the extremities is often witnessed together with these manifestations. Preeclampsia is an unpredictable disease, which may progress very rapidly and result in extremely high levels of maternal BP, convulsions and visual changes, potentially leading to organ and systemic failure. This final and very severe phase of preeclampsia denotes eclampsia. Women with eclampsia often have seizures, can fall into a coma, or at worst case the death of the mother and/or the baby. Eclampsia may occur before, during or immediately after birth, and the condition often leads to changes in the circulatory system and kidney failure, together with heart and lung failure.

## *1.2 Preeclampsia – fetal problems*

The function of the placenta is affected by preeclampsia, causing reduced blood flow and thereby lack of oxygen and nutrition to the fetus. Normally this is not life-threatening for the fetus but may lead to growth restriction. Unfortunately, preeclampsia sometimes causes death

of the baby. Women experiencing preeclampsia have an increased risk (odds ratio 1.5) of loosing their baby before, during or shortly after birth.[6] Another aspect is the fact that delivery of the baby and placenta is the only effective 'treatment' if the life of the pregnant woman is threatened, irrespective of gestational age. Therefore, preeclampsia is an important cause of premature birth, and more than 15% of all premature births in Norway have been shown to be associated with preeclampsia.[6] If preterm intervention is required by cesarean section (CS), promotion of short- and long-term complications from prematurity for the new-born baby (neonate) is evident.

### *1.3 Preeclampsia – an important health problem*

Pregnancy may be considered a maternal stress test, and the physiologic demands of pregnancy can predict a woman's health in later life and reveal risk of chronic diseases. Preeclampsia has been associated with an increased risk of developing metabolic syndrome later in life.[7-10] Metabolic syndrome refers to a group of conditions including hypertension, high blood levels of glucose and triglycerides, low levels of high density lipoprotein (HDL), too much fat around the waist, associated with increased risk of impaired glucose tolerance, type 2 diabetes, hypertension, dyslipidemia, obesity and heart disease.[11,12] The exaggerated responses reflective of the metabolic syndrome often seen in preeclampsia may herald future cardiovascular and metabolic disease risk(s).[13] In the HUNT population it appears that preexisting metabolic syndrome may predispose to preeclampsia.[14] Elevated levels of triglycerides, cholesterol, low density lipoprotein cholesterol, non-high density lipoprotein cholesterol and BP in women who later developed preeclampsia was observed,[14] in accordance with observations reported by others.[15,16] Women experiencing preeclampsia seem to be predisposed to cardiovascular disease (CVD) and vice versa, cardiovascular risk factors appear to predispose to developing preeclampsia. Could metabolic syndrome possibly be an

intermediate state between preeclampsia and CVD? Meaning that preeclampsia is an early manifestation in development of CVD?

Several studies have shown, and it is now widely accepted, that women with a history of preeclampsia have an increased risk of developing CVD later in life (reviewed by Harskamp et al.[17]). Examples of remote CVDs are hypertension, myocardial infarction, ischemic heart disease, and stroke. Women with severe preeclampsia and preterm delivery and those with recurrent preeclampsia seem to be at greater risk,[18-20] but even preeclampsia that occurs in the first pregnancy at term and never recurs is associated with increased later life CVD.[19] A study of data from the Medical Birth Registry of Norway (MBRN) show that the risk of death from cardiovascular causes among women with preeclampsia and a preterm delivery was about 8-fold higher than among women having normal pregnancies.[20] In general, patients with a history of preeclampsia seem to have higher risk of all-cause death[19,20] and significant increase of mortality from coronary heart disease (CHD) has been reported.[20-25] An increased risk of morbidity and mortality from ischemic stroke in women with a history of preeclampsia has also been reported.[26,27] These findings do not imply that every preeclampsia survivor is destined to develop CVD, but rather, a history of preeclampsia may identify a population at significantly increased risk for CVD. Whether this increased risk of CVD is due to underlying conditions that predispose women to both conditions or due to long-term sequelae of the preeclampsia syndrome is unknown.

Studies of long-term effects of preeclampsia on maternal BP have demonstrated that preeclamptic women are at increased risk of chronic hypertension.[28-30] For most women with a history of preeclampsia, BP regresses to normal after delivery. However compared with women who have experienced only normal pregnancies they have higher BPs.[31] BP regulation

and the kidney are closely linked, and renal function is altered with the dramatic hormonal and hemodynamic changes of pregnancy. Kidney disease has been suggested to be a likely long-term effect of preeclampsia. A Norwegian study reporting that preeclamptic women had a substantially increased risk for having a later kidney biopsy supported this hypothesis.[32] Recently the same research group linked data from the MBRN and the Norwegian Renal Registry and concluded that preeclamptic women do have an increased risk of end-state renal disease.[33]

## *1.4 Definition of preeclampsia*

Hypertension is considered to be the hallmark of preeclampsia by all existing classification systems, while proteinuria may be a late manifestation of preeclampsia. Although this disease has been known for a very long time, there is currently no international consensus on definition or diagnostic criteria of preeclampsia. There is a wide diversity of definitions and diagnostic criteria for preeclampsia[34,35] and these have also changed over time. Pregnancy-induced hypertension (PIH) could signify both gestational hypertension and preeclampsia to some, whereas others require PIH plus proteinuria to signify preeclampsia. Previous definitions included edema, however, since it occurs in many women with normal pregnancies it is not a suited discriminant. Thus, many classification schemes have abandoned edema as a marker for preeclampsia.[36-38] Although most define preeclampsia as a combination of hypertension and significant proteinuria today, there have been and probably still are inconsistencies in the definitions of hypertension and proteinuria. There seems to be an agreement to use absolute BP thresholds of 140 mmHg (systolic BP, SBP) and/or 90 mmHg (diastolic BP, DBP) to define hypertension in pregnancy.[39,40] Previously, an increase of DBP (15 mmHg) and/or SBP (30 mmHg) was also included in the diagnostic criteria and even

these increments in BP alone have been sufficient to define hypertension in pregnancy, even when the absolutes value remained below 140/90 mmHg.[40]

The methods/techniques (and instruments) used to perform the BP measurements probably vary significantly. This is a problem since this may lead to quite different BP readings.[35,41-43] It is recommended that gestational BP elevation should be defined on basis of at least two determinations, since measuring the BP successively may result in different readings. However, the measurements of increased BP should be no more than a week apart.[40] The 5th Korotkoff has been established as the sound closest to true diastolic pressure,[37,44,45] and it is recommended used,[45,46] also in Norway.[47,48]

A level of ≥0.3 g/L in a 24 hour urine sample has frequently been used for "significant" proteinuria, but also in measuring proteinuria there are different methods available. A qualitative ≥1+ dipstick reading usually correlates with ≥0.3 g/L in a random urine determination with no evidence of urinary tract infection, and is also used to define significant proteinuria. The standard practice of collecting 24 hour urine specimen for protein is time consuming, inconvenient and cumbersome, especially for patients who are not hospitalized. The urinary dipstick giving a qualitative result has been shown to be a poor predictor of 24 hour urine total protein level.[49-51] A dipstick value is less useful since a 1+ reading can produce many false positives and false negative results.[39,40] It is therefore recommended that the diagnosis be based on a 24 hour urine sample if possible. More recently, the use of a protein/creatinine ratio (≥0.3) has been suggested as an alternative to the standard practice of collecting a 24 hour urine for protein.[52-54] The accuracy of this test is still being investigated.

There is no doubt that an international consensus of both definition and diagnosis criteria of preeclampsia together with the use of identical methods to measure BP and proteinuria, would make comparisons between studies much easier to interpret. Table 1 summarizes the commonly used diagnostic criteria for hypertension and preeclampsia.

**Table 1: Commonly used diagnostic criteria for hypertension and preeclampsia.**

| | |
|---|---|
| Hypertension | SBP ≥140 mmHg and/or DBP ≥90 mmHg |
| Preeclampsia | Hypertension and significant proteinuria;<br>≥0.3 g/L in a 24 hour urine sample, or ≥1+ on a qualitative dipstick reading |

Attempts to categorize preeclampsia as mild or severe have also been made.[40,55] Severe preeclampsia is often defined on basis of BP levels of ≥110 mmHg diastolic and 160 mmHg systolic, severe proteinuria (defined as >3 g/ 24 hours), sudden oliguria, neurologic symptoms such as headache, laboratory tests demonstrating thrombocytopenia (defined as <100,000 per μL), hemolysis, abnormal liver function.[55,56] Efforts to recognize different subsets of women with preeclampsia, based on criteria such as gestational age at delivery and association with intrauterine growth restriction (IUGR), have also been made. Early onset preeclampsia, i.e. onset before 34 weeks of gestation, has been associated with greater morbidity than late onset preeclampsia presented at term.

### *1.4.1 Preeclampsia in Norway*

**The Norwegian Society of Gynecology and Obstetrics**

The diagnostic criteria for preeclampsia in Norway are set by The Norwegian Society of Gynecology and Obstetrics (NGF). The first written national (Norwegian) guidelines to clinicians diagnosing pregnant women were introduced in 1995.[57] Before 1995 international

classification of disease (ICD) codes were used to define preeclampsia. ICD-8 codes were available from 1965,[58] but were generally not used until 1969 in Norway.[59] Relevant ICD-8 codes are 637.0 Preeclampsia, 637.1 Eclampsia and 637.9 Toxemia, unspecified. In 1986 clinicians started to use ICD-9 codes[59,60] to define preeclampsia and used these codes until the NGF guidelines were introduced in 1995. The following ICD-9 codes were used to define preeclampsia 642.4 Mild or unspecified preeclampsia and 642.5 Severe preeclampsia. The Norwegian version of the ICD-9 code 642.2 was Hypertension in pregnancy, intrapartum or postpartum, not previously diagnosed, with albuminuria, edema or both – specified as mild or unspecified, while ICD-9 642.5 was Increased blood pressure in pregnancy, intrapartum or postpartum, not previously diagnosed, with albuminuria, edema or both – specified as severe. Since there were no written national guidelines before 1995, it is difficult to determine what diagnostic criteria were used before 1995. In the NGF guidelines from 1995 the diagnostic criteria for preeclampsia were 1) blood pressure ≥140/90 mmHg or an increase of diastolic blood pressure ≥15 mmHg compared with mean blood pressure values measured before 20 weeks of gestation and 2) proteinuria with ≥0.3 g/L in a 24 hour urine sample or ≥1+ on a dipstick reading after 20 weeks of gestation.[57] In the revised NGF guidelines published in 1998 the criteria for the BP changed to increased BP ≥140/90 mmHg measured at least two times, while the criteria for proteinuria remained the same.[61] The 1998 guidelines were used until new guidelines were introduced in 2006.[62] The criteria for mild preeclampsia in the guidelines from 2006 were 1) blood pressure ≥140/90 mmHg combined with 2) proteinuria ≥0.3 g/L in a 24 hour urine sample or a ≥1+ on a dipstick reading measured on at least two occasions with a 4-6 hour interval. The guidelines from 2006 also specified the following diagnostic criteria for severe preeclampsia by (one or more of the following manifestations) blood pressure ≥160/110 mmHg, proteinuria ≥3 g/L in a 24 hour urine sample, concentrated urine with oliguria (<500 mL/24 hours) or increased serum creatinine, thrombocytopenia

(<100 x 10*9/L) or signs of microangiopathic hemolytic anemia, increased levels of liver enzymes, severe epigastric pain, nausea and vomiting, severe headache and other cerebral/visual disturbances, pulmonary edema, cyanosis. The diagnostic criteria for preeclampsia in the 2006 guidelines (with at least two blood pressure and proteinuria measurements required) were unaltered in the latest release of NGF guidelines in 2008,[63] which to this day (April 2009) remain the guidelines used to diagnose preeclampsia among medical doctors in Norway.

### *1.4.2 Preeclampsia in the HUNT case/control cohort*

Preeclampsia in our Norwegian HUNT case/control cohort was defined according to the criteria given by National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy.[40] Preeclampsia was defined as the onset of persistent hypertension (BP ≥140/90 mmHg) in combination with proteinuria (≥0.3 g/L in a 24 hour urine sample or a 1+ on an urine dipstick reading) after the 20th week of gestation.

## *1.5 Etiology and pathogenesis of preeclampsia*

Preeclampsia is a disorder of numerous etiological hypotheses pertaining to immunological, vascular, ischemic and inflammatory biological mechanisms with genetic susceptibility and environmental risk factors (reviewed in [64-66]). The etiology of preeclampsia is likely to be a combination of all of these factors. Known risk factors for preeclampsia (reviewed in ref [67]) include: 1) maternal factors, such as primiparity, previous preeclamptic pregnancy, multiple pregnancies (e.g. twins), obesity, and pre-existing medical conditions such as chronic hypertension, diabetes, renal disease, autoimmune disease and antiphospholipid syndrome, and maternal age,[1,68] 2) family history of preeclampsia and 3) ethnicity. The time between

pregnancies and change of partner have also been suggested to associate with an increased risk for developing preeclampsia.[69-71] It is also evident that interactions between the mother and the fetus via the placenta and decidua are important.

Whilst the clinical gestational hypertensive and proteinuric manifestations usually present themselves after 20 weeks gestation, the mechanisms underlying these manifestations are likely to occur at much earlier gestational age. This dichotomy of pathophysiological events has therefore been proposed to represent a two-stage process; with poor placental perfusion (Stage 1) leading to the maternal responses evoked with varying degrees of clinical manifestations (Stage 2).[72] It has been known for nearly 100 years that preeclampsia is probably a placental condition.[73,74] Preeclampsia only develops if a placenta is present, i.e. the starting point is postulated to derive from the placenta.[75] Therefore, a central role of the placenta in the pathogenesis of this disease is undisputed. Poor placentation is a powerful predisposing factor, usually leading to the maternal syndrome of preeclampsia. But, although reduced placental perfusion seems required for development of preeclampsia, it is obviously not sufficient when exploring the linkage between reduced placental perfusion and the maternal systemic manifestations. Recently, the Two Stage Model has been modified (Figure 1a),[76] based on the hypothesis that abnormal implantation/placentation occurs before abnormal vascular remodeling of spiral arteries.[77] In addition, it is suggested that maternal constitutional factors (genetic, behavioral or environmental) may play a more extensive role than previously thought. Development of preeclampsia seems to depend on an interaction between the reduced perfusion and maternal genetic predisposition.[78] It is likely that there are several factors, which may differ in different women, serving as linkers between the two stages (Figure 1b).[76] Until recently the linking factor(s) has been considered as a pathogen or toxin, however, factor(s) modifying maternal metabolism to increase nutrient availability and

acting on the placenta to facilitate nutrient transfer is another possibility.[76] Whilst there is epidemiological evidence suggesting a paternal (fetal) contribution,[70] it is unknown what role fetal genes may play.

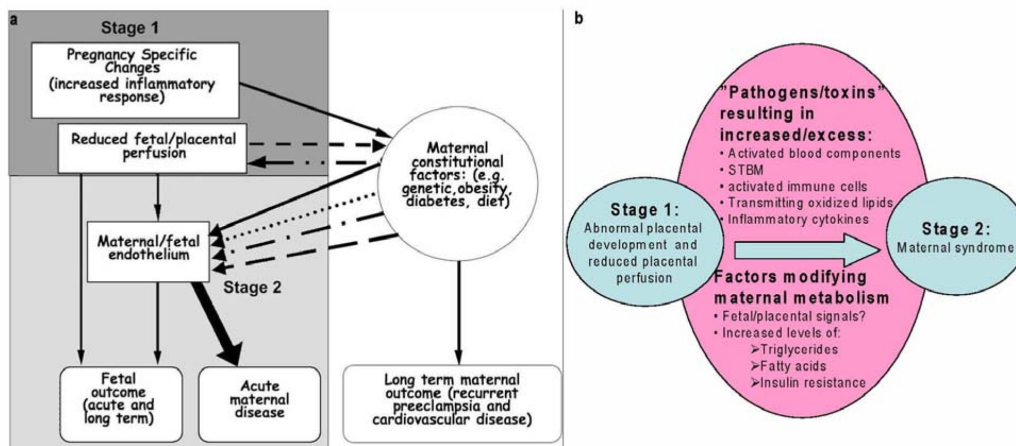


**Figure 1. Revised Two Stage Model and suggested factors linking Stage 1 and Stage 2.[76] (a)** Abnormal placentation occurs in first trimester and is the important contributor to reduced placental perfusion (Stage 1). Stage 1 results in release of factors from the fetal/placental unit ( – – – ➔) that may influence maternal physiology. Women who do not tolerate these constitutional changes develop preeclampsia, the same constitutional changes may also induce placentation abnormalities ( ⟵ · · – ). **(b)** There are likely several factors linking Stage 1 and Stage 2. Previously these were considered as pathogens/toxins, however more recently linkers have been considered as factors modifying maternal metabolism. STBM=syncytiotrophoblast microparticles.

The theories on the cause of preeclampsia are often depicted from two different views, a vascularist's or an immunologist's. However, one should bear in mind that there is no clear distinction between vascular and immune events since most, if not all, cytokines secreted within the immune system have pleiotropic properties.[79]

### *1.5.1 The vascular view*

In a normal pregnancy invading cytotrophoblasts displace vascular endothelial cells in the uterine spiral arteries by replacing muscular and elastic components with a fibrinoid layer, resulting in high capacity, low resistance tubes unresponsive to vasoreactive stimuli. Poor

placentation, revealed as insufficient cytotrophoblast invasion and defective remodeling of maternal spiral arteries, is a widely recognized predisposing factor for preeclampsia. Insufficient uteroplacental circulation leading to hypoxia, oxidative stress and, in most severe cases, infarction may be the result.[80,81] However, there are different views as to how this arises. One view is that the inadequate remodeling of the spiral arteries during placentation results in reduced blood flow,[82] which implicates chronic hypoxia in the placenta.[83] A second view is that the volume flow is normal, but ischemia reperfusion is created because of intermittent blood flow through narrow spiral arteries that retain their smooth muscle.[84] Subsequently, oxidative stress and reactive oxygen species (ROS) result from variable oxygenation in the intervillous space because of the intermittent high velocity flow.[83] Whatever mechanism at play, the end result would be placental oxidative stress and dysfunction. Reduced placental perfusion has been confirmed by Doppler ultrasound assessment of blood flow velocities in the uterine arteries which have indicated an increased incidence of patterns associated with high resistance.[85,86] Recently it was proposed that the primary placental problem generating the preeclampsia syndrome is likely to be oxidative stress rather than hypoxia.[83,84] It has now become widely accepted that the insufficient utero-placental oxygenation in preeclampsia results in placental secretion of soluble factors into the maternal system inducing maternal endothelial cell dysfunction and the clinical features of preeclampsia.[87]

The systemic inflammation that is commonly evident in all pregnancies is often exacerbated in a preeclamptic pregnancy.[64,75] Therefore the clinical features of preeclampsia always overlap with those of normal pregnancy. Inflammation is an attempt by the body to restore and maintain homeostasis after injury and is an integral part of the body defense, involving the vascular system of the body. In the late 1980s and early 1990s it became apparent that

preeclampsia, in common with many other vascular disorders, was a state of endothelial cell activation.[88] This endothelial cell activation appears to be part of a generalized intravascular inflammatory response involving maternal leukocytes, the coagulation and complement systems.[64] However, the origin of endothelial dysfunction in preeclampsia is still unknown, and it is likely to be diverse. The link between abnormalities in trophoblast invasion and generalized maternal endothelial dysfunction seen in preeclampsia may be via release of placental factors.[75]

**Trophoblast microparticles**

The placenta undergoes a continuous process of growth and apoptotic events in which numerous microparticles are produced. Microparticles are cellular, membrane-bound vesicles that mediate cell-to-cell communication with many potential roles in a number of biological processes such as inflammation, angiogenesis, hemostasis, and thrombosis.[89] In conditions associated with enhanced systemic inflammation, the levels of circulating microparticles are increased.[90,91] This is the case in the third trimester of a normal pregnancy, and to a greater degree in preeclamptic pregnancies.[92-97] Particularly syncytiotrophoblast microparticles (STBM), which form the maternal-placental interface, are suggested to directly damage endothelial cells and also to stimulate systemic inflammatory responses.[98] Several other factors, including leukocyte and platelet membrane particles, ROS, activated neutrophils, cytokines, growth factors, angiogenic factors and hormones are also released during the apoptotic cascade. These factors will then interact with maternal vascular endothelium which may already be damaged.

**Oxidative stress**

The placenta is the essential interface between maternal circulation carrying oxygen rich blood and the fetal circulation. In early pregnancy the outermost tissue of the conceptus, mainly placental syncytiotrophoblasts, contains very low antioxidant enzymes, making these cells particularly sensitive when exposed to the highest concentrations of oxygen coming from the maternal circulation. Pregnancy is therefore a state of oxidative stress arising from increased maternal metabolism and the metabolic activity of the placenta. Oxidative stress is suggested to arise from an overproduction of ROS, which exceeds the capacity of the antioxidant defences, due to hypoxia re-oxygenation (H/R) injury. There is strong evidence that preeclampsia is a state of excessive oxidative stress (reviewed in [84]), which may cause endothelial dysfunction.[99] Considering the unique role of the placenta, it is likely that this organ is the origin or major source of oxidative stress observed in preeclampsia.[100] Placental hypoxia and re-oxygenation may also stimulate placental synthesis of cytokines e.g. TNF-α,[101] and could lead to lipid peroxidation. Lipid peroxides bind to lipoproteins and are thereby transported to distant sites in the body where they may cause damage and result in systemic oxidative stress. Increased lipid peroxidation has been reported in preeclampsia.[102-106] Many triggers of placental oxidative stress have been suggested, including high concentrations of plasma endothelin-1,[107] increased superoxide generation via the enzyme NADPH oxidase,[108-110] lower levels of superoxide dismutase (SOD)[105,111] and glucose 6-phosphate-dehydrogease[111], lower activity of Cu/ZnSOD and glutathione peroxidase and lower levels of vitamin E.[112] Several markers of oxidative stress, such as malondialdehyde (MAD) and isoprostane 8-iso-PGF-2α are elevated in preeclampsia.[104,113,114] It is now widely hypothesized that placental synthesis of ROS followed by free radical-associated endothelial dysfunction may provide the link between reduced placental perfusion and the systemic maternal disease in preeclampsia.[115]

**Angiogenic factors**

Factors related to angiogenesis, the process of new blood vessel development from existing endothelium, have in the recent years been given much attention in preeclampsia research. Angiogenesis is essential for normal placental development, and is promoted by angiogenic factors released in placenta. Two extensively studied angiogenic factors, vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), are suggested to be crucial for normal trophoblast proliferation and implantation[116] and therefore normal pregnancy.[117,118] VEGF has been proposed to be an useful marker of early vascular damage since it is secreted in response to tissue hypoxia and endothelial cell damage.[119] Therefore changes in circulating levels of these angiogenic factors may be promising markers in the prediction of early-onset preeclampsia.[120] Both placental levels of VEGF mRNA and maternal serum levels of PIGF have been shown to be much lower in preeclamptic women compared to normal pregnant women.[121] However, many of the recent studies have concentrated on factors which antagonize VEGF and PIGF to assess their role in the development of preeclampsia. Two key anti-angiogenic factors, also produced by the placenta, are soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sEng). Both these factors have been implicated in the pathogenesis of preeclampsia whereby they have been shown to be over abundant in preeclamptic women compared to non-preeclamptic women.[116,122-129] However, this is not always the case, some preeclamptic women have sFlt-1 within the normal range.[72]

### *1.5.2 The immune maladaption view*

The cornerstone of the immune system is the recognition of "self" versus "non-self", implicating that donor organ transplants expressing human leukocyte antigens (HLAs) different from the recipient's will be rejected. However, in a normal pregnancy the fetal allograft presenting paternal antigens considered "non-self" by the maternal host is accepted.

An uteroplacental immune privilege is developed as a consequence of cooperative interactions between the fetus and the mother (reviewed in [130]). The tissues located at the immune privileged maternal-fetal interface (placenta, decidua) are protected against cytotoxic T lymphocyte (CTL)-mediated destruction by not expressing the polymorphic classic class-I HLA molecules, apart from HLA-C.[131,132] Instead the invading fetal trophoblasts express the non-classical molecules HLA-G,[133] -E and -F,[134] thus excluding them from being destroyed by the natural killer (NK) cells which are programmed to recognize HLA-null cells. Other factors expressed by trophoblasts, such as death-inducing members of the TNF superfamily ligands (e.g. FasL and TNF- α) also confer to immune privilege.[135-140]

In a preeclamptic pregnancy however, the maternal "acceptance" of the fetus allograft is perturbed in a manner akin to the immunological dynamics as seen in organ graft rejection.[141] The maternal-fetal immune maladaption has been suggested resulting from a disturbed interaction between the maternal NK cells and invading fetal trophoblasts, which are suggested to contribute to the depth of trophoblast cell invasion during implantation/placentation.[142] The depth of trophoblast cell invasion is pertinent to successful pregnancies whereby inadequate, shallow trophoblast cell invasion is a precursor to insufficient decidual spiral artery remodeling.

The placenta is a source of many immunomodulatory hormones and cytokines, and several of these factors are suggested to be released at the maternal-fetal interface or into the maternal circulation contributing to the regulation of the local and systemic immune changes required for a successful pregnancy.[143,144] Preeclampsia has been shown to be associated with aberrant placental production of immunomodulatory factors.[145,146] An abnormal activation of the maternal immune system has been reported in preeclampsia (reviewed in [147,148]), with an

impaired activation of immune cells like Th1/Th2 and NK cells.[149,150] This fits with the general assumption that the heterogeneous maternal symptoms of preeclampsia may all be ascribed to a generalized endothelial dysfunction,[88] which contributes to an exaggeration of the systemic inflammation that occurs in normal pregnancy.[75]

**Inflammatory mediators**

In general, cytokines which are central players of the inflammatory response are secreted by both the immune system and adipocytes.[151,152] The enhanced inflammatory state observed in preeclampsia is associated with maternal leukocyte activation, increased cytokine release and interaction between leukocytes and endothelial cells.[153] The circulating levels of TNF-α and interleukin-6 (IL-6), that are elevated in normal pregnant women compared to non-pregnant controls, are further raised in preeclamptic women.[154-158] Increased serum or plasma levels of other pro-inflammatory cytokines and of their modulators, such as IL-2, IL-8, IL-12, IL-15 and IL-18. The IL-1 receptor antagonist (IL-1Ra), soluble IL-4 receptor and soluble TNF receptor have also been observed.[155,159-164] Cytokine imbalance and elevated expression of the pro-inflammatory molecules TNF-α, IL-1 and IFN-γ are also evident in preeclamptic placentas,[165-168] and it is undoubted that a placental contribution is likely to be significant. However, the cellular source of pro-inflammatory cytokines in preeclampsia has been difficult to identify. The main pro-inflammatory cytokines TNF-α and IL-1 stimulate both structural and functional alterations in endothelial cells,[169] and placental TNF-α and IL-1 have been suggested to be potential mediators of maternal endothelial dysfunction in preeclampsia.

## 1.6 Genetics

### 1.6.1 The DNA revolution

The determination of the three-dimensional structure of DNA in 1953 by James Watson and Francis Crick[170,171] revolutionized science and the made basis for new research. Knowing the DNA's structure immediately solved one of biology's greatest mysteries - how genetic instructions are passed on from one generation to the next. This finding opened the door to what has become one of the hottest areas of medical research – **genetics**. Human genetics describes the study of inheritance as it occurs in human beings, while medical genetics seeks to understand how genetic variation relates to human health and disease. Research on the causes and inheritance of genetic disorders can be considered within both human and medical genetics.

Although the structure of DNA showed how inheritance worked, it was still not known how DNA influenced the behavior of cells, which are the fundamental working units of every living system. All the instructions needed to direct their activities are contained within the DNA. **The human genome project** was started in 1990, and among the goals of this project was to identify all of the approximately 25,000 genes in the human DNA and to determine the sequence of the approximately 3 billion chemical base pairs that make up the human DNA sequence. Sequencing of the human genome[172,173] was completed in 2003, and the technology and resources generated by this project are already having a major impact on research across the life sciences.

The genome of any two unrelated individuals is close to identical (99.9%),[174-177] but many genetic variations in the human genome have been observed. The two most important structural classes of sequence variation are microsatellites or short tandem repeat

polymorphisms (STRs) and single nucleotide polymorphisms (SNPs). Microsatellites are tandem repeats of a simple DNA sequence consisting of single repetitive nucleotide or di-, tri-, tetra-, or pentanucleotide repeats. Microsatellites are highly variable and most people are heterozygous at any given locus, meaning that one can distinguish between the maternally and paternally inherited alleles. The alleles are differentiated by the number of repeats (e.g. $(CAG)_n$ indicates that the CAG 3-base sequence is repeated n times). Microsatellite sequences tend to appear in non-coding regions, while SNPs, representing variation at a single nucleotide position, are distributed throughout the genome in both coding and non-coding regions. SNPs are the most abundant form of genetic variation and at present there have been identified more than 14.5 million RefSNP in the human genome, approximately 6.5 million being validated.(www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi) The genetic differences observed between humans are caused by genetic recombination and various mutational events. Two unrelated individuals are expected to differ at approximately 1 in 500-1,000 nucleotides[174-177] and these differences provide a wealth of information regarding the elements responsible for the phenotypic differences among them. Natural selection and a high degree of neutral mutations mainly due to genetic drift are causes for the genetic variation observed at a population level. A central goal of genetics is to pinpoint the DNA variants that contribute most significantly to population variation in various traits. Human genetic diversity appears to be limited not only at the level of individual polymorphisms, but also in the specific combinations of alleles (haplotypes) observed at closely linked sites.[178-182] An important aspect of SNPs with regard to their use in whole-genome scans is that they can be inherited together in haplotype or linkage disequilibrium (LD) blocks.[183,184] LD describes a situation in which some combinations of alleles or genetic markers occur on the same haplotype more or less frequently in a population than would be expected. Non-random associations between polymorphisms/alleles at two or more different loci are measured by the

degree of LD. Haplotype or LD blocks consist of SNPs in LD with each other where little historical recombination has occurred. Therefore, as genotypes of SNPs in the same block tend to be correlated, not all SNPs in a block need to be directly assayed. **The International HapMap Project**[185] aimed to develop a haplotype map of the human genome thereby characterizing the structure of sequence variation throughout the genome of various populations and to make this information freely available in a public domain. This has provided a guide for selecting SNP markers for mapping genes underlying common, multifactorial disorders. Regions of LD were mapped out, leading to the opportunity of performing whole genome scans based on indirect association. The use of SNPs has revolutionized human genetics and a new paradigm has been introduced into genomic research with the HapMap, by making possible the cost-efficient assessment of much of the common genomic variation within an individual.[186,187] In regions of high LD, i.e. strong association between SNPs, it is appropriate to genotype only a few, carefully chosen haplotype tagging SNPs (tag SNPs) since these will provide enough information to predict much of the information about the remainder of the common SNPs in that region. This together with development of appropriate genotyping methods and analysis has now made it possible to perform a genome-wide association study (GWAS) for up to 1 million SNPs at a time.

**Hardy-Weinberg equilibrium** (HWE) is the fundamental starting point for all population-based genetic investigations, whether the goal is detection or estimation of the effects of all the factors that disrupt HWE. HWE states that both allele and genotype frequencies in a population normally remain constant from generation to generation.[188] The principle of HWE provides a baseline to determine whether or not gene frequencies have changed in a population and thus whether evolution has occurred. Deviations from HWE can be due to

non-random mating (inbreeding), population admixture or stratification, limited population size, mutations, random genetic drift and gene flow. Random genetic drift refers to evolution occurring through random changes in allele frequency over time, while gene flow refers to evolution that occurs because individuals move among populations. Sampling error including genotyping error can also lead to deviation from HWE, and researchers often test for HWE as a data quality check. However, deviation from HWE can also result from disease association.[189] The possibility that a deviation from HWE is due to a deletion polymorphism[190] or a segmental duplication[191] that could be important in disease causation, should be considered before discarding loci.

### *1.6.2 Study design*

The study design is crucial for the chances of a successful outcome in genetic studies. Attention should be paid to both ascertainment and experimental design, in order to maximize the power of a study. Knowledge about the phenotype of interest is also important. Cost has been and still is an important limiting aspect when planning a genetic study, especially association studies. The cost of a genetic study is largely determined by the number of individuals to be recruited, phenotyped, and genotyped, however the number of markers to be typed also plays a role. Thus an optimal strategy for balancing cost and power of the study should be determined. Until recently investigators were not able to perform GWA studies, due to the limited access to affordable genome-wide SNP typing technology. Although, genotyping technology has considerably improved and become cheaper, investigators are now faced with new challenges such as choosing platforms and products best suiting their research. In addition to overall cost, one must consider the coverage, efficiency, and redundancy of a platform.

**Genome-wide linkage analysis**

A genome-wide scan (genetic linkage analysis) to map regions of the genome (loci) that contain genes that predispose to disease is generally the first step in unraveling genetic patterns and inheritance. Linkage extends over much longer regions of the genome than does LD, and linkage analysis often identifies broad genomic regions that might contain a disease gene or genes. Linkage describes the association of two or more loci on a chromosome with limited recombination between them. In other words, two genetic loci are linked if they are transmitted together from generation to generation more often than expected under independent inheritance (i.e. more than 50% of the time). The reason alleles at two linked loci tend to be co-inherited is that recombination is unlikely to occur between them in a given meiosis, because of their physical proximity. Families with increased occurrence of the disease of interest are very useful in linkage studies. Multiple-case families are most likely to carry a strong genetic predisposition. In addition, related individuals are very similar in their genetic material (DNA) making the search for regions containing disease genes easier to detect when comparing affected and healthy individuals.

By genotyping genetic markers (microsatellites or SNPs) at regularly spaced intervals across the genome and studying their segregation through families, it is possible to infer their position relative to each other on the genome. If a given marker tends to be passed down through a family in conjunction with a disease, this suggests that a gene with a functional effect, potentially due to causative genetic variant(s), is located in close proximity to that marker. However, this does not mean that the marker and the causative variant(s) are within the same gene but may lie within a particular genomic interval. Linkage analysis, particularly in studying complex diseases, can only identify large genetic regions. These linkage regions often contain hundreds of genes, many of which may seem like plausible biological

candidates. The identification of genomic regions linked to a particular disease posits the next challenge to prioritize positional candidate genes for further investigation.

### Genetic association analysis

Genetic association studies aim to identify loci that contribute to disease susceptibility by comparing patterns of genetic variation between individuals with a disease (cases) and those without (controls).[187] The principle aim of this approach is to detect association between genetic polymorphism(s) within gene(s) and a trait or disease of interest. There are two types of association studies: the candidate-gene approach (hypothesis driven) and the genome-wide approach (hypothesis generating). In this section, the candidate-gene association approach is in focus, whereas the genome-wide approach is described in chapter 6. In genetic association studies an *a priori* assessment of candidate genes relevant to the pathophysiology of the disease of interest are made, usually on the basis of biological hypotheses or the location of the candidate within a previously determined region of linkage. Association differs from linkage in that the same allele(s) is associated with the trait in a similar manner across the whole population, while linkage allows different alleles to be associated with the trait in different families.[192] The fact that association operates only over short distances in the genome has for long guaranteed association studies an important place in fine mapping genetic loci initially detected by linkage. It is now widely accepted that genetic susceptibility to common complex disorders probably involves many genes, most of which have small effects. Association studies have greater power than linkage studies to detect small effects.[192] In addition, a large number of SNPs throughout the genome has been identified and development of technology enables genotyping of numerous SNPs (≤1,000,000) simultaneously. Another fact is that genotyping costs are continuously falling. Taken together,

this has led to an increasing importance of association studies in genetic epidemiology and biomedical research.

There are two types of association analysis; 1) direct which is based on a functional genetic variant and 2) indirect which is based on a non-functional variant being in LD with a functional variant (Figure 2). In fact, the indirect association analysis (LD analysis) is a form of linkage analysis since it is hypothesized that affected individuals share their phenotype because they also share some disease-predisposing allele identical by descent from a common ancestor. LD analysis allows finer mapping than conventional linkage analysis. In situations where the locus variation itself has a direct effect on a trait variation, association studies are more powerful than linkage studies because the causal risk factor is measured.[192] However, with modern exploratory strategies it is less likely that causal variants are measured directly, and more likely that neighboring polymorphisms in LD are found to be associated. In these situations the power to detect a trait locus is dependent on the strength of the LD and similar to the recombination fraction in linkage analysis.[193]

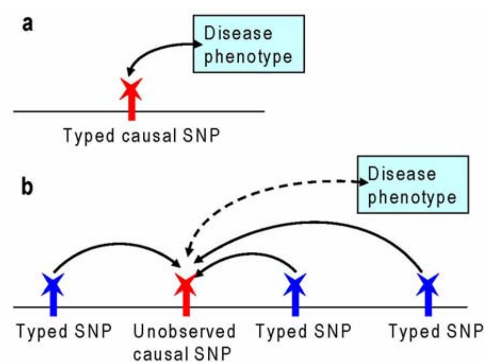


**Figure 2. Direct and indirect association analysis. (a)** The causal SNP (red) is directly tested for association with a disease phenotype. **(b)** The causal SNP (red) is tested for association indirectly, as it is in LD with the SNPs typed (blue).

In association studies the standard study design is to collect a number of unrelated affected (case) and unaffected (control) individuals from a homogeneous population. Then the genotypes of a marker locus or loci are tested to evaluate their frequencies between the case and control groups. An alternative design is to collect triads, consisting of two parents and one offspring. Depending on the trait/disease of interest either one of the parents or the offspring may be affected, or one may compare case triads to control triads. Association studies can also be carried out in multiple families, however family-based studies suffer from several disadvantages such as potential ascertainment bias toward early age of onset if living parents are required, difficulty of recruiting related individuals, and they require different analytical methods that account for the expected correlation in genotypes between related individuals.[194]

Finally, to evaluate whether candidate genes showing a nominal association with a disease exhibit a general role in the development of disease, the same, associated, variant needs to be tested in several independent populations, so-called replication studies.

### *1.6.3 The role of genetics in preeclampsia*

Studying the familial aspects of preeclampsia led to the discovery of preeclampsia as a disease exhibiting heritable characteristics, which means it has a genetic basis. Adams et al. observed a strong familial tendency to preeclampsia and hypertension in pregnancy, finding that 49% of the sisters of preeclamptic women had themselves experienced these conditions.[195] Several studies have since then confirmed these findings and observed an increased frequency of preeclampsia among mothers, daughters, sisters, and granddaughters of women who had this syndrome.[195-202] In addition to the maternal genetic factors, fetal genetic factors also appear to contribute to preeclampsia susceptibility, via paternal

transmission.[70,203] In a retrospective Norwegian study of births (data from the MBRN) it was shown that males born from a preeclamptic pregnancy presents a moderate risk (1.5-fold) to father a preeclamptic pregnancy.[70] Genetic factors are suggested to be responsible for more than 50% of the liability to preeclampsia[196,204,205] but the exact pattern of inheritance is still unknown.

Efforts to elucidate genetic susceptibility for preeclampsia has uncovered evidence for maternal susceptibility loci and locus heterogeneity amongst Icelandic,[206] Australian/New Zealand (Aust/NZ),[204,207-209] Dutch[210,211] and Finnish[212] family cohorts (Table 2).

**Table 2: Genome-wide linkage studies in preeclampsia families.**

| **Country** | **Chromosome with significant linkage** | **Chromosome with suggestive linkage** | **Publication(s)** |
|---|---|---|---|
| Iceland | 2p13 | 2q23 | Arngrimsson et al. 1999[206] |
| Australia/ New Zealand | 2q22, 5q, 13q | 4q34, 11q23 | Moses et al. 2000,[207] Johnson et al. 2007,[209] Harrison et al. 1997[213] |
| Netherlands | | 10q, 12q, 22q | Lachmeijer et al. 2001[211] |
| Finland | 2p25, 9p13 | 4q32 | Laivouri et al. 2003[212] |

The genome-wide scan in the Icelandic families revealed a maternal susceptibility locus for preeclampsia on chromosome 2p13.[206] In the Aust/NZ family cohort linkage to chromosome 2 was confirmed by identification of significant linkage on 2p12 and suggestive linkage on 2q23.[207] It was suggested that the Icelandic and Australian findings represented the same chromosomal region on 2p. But subsequent analyses of the Aust/NZ genome-wide linkage scan data set, applying a variance components-based procedure, resolved and strengthened the chromosome 2 linkage signal to 2q22.[204] This analysis approach also identified two novel preeclampsia susceptibility quantitative trait loci (QTLs) on chromosomes 5q and 13q.[209] A quantitative trait, also called continuous trait, varies over a continuous range of phenotypes

and stands in contrast to qualitative, or discontinuous, traits that are expressed in the form of distinct phenotypes chosen from a discrete set. The appearance of a quantitative trait usually signifies the involvement of multiple genetic loci, although this does not need to be the case. In particular, a single polymorphic locus with multiple, differentially expressed alleles can give rise to continuous variation within a natural population. Multifactorial traits outside of illness contribute to what we see as continuous characteristics such as blood pressure and height which can be measured by some quantitative means. These traits generally exhibit a normal continuous distribution. A genome-wide linkage scan of the Finnish families has also confirmed linkage on chromosome 2, however at a different locus, 2p25, than the Icelandic and Australian loci.[212] In addition, significant linkage was identified on chromosome 9p13 in the Finnish families, which has been shown to be a candidate region to type 2 diabetes in Finnish and Chinese families.[214,215] The genome-wide scan of Dutch affected sib-pair families did not confirm linkage on chromosome 2, but revealed two peaks with suggestive evidence for linkage on chromosome 10q and 22q.[211]

While the familial association of preeclampsia has been recognized for decades, identification of susceptibility genes for preeclampsia has proven to be more difficult. A reason for this is that the genetics of preeclampsia fits the disease characterization of complex human disorder with multiple genetic effects (e.g. more than one gene) with no clear pattern of genetic inheritance in combination with environmental factors (e.g. lifestyle). It has been said that the cause of “any” disease is immunological, vascular, metabolic, genetic or a consequence of trauma.[216] In the etiology of preeclampsia all of these causes except for the last one have been considered central.[217-220] However, there is a growing acceptance that related common diseases previously considered to be multi-factorial and primarily immunological, vascular or metabolic (e.g. multiple sclerosis, essential hypertension and type 2 diabetes, respectively),

are in fact genetic.[221] In complex human disorders like preeclampsia the end phenotype is a result of multiple susceptibility loci each having a modest contribution and highly likely an interactive effect.[222] Figure 3 (modified after [223]) gives an overview of interactions between causal factors resulting in preeclampsia, and shows that all these factors could potentially be due to genetic predisposition.

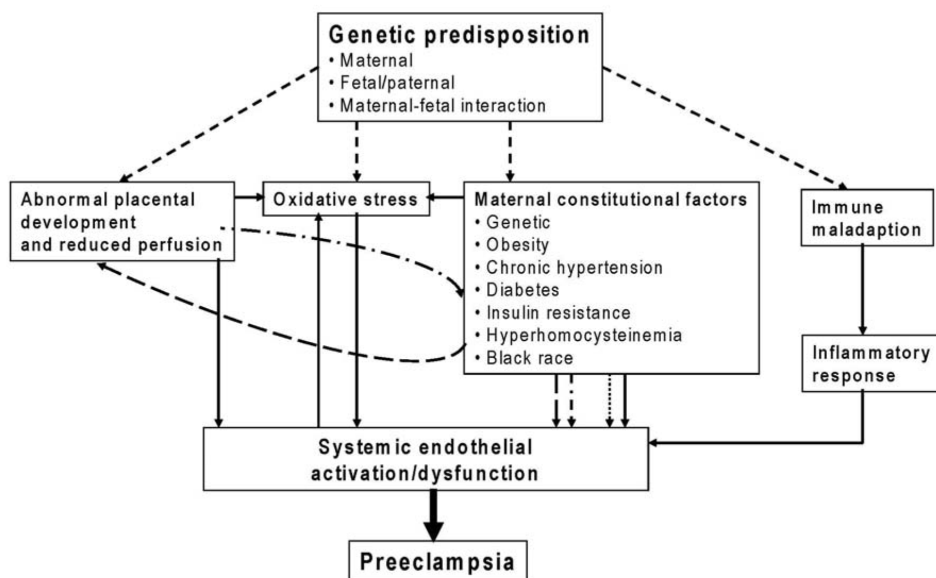


**Figure 3. Schematic overview of factors associated with development of preeclampsia (modified after [223]) and interactions between these.** Solid arrows indicate interactions accepted to be involved in preeclampsia pathogenesis, whereas broken arrows indicate suggested interactions.

### *1.6.4 Preeclampsia candidate genes*

Studies of more than 50 candidate genes have been reported, but these have so far failed to identify universally accepted susceptibility genes (reviewed in [224,225]). Approximately 70% of the published candidate gene studies in preeclampsia have focused on only eight genes.[226] The majority of the candidate gene studies have also addressed the role of maternal genotype,

however one large multicenter study addressed the contribution of both maternal and fetal genes.[227] Major risks associated with a number of SNPs in the candidate genes that have dominated the studies of the genetics of preeclampsia were excluded by this study.[227] Unraveling the genetic contribution of preeclampsia still remains one of the most fundamentally important challenges in obstetric medicine.[228,229]

The candidate genes studied in preeclampsia can be subdivided into different groups according to their assumed role in the pathophysiology; genes encoding 1) proteins that are vasoactive and involved in vascular remodeling, 2) proteins involved in thrombophilia, 3) proteins involved in oxidative stress, lipid metabolism and endothelial injury, 4) immunoregulatory proteins, 5) proteins involved in placentation and 6) growth factors.[224] In addition, there is a small number of promising positional candidate genes that has been studied. Positional candidate genes are genes residing within a chromosomal region previously shown to be in linkage with disease susceptibility.

**Vasoactive proteins and vascular remodeling**

Genes encoding components of the renin-angiotensin-aldosterone system (RAAS) seem to be plausible candidates for involvement in the etiology of preeclampsia, due to the central role of RAAS in BP regulation, vascular remodeling and body-fluid volume during pregnancy.[66,230] Genetic polymorphisms in angiotensin converting-enzyme (*ACE*), angiotensinogen (*AGT*) and angiotensin II type 1 and 2 receptors (*AGTR1, AGTR2*) have been studied extensively with inconsistent results (reviewed in [224]). Dysfunction of another system, the VEGF system, has been associated with development of preeclampsia. The soluble form of the VEGF receptor, sFlt-1, acts as an antagonist for VEGF and PIGF[129,231] and has been shown to be up-regulated in preeclampsia.[116,122-124,126,128,129] It has been proposed that sFlt-1 might trigger

endothelial dysfunction, and that this factor might link placental ischemia and the maternal disease since its production is induced by hypoxia. Dysfunction of the VEGF system has also been suggested to contribute to reduced invasion of trophoblasts.[116,117,232,233] At the mRNA and protein level there is evidence of association between factors belonging to the VEGF system and preeclampsia,[121] but no polymorphisms in these genes that could possibly be related to this effect have been identified so far. Other vasoactive proteins that have been suggested to be involved in development of preeclampsia are sEng, endothelin-1, estrogen receptors α and β and endothelial nitric oxide synthase (eNOS). However, the number of studies that have investigated SNPs in the genes encoding vasoactive proteins is rather small and to date none of them have been identified as major determinants of preeclampsia risk.

**Thrombophilia**

Association between inherited thrombophilia and severe preeclampsia has been reported.[234] Due to the characteristic features of placental infarctions and villous thrombosis in preeclampsia, thrombophilic gene mutations have been suggested as predisposing factors. Functional mutations in the Factor V Leiden (*FVL*), prothombin and methylenetetrahydrofolate reductase (*MTHFR*) genes have been discovered.[235] However, the studies of maternal genotypes of these genes published are controversial. Fetal trombophilic mutations have also been suggested to be associated with preeclampsia, but have not yet been detected with statistical significance.[196,236-242]

**Oxidative stress, lipid metabolism and endothelial injury**

The evidence that placental oxidative stress play a central role in the pathogenesis of preeclampsia is increasing.[243] Genes involved in generation of or protection against ROS

therefore seems like plausible candidates for preeclampsia. Only a few studies have addressed the possible relation between preeclampsia and oxidative stress genes, such as epoxide hydrolase,[244-246] glutathione S-transferase,[247-250] cytochrome P4501A1[248,250] and superoxide dismutase (*SOD*)[246]. Only polymorphisms in the epoxide hydrolase and glutathione S-transferase genes have shown an association with risk for preeclampsia susceptibility,[244-246,250], however, these results are conflicting.[246,247]

Abnormal lipid metabolism is associated with oxidative stress and an abnormal lipid profile, potentially contributing to endothelial dysfunction, has been shown in women with preeclampsia.[251] Lipoprotein lipase and apolipoprotein E (ApoE) are two major regulators of lipid and lipoprotein metabolism, thus these genes have been proposed as possible candidates for preeclampsia.[66,252] Studies of maternal alleles and polymorphisms in the lipoprotein lipase and *ApoE* genes have given inconsistent results regarding their association with preeclampsia.[252-260] A recent study proposes that the fetal genotype in the lipoprotein lipase and *ApoE* genes is important to the metabolism of maternal lipoproteins,[261] suggesting that future investigations should focus on both maternal and fetal genotype.

**Immunoregulators**

Genes encoding the various aspects of the immune system may be good candidates for involvement in preeclampsia development, due to the central role the maternal-fetal immune maladaption has been ascribed in the pathogenesis (reviewed in [66,220]). Several HLA molecules (HLA-DR, -A, -B, -DQ, -G, -C) have been suggested as candidate genes for preeclampsia, but the data remain inconclusive (reviewed in [262]). Maternal-fetal allele sharing is proposed to be a focus in future studies of HLA molecules. Another immunoregulatory protein suggested to play a role in the development of preeclampsia is TNF-α. TNF-α is

suggested to be important in early pregnancy by promoting growth, differentiation and normal placentation.[158,263,264] Elevated levels of TNF-α and TNF receptors in plasma and amniotic fluid of preeclamtic women[157,158] may contribute to vasoconstriction and platelet aggregation via increasing levels of thromboxane.[263,265] In addition TNF-α is involved in generation of ROS and may influence the susceptibility of oxidant-mediated injury in the endothelium.[265] Several studies have investigated TNF-α promoter polymorphisms, with some reports showing association with preeclampsia[265-268] or eclampsia[269] with as many other studies finding no association[227,263,268,270-273]. Genes encoding interleukins have also been suggested as candidate genes for preeclampsia. Polymorphisms in the genes encoding IL-1β and IL-1Ra have been shown to alter protein expression,[274,275] but there seems to be a lack of association with preeclampsia in the few studies performed.[267,274,276,277] Gene expression (mRNA) of the anti-inflammatory cytokine IL-10 have been shown to be reduced in preeclamptic placentas,[267,278] and some studies have identified association between variants in this gene and preeclampsia risk.[270,279,280] However, similar to many of the other candidate genes described the results for the *IL-10* polymorphisms remain controversial.[267,273,279,280]

**Placentation**

Genes involved in placentation have been suggested to be good candidate genes for predisposing to preeclampsia due to their obvious connection to placental function (reviewed in [229]). Based on studies in mice, the best candidates for preeclampsia development seem to be genes that are involved in differentiation and maintenance of trophoblast cells.[66,281,282] However, the role of these genes in preeclampsia remains to be investigated. Transcriptional profiling in placentas from preeclamptic women has also identified genes likely to be involved in placentation as relevant genes in the pathogenesis of preeclampsia. Among these are trophoblast-invasion associated genes, such as matrix metalloproteinases (MMPs)[283] and

apoptosis related genes, such as cyclin and p53,[284]. Also leptin, shown to have a stimulatory effect on trophoblast invasion *in vitro*,[285,286] and the potential leptin receptor siglec-6 has been proposed to affect trophoblast invasion.[287] Increased circulating levels of leptin have been reported to be associated with preeclampsia in several studies,[8,288-295] and a leptin gene polymorphism has been linked to preeclampsia susceptibility.[296] Interestingly, singlec-6 is restricted to B lymphocytes and human placenta, in other species placental cells lack singlec-6 expression.[297] Since preeclampsia is thought to be a disease unique to humans this is extremely intriguing. The present data on placentation genes is scarce, but current findings suggest that research in this area deserves attention and should encourage intense investigation.

**Growth factors**

Insulin-like growth factors (IGFs) are involved in regulation of fetal and placental growth throughout gestation,[298] and IGF-II may be important for both trophoblast and decidual function.[299] In normal pregnancies, maternal serum levels of IGF-I increase from midgestation.[300] However, an increase in serum concentration of IGF-I from first to second trimester has been shown to be associated with an increased risk of developing preterm preeclampsia.[301] Insulin-like growth factor binding protein-1 (IGFBP-1) is thought to inhibit local IGF activity and to reduce trophoblast invasion early in pregnancy.[302,303] In a recent study, low levels of IGFBP-1 in first and second trimester were shown to be associated with an increased risk of term preeclampsia.[301] A congenital disorder involving fetal and postnatal overgrowth, Beckwith-Wiedemann syndrome, has been associated with both IGF-II over expression and severe early-onset preeclampsia. This suggests a possible link between genetic variations in *IGF-II* and development of preeclampsia by restricting intrauterine fetal growth.[66,304,305] Genotyping of a *ApaI* restriction-fragment-length polymorphism in *IGF-II*

showed no association with preeclampsia.[304] Contribution of an intronic SNP in the *IGF-I* gene has recently been suggested, although the result was not statistically significant after adjustment for multiple hypothesis testing.[273]

**Positional candidate genes**

A gene coding for a transcription factor that is expressed in EVTs, storkhead box 1 (STOX1), was identified as a promising positional candidate gene for preeclampsia.[306] This gene is located within a region on chromosome 10q that showed suggestive linkage to preeclampsia in Dutch families,[210] and is known to be expressed in the placenta during invasion of the uterus in early pregnancy. It was concluded that *STOX1* is a maternal effect gene due to the observation that women with preeclampsia inherited a version of the gene with reduced function from their mothers. Over the past decade genomic imprinting has been strongly suggested to play a role in the preeclampsia pathogenesis, due to its role in placental and embryonic/fetal development.[307,308] Due to mutation or loss of a single active copy with an unusual mode of inheritance imprinted genes may influence trophoblast growth or fetal development that causes disease. Subsequent studies have not been able confirm an association of *STOX1* with preeclampsia.[309,310] Also our genotyping results in the HUNT preeclampsia case-control cohort contradict an association between *STOX1* and preeclampsia (Fenstad et al., *in preparation*).

Activin receptor type II A (*ACVR2A*) is another gene that has been proposed as a positional candidate gene for preeclampsia.[204] This gene resides within chromosome 2q22 which has been shown to be in significant linkage with preeclampsia in Aust/NZ families. Moreover, a gene expression study in decidual tissue showed *ACVR2A* to be more than 10-fold down-regulated in preeclamptic compared to normotensive women.[204] ACVR2A is a receptor for

activin A which appears to have a fundamental function during implantation and decidualization,[311,312] and modulates placental production of hormones[313] and secretion of uterotonin[314]. In preeclamptic women elevated serum levels of activin A have been detected.[315-320] Altered expression of ACVR2A, possibly due to polymorphisms in this gene, and its effect on activin A function may influence the central pathophysiologic features of preeclampsia by impairment of trophoblast invasion and remodeling of spiral arteries.[204] Results from genotyping five *ACVR2A* SNPs in Aust/NZ families showed preliminary evidence of association with preeclampsia for three of them.[204]

In addition positional candidate genes encoding the endoplasmic reticulum aminopeptidase enzymes (*ERAP1* and *ERAP2*) and a placental peptide hormone corticotrophin-releasing hormone-binding protein (*CRHBP*) on the Australian 5q QTL and genes encoding type IV collagens, alpha-1 (*COL4A1*) and alpha-2 (*COL4A2*) on the Australian 13q QTL have been suggested as plausible candidates.[209] These genes are all positional candidate genes with major "hit-scores" obtained from a computer program used to objectively prioritize genes under defined QTLs. All these genes are expressed in the placenta, and thus, are potentially involved in preeclampsia development.

**The current status of candidate gene studies in preeclampsia**

Very few of the candidate genes studied to date are located within chromosomal regions shown to be in significant linkage with preeclampsia. The majority of the candidate genes have rather been selected based on biological hypotheses and their role in biological pathways relevant to the pathology of preeclampsia. Most of these genes have shown inconsistent association with preeclampsia. To date, the current literature suggests two promising positional candidate genes, *STOX1* and *ACVR2A*, have been reported after follow-up studies

on linkage detected on chromosome 10q[210,306] and 2q[204,321,322], respectively. In conclusion, data remain inconclusive for the large panel of candidate genes for preeclampsia tested so far. However, genome-wide linkage analysis and subsequent analysis using novel approaches such as objective prioritization of positional candidate genes, have generated encouraging results.

# 2 AIMS OF THE STUDY

The aim of this dissertation was to identify genes contributing to increased maternal risk of developing preeclampsia, particularly focusing on:

I. Positional candidate genes
   a) within the Australian 2q QTL
   b) within the Australian 5q QTL
   c) within the Australian 13q QTL

II. Candidate genes involved in inflammation and oxidative stress

# 3 MATERIALS AND METHODS

## 3.1 The HUNT population

The second Health Study of Nord-Trøndelag (HUNT2), a multipurpose health survey focusing on the total population in the rural county of Nord-Trøndelag, Norway, took place during 1995-1997. Nord-Trøndelag County is located in the middle of Norway (Figure 4), and is a representative of Norwegian geography, economics, industry, age distribution, morbidity and mortality.[323] The population in Nord-Trøndelag is ethnically homogenous with less than 3% non-Caucasians, making it suitable for epidemiological genetic studies.[323,324] All county citizens aged 20 years or older (n=92,566) were invited and 71.2% participated (n≈65,900). The invitation comprised a health questionnaire, a clinical examination and blood tests (including peripheral leukocyte sampling).[323]

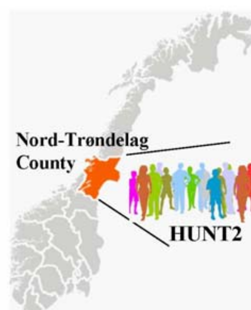


**Figure 4.** HUNT2, with approximately 65,900 participants, was conducted in Nord-Trøndelag County (orange section in the map) which is located in the middle of Norway.

### *3.2 Identification and classification of preeclamptic cases and controls*

In order to identify women who had been pregnant and if they had a normal or preeclamptic pregnancy in the HUNT population we had to use the Medical Birth Registry of Norway (MBRN). Since 1967 all deliveries in Norway after 16 weeks of gestation, more than 1.8 million births, have been recorded in the MBRN.[325] The registration is based on standardized forms completed by midwives and doctors at the delivery ward within one week (9 days) after delivery. All diagnoses recorded before discharge from the hospital are included in the registry. In the MBRN preeclampsia is usually recorded as a specified diagnosis. The medical birth registration form may also hold information about specific symptoms of preeclampsia, such as hypertension, proteinuria, or edema during pregnancy. From 1967 to December 1998 women registered with ICD-8 codes. MBRN made extensions of the ICD-8 codes[326] and these diagnosis codes or combinations of them defined preeclampsia in the present work (Table 3).

**Table 3. Preeclampsia ICD-8 codes and definition of preeclampsia in the present work.**

| | |
|---|---|
| International ICD-8 codes | 637.0 Preeclampsia<br>637.1 Eclampsia<br>637.9 Toxemia, unspecified |
| MBRN ICD-8 codes | 637.0 Hypertension developed during pregnancy<br>637.2 Hypertension and edema<br>637.3 Proteinuria without previous kidney disease<br>637.4 Hypertension and proteinuria<br>637.5 Hypertension, proteinuria and edema<br>637.6 Threatening eclampsia<br>637.9 Toxemia, intoxication, preeclampsia ina |
| MBRN ICD-8 codes and combinations of these defining preeclampsia in the work presented in this thesis | 637.0 + 637.3<br>637.2 + 637.3<br>637.4<br>637.5<br>637.6<br>637.9 |

After the 1st of December 1998 the MBRN changed their routines and the registrations were thereafter based on ICD-10 codes. These are: O13 Hypertension during pregnancy without considerable proteinuria, O14.0 Moderate preeclampsia, O14.1 Severe preeclampsia and O14.9 Unspecified preeclampsia. After 1998 subgrouping of preeclampsia cases was performed using the ICD-10 codes, with information about clinical manifestations (severe, moderate) and time of onset (before or after 34 weeks of gestation).

In Norway, every individual has a unique 11-digit personal identification number given at birth. This allows cross linkage of individuals between health registries and other databases. In the work presented in this thesis, women with preeclamptic (cases) and non-preeclamptic singleton pregnancies (controls) in the HUNT population were identified by staff at MBRN by linking the HUNT database to the database at the MBRN. The data was subsequently made anonymous and linked to the biological samples by staff at HUNT biobank. Women registered with multiple pregnancies (twins, triplets etc.) were excluded since this is known to be a risk factor for preeclampsia. The HUNT database included 1,179 women registered with preeclampsia (cases), and 2,358 women with a history of non-preeclamptic singleton pregnancies (controls) were selected. The controls were selected as the two non-preeclamptic women subsequent to a women registered with preeclampsia in the linked data file. Blood samples were available from 1,139 cases and 2,269 controls at the HUNT biobank.

## *3.3 Candidate gene prioritization and selection of SNPs*

We used the GeneSniffer (www.genesniffer.org) computer program to interrogate the LOD drop (99% confidence) intervals at the Australian 2q, 5q and 13q preeclampsia QTLs to prioritize positional candidate genes *in silico*. GeneSniffer is a tool that automates bioinformatics database mining from National Center for Biotechnology Information's

(NCBI's) Entrez Gene, Online Mendelian Inheritance in Man (OMIM) and PubMed databases to objectively identify plausible positional candidate genes residing under defined QTLs. Interrogation of these databases is performed using a set of key words specific for the disease of interest as input. The input key words are assigned a weighting score of 10, 5 or 1 (10 being the highest) based on their relevance and significance to the studied disease. The keywords assigned a score of 10 were pre-eclampsia, preeclampsia, eclampsia, pregnancy-induced hypertension, pregnancy induced hypertension, pregnancy hypertension, gestational hypertension, PIH, toxaemic pregnancy and decidua. The keywords assigned a score of 5 were toxaemia, pregnancy, placenta, spiral artery, glomerular endotheliosis, implantation, trophoblasts, cytotrophoblasts, proteinuria and placental bed. The keywords that assigned a score of 1 were uterus, uterine, amnion, chorion, NK cell, hypertension, gestation, gestational, oedema, edema, maternal, endothelial, endothelium, haemostasis, coagulation, coagulopathy, thrombophelia, ischemia, hypoxia and allograft. Identification of homologues of each gene, under the linkage peak, was performed by BLAST and scored for content of their Entrez Gene, OMIM and PubMed entries. Each score was weighted in accordance with the degree of homology. For each gene a cumulative "hit-score" was calculated and shown as an output. The output was provided in an HTML format documenting all genes under an observed QTL region with the source of database hits and links to external databases for additional information. GeneSniffer can also incorporate observed logarithm of the odds ratio (LOD) score function within a QTL region to use the localization data as an additional weighting function in which genes are considered more relevant the closer they are to the observed QTL peak.

We used this data along with knowledge on current and emerging concepts of the pathophysiology of preeclampsia[327] to select genes for further analysis. For the prioritized

genes we selected validated SNPs from NCBI's SNP database (*Homo sapiens* dbSNP build 125) for further genetic analyses. In selecting these SNPs our initial focus was on likely functional variants (coding sequence SNPs) and those in known gene regulatory regions (proximal promoter, 5 prime (5') untranslated region (UTR) and 3 prime (3') UTR). In order to sufficiently cover known SNP variation within some of the genes we also selected validated intronic SNPs.

## *3.4 Genotyping*

Genotyping refers to the process of determining the genetic make-up of an individual at a specific locus. The use of SNPs has revolutionized human genetics, and several platforms have been designed for the analysis of SNP genotypes, including gel and capillary (fluorescent) based electrophoresis, fluorescent read-out, oligonucleotide (bead-based) microarrays and mass spectrometry. Single SNP genotyping assays have the longest history, but the need for genotyping technologies in which one can study several SNPs more effectively has evolved as a result of The Human Genome project and the International HapMap project. Several high-throughput genotyping approaches, with a variety of platforms and chemistries, are now available so that researchers can study from several up to 1 million SNPs simultaneously. Three different assays for three different genotyping technologies were used to genotype samples in the Norwegian case/control cohort presented in this thesis; the Applied Biosystems' TaqMan (*paper III*) and SNPlex assay (*papers I, II, IV, V*) and Sequenom's iPLEX Gold assay (*paper IV*). An additional genotyping technology, Illumina's GoldenGate assay, was used for genotyping samples from the Aust/NZ family cohort for data presented in *papers IV and V*.

### *3.4.1 TaqMan assay*

The TaqMan assay is a single tube and single SNP real-time PCR based genotyping assay which exploits the 5’ to 3’ exonuclease activity of Taq DNA polymerase. The reaction utilizes two PCR primers and two allele specific oligonucleotide probes, each labeled with a different fluorophore (FAM and VIC) at the 5’ end and a non-fluorescent quencher (NFQ) at the 3’ end. During the PCR a perfectly matching probe which hybridizes to its target allele will be displaced and cleaved by the exonuclease activity of the Taq polymerase, resulting in the release of the fluorophore away from the NFO. A mismatched probe will not hybridize to its target and remain intact, free floating in solution with its specific fluorophore and NFQ. At the end point of the PCR fluorescent emission of one dye over the other indicates a homozygous sample, whereas fluorescent emission of both dyes indicates a heterozygous sample. An intact, non-hybridized probe emits no fluorescent signal when excited due to the NFQ via Förster resonance energy transfer (FRET). An overview of the TaqMan technology is showed in Figure 5[328]. In the work presented, the TaqMan assay was used to genotype a known functional SNP within a potential candidate gene, catechol-*O*-methyltransferase (*COMT*), for preeclampsia (*paper III*).

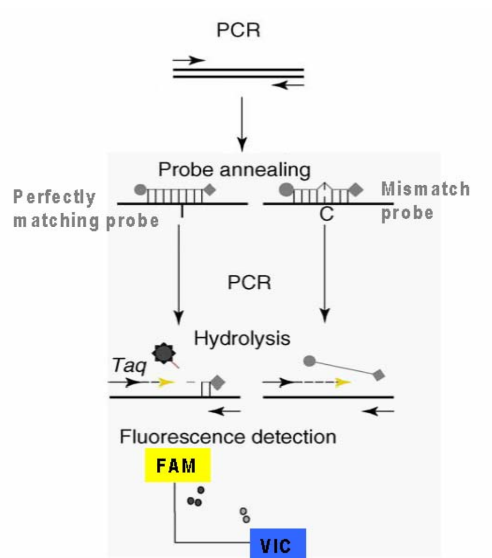


**Figure 5.** Schematic overview of basic molecular biology steps in the TaqMan assay.[328]

### *3.4.2 SNPlex assay*

The SNPlex™ Genotyping System is a migration specific assay designed to discriminate alleles by the application of three SNP specific ligation probes. Two of the probes are allele specific oligos (ASOs) that are designed to discriminate the two alleles at each SNP locus and the third probe is a locus specific oligo (LSO) that is common to both SNP alleles. All three probes house an universal PCR priming site whilst the ASOs are designed with unique ZipCode identifiers that are used to hybridize to complementary ZipChute probes. The ZipChute probes are designed with mobility modifiers that provide unique migration patterns for each specific SNP within the ASO/LSO probe pool. The SNPlex system allowed multiplexing of up to 48 SNPs in one SNP pool (panel), at the time the work presented in this thesis was performed, meaning that one could examine up to 48 SNPs simultaneously for each individual DNA sample. Fluorescent intensity signals for each individual sample are

interpreted and subsequently calculated into three genotype clusters using Applied Biosystems'GeneMapper® Software (version 4.0). An overview of the SNPlex technology is shown in Figure 6[328]. The SNPlex assay was used to genotype SNPs within positional candidate genes within the chromosome 2q, 5q and 13q preeclampsia QTLs (*papers II*, *IV* and *V*), and also a known functional SNP within a potential preeclampsia candidate gene involved in inflammatory response, Selenoprotein S (*SEPS1*) (*paper I*).

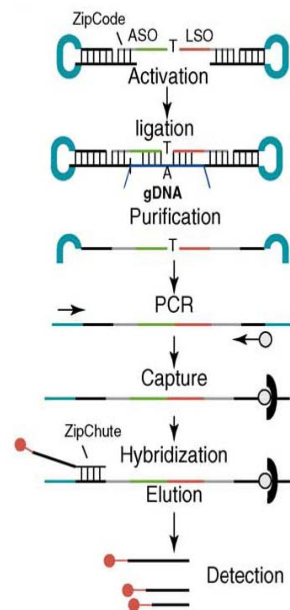


**Figure 6.** Schematic overview of basic molecular biology steps in the SNPlex assay.[328]

### *3.4.3 iPLEX Gold assay*

The iPLEX Gold assay on the Sequenom MassARRAY® Platform combines the benefits of robust single-base primer extension biochemistry with the sensitivity, speed and accuracy of

Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS). Sequenom SNP genotyping uses a bead-less and label-free primer-extension chemistry to generate allele-specific products which are separated by their distinct mass.[329] Differences in mass are automatically translated by the MassARRAY Typer software into specific genotype calls. The iPLEX Gold genotyping assay allows multiplexing of up to 40 SNPs simultaneously, where differentiation of genotypes for each of the SNPs in one assay is a result of unique mass ranges for the extension primers. This assay is widely used for fine mapping and validation studies and for routine applications that employ fixed SNP panels. An overview of the iPLEX Gold assay technology is shown in Figure 7[328]. The iPLEX Gold assay was used to perform replication genotyping of SNPs within positional candidate genes within the chromosome 5q preeclampsia QTL (*paper IV*) showing nominal association with the SNPlex genotyping.

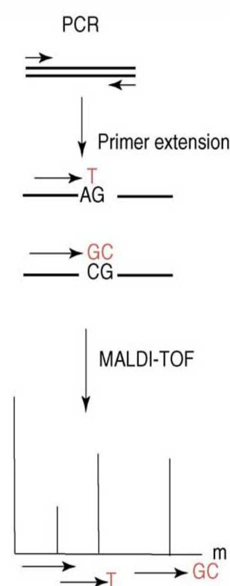


**Figure 7.** Schematic overview of basic molecular biology steps in the iPLEX assay.[328]

### 3.4.4 GoldenGate assay

The GoldenGate assay is based on a combination of oligo ligation assay (OLA), allele-specific extension reactions and universal PCR. Three oligonucleotides, two ASOs and the third is a LSO, are designed for each SNP. All oligos contain universal PCR primer sites and the LSO also contains a sequence complementary to tags present in a particular bead type.[330] These three oligonucleotides hybridize to the DNA once it binds to the paramagnetic particles, successfully extended and ligated products are amplified by PCR with fluorescently labeled universal primers. Following PCR and some downstream processing, single-stranded, dye-labeled DNAs are then hybridized to their complement bead type on an array of beads carrying the sequences complementary to the locus specific tags. This hybridization step allows for the assay products to be separated for individual SNP readout. A high-resolution scanner detects the fluorescent beads and decodes the information used to generate the genotype calls. The GoldenGate assay is capable of multiplexing up to 1,536 (96-1,536) SNPs in a single tube with very high pass rates and accuracy.[331] An overview of the GoldenGate assay technology is shown in Figure 8[328]. The GoldenGate assay was used to genotype SNPs within positional candidate genes within the chromosome 2q, 5q and 13q preeclampsia QTLs in the Aust/NZ family cohort (*papers IV* and *V*).

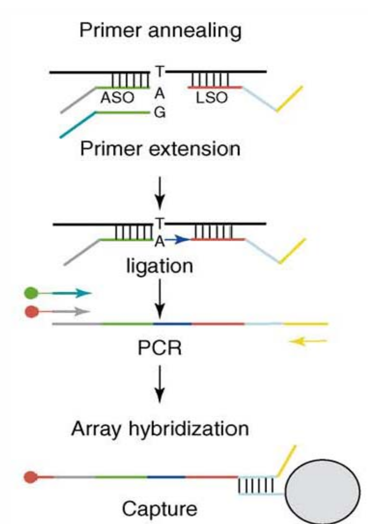


**Figure 8.** Schematic overview of basic molecular biology steps in the GoldenGate assay.[328]

### 3.5 *Statistical analysis*

The statistical analysis of genetic association data depends crucially on the study design. When deciding what type of data to collect, e.g. family or population, one to some extent predetermine what kind of statistical analysis will be most appropriate. This means that also statistical geneticists should also focus on study design and ascertainment, in order to improve power of studies so that the genetic basis of complex phenotypes can be better understood. Prior to our genotyping experiments power calculations were performed. Typing 144 SNPs in 1,086 preeclamptic and the same number of control samples gave us 80% power to detect a marker that accounts for 2% of the variation.

The increased focus on SNP genotyping has made it clear that new statistical methods are needed for LD mapping of complex trait genes,[332-335] and has led to re-examination of mapping methodologies and study designs.[332,336-339] Developments in biostatistics have been

lagging behind the capacity to generate SNP genotypes,[340,341] and what the best way to apply SNPs and LD mapping data to the genetic epidemiology of common diseases has been unclear. However, in the recent years quite a few GWAS have mapped and replicated candidate SNPs by LD mapping, and this approach seems very promising.

### *3.5.1 Association analysis*

Methods such as logistic regression, chi-squared ($\chi^2$) tests of association, and odds ratios may be suitable in simple cases, e.g. when studying a single marker and are available from standard statistical packages.[192] Logistic regression model log odds of disease as a linear function of underlying genotype variables, while a $\chi^2$ test of association tests for independence of disease status and a genetic risk factor. Pearson's $\chi^2$ test was used in *papers I* and *III* presented in this thesis. Tests of dichotomous traits in unrelated individuals involve testing for different allele or genotype frequencies among affected versus unaffected individuals. To test for association with quantitative traits in unrelated individuals linear regression methods are commonly used.[194]

A **measured genotype test,** which directly tests the effect of measured genotype on the phenotypic outcome,[342] has been used in papers *II, IV* and *V* included in this thesis. In principle, the effect of measured genotypes can be assessed for any given phenotypic outcome, either qualitative (e.g. disease presence or absence) or quantitative. The measured genotype test used in *paper II, IV* and *V* uses a standard threshold model assuming an underlying normal distribution of liability. This threshold model and its assumptions are near identical to those used in standard logistic regression but benefits from the ease of interpretation with regard to genetic effects. The measured genotype test of association can

assess the extent of genotypic mean differences (or the liability or risk scale) between case and control singletons assuming a model of additive gene action.[342]

In studies in which LD between typed markers and causal locus is exploited it is preferable to use multilocus, also called multivariant, approaches to analysis instead of analysis of marker loci one at a time. Combining genetic information from multiple markers simultaneously provides greater power to detect QTLs than do tests of individual markers, unless there is large degrees of freedom. Multivariant approaches generally involve consideration of haplotypes which are promising in the study of the genetic basis of complex traits. But since the field of haplotype analysis is relatively new and continuously evolving the development of multilocus approaches has been challenging. However, in the recent years novel multilocus approaches combating the problem with large degrees of freedom have been developed.

**Transmission disequilibrium test** (TDT) is one of the best known family-based association tests, and was first developed to test for linkage between alleles and phenotypes that is either causal or due to LD in qualitative (dichotomous) traits.[343] The underlying presumption of the TDT is to assess the transmission of alleles from two heterozygous, unaffected parents to an affected offspring. In a basic triad pedigree-structure the transmission of one parental and one maternal allele is measured against the non-transmitted parental and maternal alleles, respectively. Transmitted alleles favoring affected offspring greater than 50% therefore indicate evidence of association with the studied phenotype or trait. The TDT alleviates potential population stratification problems that may arise in case/control studies and has been extended for use in studies of qualitative traits with various family designs, such as sib-ships with no parental genotypes and families with one available parent. It has also been extended for use in studies of quantitative traits, named **quantitative transmission disequilibrium test**

(QTDT).[344,345] Both the TDT and QTDT can be used in studies of genetic markers with multiple alleles.[345,346] QTDT is LD analysis of quantitative and qualitative traits based on variance components. In *paper IV* and *V* presented in this thesis a QTDT[347] for SNP association in the Aust/NZ familial cohort was conducted in SOLAR[348]. The QTDT procedure scores allele transmissions throughout the entire pedigree structure for quantitative or qualitative traits[347] and it has been modified in SOLAR to work with discrete traits using a threshold model.[349]

### *3.5.2 Multiple testing*

Testing large numbers of markers (many different loci) for association with a particular disease or trait gives rise to the multiple testing problem, i.e. it is likely that some associations are found by chance and one obtains false positive results (type I error). The traditional solution is to use a **Bonferroni correction**, where the basic premise is to ensure that the overall probability of declaring any significant differences between all possible pairs of groups is maintained at some fixed significance level (α). The Bonferroni method relies on the assumption that all tests are independent, however this is not likely to be true in genetic mapping studies due to LD and correlations among subsets of SNPs. In addition, the Bonferroni correction fails to consider the correlation among related phenotypes. Bonferroni correction works well with small numbers of tests. However, association studies have exploded, with hundreds of thousands of tests as in GWAS with 500,000 to 1 million SNPs, and at such a large scale, a Bonferroni type correction is not an attractive solution because of LD between SNPs close to each other. In fact Bonferroni correction will likely result in a large false negative rate (type II error). The Bonferroni method is considered excessively stringent. Due to this, various, less conservative methods have been devised in order to control for multiple testing issues and attempt to balance between the type I and type II errors.

To accommodate for multiple hypothesis testing in *papers II*, *IV* and *V*, the approach of Li and Ji[350] was used to determine the effective number of independent SNPs (and tests) in combination with applying a false discovery rate (FDR) or permutation analysis. The algorithmic approach of Li and Ji evaluates the strength of correlation amongst the observed genotypes at each SNP locus within a gene and has been implemented into SOLAR.

**Permutation testing** is a simulation-based re-sampling technique that compares observed *p*-values with *p*-values calculated from simple repeated perturbations of the data. In this approach, two files are created, one with all of the genotypes for each individual, and one with all of the phenotypic data. By randomly permuting just the individual identifiers for one of the two files, the correlation among genotypes is preserved, as is the correlation among phenotypes, but any association between genotype and phenotype is broken. The complete set of association tests is then performed on the permuted data, and the permutation process is repeated thousands of times. This process generates a distribution of the best *p*-value expected in the entire experiment under the null model of no association between genotype and phenotype, allowing estimation of "experiment-wide significance." If an association has a nominal *p*-value of 0.001, and a *p*-value of 0.001 or lower is observed 60 times in 1000 permutations, then the corrected, empirical experiment-wide *p*-value is 0.06. The advantages of permutation testing are that these methods are robust and useful in achieving good type I rates and power, and appropriate for any test statistic regardless of whether the underlying distribution is known. A major disadvantage of these methods is that they can be computationally intensive and may require complex programming skills and statistical knowledge. Permutation testing is available within some genetic software packages, including SOLAR. In *paper IV* and *V* permutation testing was performed to generate experiment-wide *p*-values. Observed genotypes were used and one third of the HUNT case/control cohort was

randomized as being affected. For example, for permutation number 1, individual number 1 may be affected with genotype AA but for permutation number 2, individual number 1 may not be affected with genotype AA, and so on. In practice, for each of the 10,000 permutation tests there was a different set of preeclamptic women (~1.100) and a different set of control women (~2.200). The SNP genotypes were held constant, while the individuals coded as affected or not, changed. Then the normal measured genotype test was run.

**False discovery rate (FDR),** developed by Benjamini and Hochberg[351], attempts to control the proportion of false-positive results among reported statistically significant results. In other words, FDR is the expected proportion of false rejections within the class of rejected null hypotheses. Some advantages of applying a FDR include formal consideration of an alternative error rate, increased statistical power, and consistency of findings over differing number of inferences under consideration. In addition, this approach is less computationally intensive than permutation methods, and less conservative than the Bonferroni correction. As a result of this, FDR yields more power to detect genuine positive effects. A FDR of 5% was used in *paper II*.

The "gold standard" for identifying and eliminating false positives from the true association signals is independent (technical and biological) **replication**.

# 4 SUMMARY AND DISCUSSION OF PAPERS

## *4.1 Paper I*

***Genetic association of preeclampsia to the inflammatory response gene SEPS1.***

Selenoprotein S (SEPS1, also known as SELS and VIMP)[352,353] is a newly discovered inflammatory response gene, which plays a crucial role in protecting the functional integrity of the endoplasmic reticulum (ER) against potential metabolic stressors and deleterious effects of oxidative stress.[353] This novel ER and plasma membrane-located selenoprotein participates in the processing and removal of misfolded proteins from the ER to cytosol where they are degraded. The transcription of the *SEPS1* gene is induced by ER stress and inflammatory cytokines, and impaired expression of *SEPS1* has been associated with the A allele of a polymorphism in the promoter region in *SEPS1* (-105G>A).[354] Genetic variation in *SEPS1* was recently shown to be strongly associated with circulating levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1).[354] It has been suggested that *SEPS1* may be a crucial genetic link influencing the pro-inflammatory cytokine profiles observed in common human disorders influenced by inflammation such as atherosclerosis, diabetes mellitus and cancer.[355-357] With respect to a genetic contribution to the inflammatory response seen in preeclampsia, most attention has been given to polymorphisms in the pro-inflammatory cytokine genes, arguing that *cis*-acting genetic regulation may be at play. The TNF-α gene has been the most widely studied, with some reports showing association of promoter polymorphisms with preeclampsia[265,266,268] or eclampsia[269] with as many other studies finding no association[263,270-272,358]. Conflicting association data has also been reported for IL-6[271,359].

In ***paper I*** we aimed to test for a genetic association between *SEPS1* and preeclampsia by genotyping the -105G>A promoter polymorphism in 1,139 preeclamtic cases and 2,269

controls identified from the HUNT2 study population. Our results showed that women with preeclampsia were 1.34 times more likely to have the GA or AA genotype (P=0.0039; odds ratio (OR); 95% confidence interval (CI) 1.09-1.64) under a dominant genetic model and 1.22 times more likely to carry the A allele (P=0.023; OR 1.22; 95% CI 1.02-1.46) under an additive genetic model. (Both models were adjusted to account for maternal age and primiparity using multivariate regression analysis).

Multiple disturbances, including ischemia reperfusion injury, can cause an accumulation of unfolded proteins in the ER triggering an ER stress response called the unfolded protein response (UPR).[360,361] In preeclampsia, ischemia reperfusion injury to the placenta results in placental oxidative stress which is widely thought to be involved in the increased lipid peroxidation and endothelial cell dysfunction associated with the pathophysiology of preeclampsia.[362,363] Evidence of ER stress is observed in situations of altered vascular reactivity such as atherosclerotic lesions, neurodegenerative disorders, and metabolic syndrome, and has been implicated in placental dysfunction.[364,365] The involvement of ER stress in endothelial dysfunction/injury has been explored in heart disease, with relevance to myocardial ischemia, cardiac hypertrophy, heart failure and possibly atherosclerosis (reviewed in [366,367]). From the existing research in this field it seems possible that ER stress response (or UPR) can mediate both protective and damaging effects in the heart, depending upon the context. The general view is that UPR during the initial phases of ER stress mediates induction and activation of protective genes and proteins, whereas continued ER stress activate the pro-apoptotic machinery.[367] However, involvement of ER stress has not been extensively studied in preeclampsia or in the maternal vasculature. When protein folding is persistent or excessive, due the UPR being perturbed or not sufficient to deal with the stress conditions, apoptotic cell death is initiated.[368,369] Increased placental apoptosis is a feature of

preeclampsia,[363] and we therefore speculate that SEPS1 may contribute to preeclampsia risk via a role in the ER stress response to reduced placentation.

It is widely accepted that inflammation plays an important role in preeclampsia.[64,370] Circulating levels of many pro-inflammatory cytokines and their modulators have been shown to be elevated in preeclamptic women compared to normal pregnant women.[154-164,371] Cytokine imbalance and elevated expression of the pro-inflammatory molecules is also evident in preeclamptic placentas.[165-168] In this large case-control association study, we have for the first time demonstrated association of a polymorphism in the recently discovered inflammatory response gene *SEPS1* and preeclampsia. Based on the observation of association between genetic variations in *SEPS1* and circulating levels of pro-inflammatory cytokines,[354] and the evidence obtained in ***paper I***, one may now speculate if SEPS1 also contributes to the perturbed circulating levels of pro-inflammatory cytokines and other inflammatory markers, including C-reactive protein and calprotectin, that have been reported in women with preeclampsia.[370,372]

In conclusion, the A allele of the *SEPS1* -105G>A polymorphism is a risk factor for preeclampsia in the Norwegian population studied, possibly due to the effect this allele has on expression of *SEPS1* and thereby the ER stress/inflammatory response.

## *4.2 Paper II*

***Association between the candidate susceptibility gene ACVR2A on chromosome 2q22 and pre-eclampsia in a large Norwegian population-based study (the HUNT study).***

Three independent and distinct genome-wide scans in families from Iceland, Aust/NZ and Finland have provided evidence for maternal susceptibility loci for preeclampsia on

chromosome 2, although at different positions.[206,207,212] The Aust/NZ linkage signal was further resolved and strengthened to chromosome 2q22, by use of a variance components-based linkage approach. In ***paper II*** we aimed to further explore the association between positional candidate genes on chromosome 2q and preeclampsia by genotyping SNPs within seven genes at this locus in 1,139 cases and 2,269 controls identified from the HUNT2-study population. Our results showed evidence for association between preeclampsia and SNPs within one of these positional candidate genes, the activin receptor type II A (*ACVR2A*) gene. In the Australian study *ACVR2A* was identified as a high priority positional candidate gene at the 2q preeclampsia QTL by fine mapping in combination with an objective positional candidate gene prioritization strategy.[204,208] A bioinformatics assessment of the Australian 2q QTL prioritized *ACVR2A* as the top positional candidate gene and gene expression profiling in decidual tissues from preeclamptic and normotensive women identified *ACVR2A* was one of the most differentially expressed genes, exemplifying the importance of this gene.[204] ACVR2A is a key receptor for the cell signaling protein, activin A, which has long been recognized as an important regulator of human pregnancy.[373] Therefore, altered expression of ACVR2A has been proposed to influence activin A concentrations and action with consequences on trophoblast invasion[311] and remodeling of the spiral arteries[312]. The Australian preeclampsia study also reported preliminary evidence of an association between three SNPs in the *ACVR2A* gene and preeclampsia in a family-based cohort.[204]

In addition to promoting trophoblast proliferation and differentiation[311] and having a fundamental function during implantation and decidualization,[312] activin A modulates placental hormonogenesis[313] and uterotonin secretion[314]. Activin A, like most members of the TGF-β superfamily, has a broad range of physiological actions and roles during development. Nevertheless, one of the unique properties of activin molecules, that have emerged relatively

recently, is their involvement in inflammation and immunity. Activin modulates several aspects of the inflammatory response, including release of pro-inflammatory cytokines, nitric oxide production and immune cell activity (reviewed in [374]). In preeclamptic women elevated levels of activin A have been observed,[314-318,320,375-378] and the placenta has been suggested to be the main source of activin A in maternal circulation.[379] However, activated monocytes and endothelium (both components of the preeclampsia syndrome) are also potential sources of activin A, which means that not all of the rise is necessarily derived from the placenta.[380] Given that preeclampsia is characterized by systemic inflammation, it is not surprising that activin A is increased in established preeclampsia,[318] and in some cases, particularly in women with an early onset disease, before the disease manifests itself.[376] The existing evidence suggests that activin A is an anti- rather than a pro-inflammatory molecule.[381] In addition, activin seems to be anti-angiogenic since it inhibits endothelial cell proliferation.[382] There is modest *in vitro* evidence that activin A can suppress certain components of the acute phase response,[383] likewise hypoxic culture of placenta explants inhibits rather than increases the production of activin A.[384,385] A study of acute coronary syndrome also proposes a potential anti-inflammatory role of activin A.[386] Recently, activin A has been suggested to have a significant function in inflammatory diseases[387-389] and atherogenesis[390,391]. Similar to preeclamptic patients, elevated serum levels of activin A have been reported in patients with CVDs.[386,392,393] Interestingly, down-regulated expression of ACVR2A has been shown in patients with unstable CVD, suggesting a dysregulated receptor expression.[386] It is known that women with preeclampsia have an elevated risk of later life CVD[394] and women with existing CVD risk profiles (e.g. chronic hypertension and obesity) have an elevated risk of developing preeclampsia.[14,395,396] Thus, the hypothesis may be raised that the linkage between ACVR2A and preeclampsia may be ascribed to the role of activin A in endothelial activation and systemic endovascular inflammatory responses. It is not clear whether the increased activin A

concentration/reduced ACVR2A expression is a cause or a consequence of placental dysfunction. Thus, the activin pathway is a plausible biological candidate for a role in mechanisms perpetuating preeclampsia pathogenesis.

## *4.3 Paper III*

***Evaluation of COMT as a preeclampsia candidate susceptibility gene, assessed by genotyping of the Val158Met polymorphism and transcriptional profiling in decidual tissue.***

Several candidate genes for preeclampsia have been studied, with inconsistent results. Therefore the specific causative genes involved in preeclampsia still remain to be identified. A recent study put forward that deficiency in catechol-*O*-methyltransferase (COMT) is associated with preeclampsia.[397] COMT is a key enzyme in the degradation of both catecholamines and estrogens[398] which are associated with hypertension (reviewed in [399,400]). High- and low-activity variants of COMT, due to single base changes, have been discovered.[401] One polymorphism with functional implications is a non-synonymous G to A base change, the *COMT* Val158Met polymorphism. The Met-allele of this polymorphism is associated with a three- to four-fold decrease in COMT enzyme activity,[402] and several clinical conditions such as pain perception,[401,403] psychiatric disorders,[404-406] hypertension,[407-409] and heart disease[410-412] have been reported to be associated with this single base change. Inspired by Kanasaki et al.'s hypothesis that COMT deficiency is associated with preeclampsia, and the recent discussion of the relevance of their mouse model for preeclampsia[413] in humans, we aimed to evaluate *COMT* as a candidate susceptibility gene in women in ***paper III***. This was done by examining the potential role of the functional *COMT* Val158Met polymorphism by genotyping this SNP in 1,134 preeclamptic cases and 2,263 controls identified from the HUNT2-study population. In addition, since the maternal-fetal

interface is a likely site for the abnormal mechanisms in the pathogenesis of preeclampsia,[414] *COMT* gene expression in decidual tissues from preeclamptic (n=37) and normal pregnancies (n=57) was also investigated. Our results showed no association between the *COMT* Val158Met polymorphism and preeclampsia, and no differential expression of *COMT* transcripts in the decidual tissues from preeclamptic women and controls was found.

COMT is not a positional candidate gene, i.e. it is not located within linkage regions reported in preeclamptic families, but rather a candidate gene selected on basis of its biological function. The generalized endothelial dysfunction resulting in vasoconstriction and end-organ ischemia is attributed in all of the clinical aspects of the maternal syndrome in preeclampsia. Recent experiments have strongly suggested that several soluble factors affecting the vasculature are probably elevated because of placental hypoxia in the preeclamptic women, indicating that upstream molecular defects may contribute to preeclampsia. The search for a unique circulating factor has resulted in identification of multiple factors of endothelial dysfunction, activation and oxidative stress. A promising factor fulfilling the expectations of the "mysterious circulating factor" was sFlt-1, as it linked the placenta with various features of the maternal endothelial dysfunction. Elevated levels of sFlt-1 have been observed in women with preeclampsia, but not in all of them. Thus, sFlt-1 alone is not capable of inducing a generalized endothelial dysfunction. In the recently published study by Kanasaki et al. it was showed that pregnant mice deficient in COMT *(Comt-/-)* developed multiple functional and structural features of a preeclampsia-like phenotype. The *Comt-/-* mice delivered preterm with higher wastage of fetuses and showed a higher BP and a higher urinary albumin excretion in comparison with wild-type mice. Elevated levels of HIF-1α protein in the placenta and significantly increased sFlt-1 plasma concentrations in pregnant *Comt-/-* mice compared to controls were also observed.[397] HIF-1α elevation may lead to a shallow invasion

of trophoblasts into the spiral arteries and uterine wall, resulting in vascular defects, hypoxia and inflammation.[415,416] Kanasaki et al. observed that administration of 2-methoxyestradiol (2-ME), a natural metabolite of estradiol that is elevated during the third trimester of normal human pregnancy, rescued the *Comt-/-* mice from the preeclampsia-like syndrome without toxicity. Levels of 2-ME vary throughout gestation, with extremely low levels early in pregnancy and increasing levels towards term.[417,418] 2-ME acts like a pro-oxidant and has direct involvement in redox-regulated signaling,[413] thus the mouse model for preeclampsia used by Kanasaki et al. provided a link between redox-regulated signaling and human pregnancy pathology.[397] Moreover, it is known that 2-ME degrades HIF-1α,[419,420] and since HIF-1α is essential in angiogenesis this activity may inhibit angiogenesis. Lack of 2-ME, as in the *Comt-/-* mice, may result in stable HIF-1α and thus, increased oxidative stress and vascular pathology. Due to this absence of 2-ME in *Comt-/-* mice, the relevance of this model for preeclampsia in humans has been questioned.[413] Taken together with the additional potential role of HIF-1α in inflammation[421] and lipopolysaccharide-induced sepsis[422] it has been suggested that stable HIF-1α alone at late pregnancy could elicit the preeclampsia-like phenotypes in *Comt-/-* mice.[413]

Supporting their findings in mice Kanasaki et al. observed lower levels of placental COMT and circulating 2-ME in women with severe preeclampsia.[397] However, their study groups were rather small with 6 cases/6 controls and 8 cases/13 controls, respectively. Our *COMT* transcriptional profiling results in decidua basalis tissue, from a relatively large number of preeclamptic and control women (37 and 57, respectively), are inconsistent with the existing observations, thus we can only speculate about the cause of these divergent observations. Possibilities include, a different function of COMT in the placenta, which is of fetal origin, and decidua basalis, representing the maternal side, the mRNA level does not correlate with

protein levels, or the case groups of the previous studies[397,423] are simply too small with insufficient statistical power.

Hypertension is a major feature of preeclampsia, and studies have reported association between *COMT* SNPs, e.g. Val158Met, and BP in humans,[407-409,424] but such an association has not been found in pregnancy-induced hypertension,[425] suggesting that mechanisms underlying hypertension in the pregnant and non-pregnant state may differ. Finally, it should be addressed that BP regulation apparently differs in Kanasaki et al.'s mice and humans. An association between *COMT* and BP in non-pregnant women has been revealed.[408] In contrast, BP did not differ between non-pregnant *Comt-/-* and wild type (*Comt+/+*) mice.

To summarize, the results from our highly powered case/control cohort study does not support Kanasaki et al.'s hypothesis that an association between COMT/2-ME deficiency and preeclampsia is due to variations in maternal *COMT* genotype. However, since only one single polymorphism has been evaluated in the present study it may be too early to draw final conclusions about the role of this enzyme in development of preeclampsia.

## *4.4 Paper IV*

***The ERAP2 gene is associated with preeclampsia in Australian and Norwegian populations.***

Several loci most likely to harbor maternal susceptibility genes have been identified (reviewed in [224]) by examining the probability of co-segregating loci within a familial cohort. The original Aust/NZ genome-wide linkage scan data set[207,208] has been re-analyzed with an approach allowing a more refined variance-components-based procedure utilizing a biological threshold model for the preeclampsia phenotype. Application of this efficient genetic linkage

analysis method resolved and the strengthened the chromosome 2 linkage signal to 2q22,[204] and revealed two novel maternal preeclampsia susceptibility QTLs to chromosomes 5q and 13q.[209] In ***paper IV*** we aimed to identify the susceptibility gene(s) for preeclampsia at the 5q QTL by genotyping SNPs within positional candidate genes in both an extended Australian family cohort (The 74 Family Cohort) and our Norwegian case/control cohort. Positional candidate genes residing within the bounds of the 5q QTL were objectively prioritized using the same computer program as in ***paper II,*** described in Moses et al. (2006)[204] and Johnson et al. (2007)[209]. We used this data along with knowledge on current and emerging concepts of the pathophysiology of preeclampsia[327] to select 10 genes for further analysis. Three of the genes, corticotrophin releasing hormone binding protein (*CRHBP*) and two endoplasmic reticulum aminopeptidase genes (*ERAP1* and *ERAP2*), presented an association with preeclampsia and are all plausible candidates due to their biological roles. When we accounted for multiple hypothesis testing and the non-independence of genotyped SNPs, the *CRHBP* and *ERAP1* SNP associations were borderline ($0.05 \leq p_{corr} < 0.10$) whilst the *ERAP2* SNP associations remained significant ($p_{corr} < 0.05$) in both the Aust/NZ familial and Norwegian case/control cohorts. We may also have found evidence of allelic heterogeneity for SNPs with the *ERAP1* and *ERAP2* genes ($p_{uncorr} < 0.05$) within the 5q preeclampsia susceptibility QTL in the Aust/NZ and Norwegian cohorts.

The associated SNPs in *ERAP2* are missense, population specific, independent of each other and could possibly be playing a functional role, although it is not obvious what effect these amino acid changes have on the activity of the ERAP2 enzyme. Bioinformatic prediction of the importance of the borderline associated *ERAP1* and significantly associated *ERAP2* SNPs and how these may affect the protein folding kinetics was performed. The reported *ERAP1* and *ERAP2* SNPs reside within regions most likely to affect the three-dimensional (3-D)

structure and function of the proteins. SNP variants in *ERAP1* were predicted to have a benign effect on the ERAP1 protein, while SNPs in *ERAP2* were predicted to damage the 3-D protein structure and function of the ERAP2 protein. One of the *ERAP2* SNPs resides within a motif essential for the enzymatic activity.[426]

ERAP1 and ERAP2 are categorized into the oxytocinase subfamily of the M1 (metallo-type) zinc-dependent aminopeptidase gene family, and there is a 49% amino acid homology between these enzymes.[426,427] A third oxytocinase subfamily member of the M1 zinc-dependent aminopeptidase gene family, leucyl-cystinal aminopeptidase (*LNPEP*), is clustered with and shares ~43% amino acid homology with both ERAP1[428] and ERAP2[427]. This family of enzymes cleaves the N-terminus of numerous proteins thereby enabling them to play an important role in a plethora of biological functions including protein maturation and protein stability, cellular maintenance, growth and development, and defense mechanisms.[429] Of major relevance to preeclampsia are their roles in BP regulation, immune recognition and the inflammatory response during pregnancy.

LNPEP has been identified as an important placental peptidase involved in regulating the peptide hormones oxytocin, vasopressin and angiotensins.[430] Marked reduction in serum protease levels has been observed as preeclampsia progresses, and this may facilitate inadequate degradation of vasopressin and angiotensin II and thereby increase placental vascular resistance.[430] *ERAP1* and *ERAP2* also play a key role in BP regulation via their involvement in the RAAS. The ERAP1 enzyme has been characterized to effectively cleave and inactivate angiotensin II in addition to converting kallidin to bradykinin *in vitro*.[431] Angiotensin II is an integral link in the RAAS, a physiological system with a long and well implicated history with preeclampsia pathogenesis.[432,433] The characterization of the ERAP2

enzyme identified the ability to cleave angiotensin III to angiotensin IV and the conversion of kallidin to bradykinin with no hydrolytic activity against oxytocin, vasopressin or angiotensin II.[427] A previous genetic study investigating known and novel *ERAP1* SNPs in a Japanese case/control cohort identified a significant association of a missense SNP with essential hypertension.[434] This SNP was subsequently shown to reduce the enzymatic activity of ERAP1 in its efficiency to cleave angiotensin II to angiotensin III by 60% and the enzymes' ability to convert kallidin into bradykinin by 70%.[435]

CRHBP is a placental peptide hormone which exhibits an inhibitory effect when bound to corticotrophin releasing hormone (CRH), a prominent regulatory hormone with a diversified role in female reproductive physiology. Circulating CRH influences embryo implantation,[436] parturition[437] and an integrated stress response.[438] After the 20$^{th}$ week of gestation the amount of CRH produced by the syncytiotrophoblasts in the placental bed increases and is released into the maternal circulation.[439,440] This increase coincides with a decrease in CRHBP levels in a normal health pregnancy,[441] but this indirect relationship is exacerbated in complicated pregnancies such as preeclampsia.[442]

A critical component in the development of preeclampsia is a maladaptation of the maternal immune response to the developing fetal allograft. The recent reported association between the *ERAP1* gene and the HLA class I mediated autoimmune disease ankylosing spondylitis adds another plausible role for ERAP enzymes in the etiology of preeclampsia.[443] ERAP1[444] and ERAP2[427] are co-localized within the ER, and have complementary functions in HLA class I peptide presentation.[445] These enzymes are involved in trimming of HLA class I ligand precursors.[446] ERAP1 has a unique property of trimming N-extended antigenic peptides to eight or nine residues,[447] thus producing epitopes that are bound to most HLA class I molecules.[448] The elimination of ERAP1 in cultured cells[444,447] or mouse knockout models[449-

[451] have shown to either enhance, inhibit or have no effect on the presentation of trimmed peptides bound to the MHC class I molecules. During early human pregnancy non-classical (class I) HLA-C, -E, -F and -G antigens expressed by invading trophoblast cells at the site of placentation are involved in a maternal-fetal immunological interaction with decidual (NK) immune cells via the killer immunoglobulin-like receptors (KIR).[370] This is thought to be crucial for the establishment of immune tolerance to the fetal allograft and successful placentation. Much attention has been focused on this interaction in preeclamptic pregnancies, arguing that unfavorable genotype combinations of both the HLA and KIR molecules may be at play.[452] The possible involvement of genetic variation in *ERAP1* and *ERAP2* in this interaction is now of great interest.

In a preeclamptic pregnancy there is an exacerbated systemic inflammatory response, resulting in increased levels of circulating pro-inflammatory cytokines. *ERAP1* cleaves cell surface receptors for the pro-inflammatory cytokines IL-1 (IL-1Rα), IL-6 (IL-6Rα) and TNF (TNFR1), which subsequently down-regulates their signaling.[453] Thus genetic variants in ERAP1 could therefore have pro-inflammatory effects through this mechanism in light of the increased circulating IL-6 levels during pregnancy (reviewed in [454]) ERAP2 has a much more limited pattern of expression in tissues than ERAP1,[427] and the molecular genetic or functional information pertaining to *ERAP2* is limited. Thus, we can only speculate on its role in immunological pathways based on its high homology with *ERAP1* and proposed divergence from a common ancestral gene.[427]

The vascular, immunological and inflammatory responses are considered important physiological mechanisms in preeclampsia pathogenesis. Although we did not find significant associations between *LNPEP*, *ERAP1* and *CRHBP* and preeclampsia it would be premature to

exclude these genes as risk factors for preeclampsia due to their biological functions. In addition, *LNPEP* and *ERAP1* are in physical proximity to *ERAP2*, and only three *CRHBP* SNPs were efficiently interrogated. The significant *ERAP2* association results observed in two independent cohorts discloses a novel maternal preeclampsia risk locus in a region of known genetic linkage to this pregnancy specific disorder.

## *4.5 Paper V*

***Identification of TNFSF13B as a genetic risk factor for preeclampsia: replication of association in Australian and Norwegian populations.***

By re-analyzing the original Aust/NZ genome-wide linkage scan data set,[207,208] the chromosome 2 linkage signal was strengthened and resolved to 2q22.[204] In addition, this analysis approach identified two novel preeclampsia susceptibility QTLs to chromosomes 5q and 13q.[209] In ***paper V*** we aimed to identify the susceptibility gene(s) for preeclampsia at the 13q QTL by genotyping SNPs within positional candidate genes in both The 74 Family Cohort and our Norwegian case/control cohort. Only one of the four genes selected for genotyping, the tumor necrosis factor ligand superfamily, member 13B (*TNFSF13B*), showed association with preeclampsia in the two independent cohorts. In fact, this association pertained to an identical SNP in *TNFSF13B* in both the Aust/NZ and Norwegian cohorts. Our combined cohort association analysis showed significant association with preeclampsia susceptibility (p=0.002), which remained significant also after correcting for the total experiment (p=0.047).

Ligands and receptors of the TNF superfamily are involved in signaling pathways important during development and host defense, they also have crucial roles in the regulation of cell survival and death in immune tissues. TNFSF13B is among the non-apoptosis-inducing

ligands playing a vital role in immune responses, in both the adaptive[455,456] and innate[457] immune systems. Non-apoptosis-inducing ligands like *TNFSF13B* have also been suggested a role in conferring immune privilege.[140,458] Studies have demonstrated that both TNFSF13B gene and protein are well expressed in cytotrophoblasts.[459,460] The observation of expression of TNFSF13B in villous cytotrophoblasts and in addition placental mesenchymal cells suggests that this protein may drive placental development, cell differentiation and/or other functions in the human placenta.[460] Expression of *TNFSF13B* has been suggested to navigate maternal leukocytes toward a favorable maternal immune response, being beneficial for the fetus, instead of a harmful one.[459] TNFSF13B has also been proposed a role in angiogenesis based on the observation that TNFSF13B is absent in the blood vessel endothelium of abortion patients whilst it is detected in normal pregnant women.[459] Angiogenesis is essential for normal placental development, and abnormal angiogenesis with insufficient remodeling of spiral arteries is a hallmark of preeclampsia.

The associated *TNFSF13B* SNP is a rare intronic SNP. However, this does not detract from its potential in playing a functional role in disease pathophysiology.[461-465] It is well known that SNPs within regulatory regions (such as regions for transcription factor binding sites, exon splicing enhancers, conserved regions, splice sites and 3'UTR microRNA binding sites) of the genome can affect gene transcription. In the recent years, several studies have shown association between intronic SNPs and various complex diseases and it has become evident that rare variants (variants with minor allele frequency less than 1%) may play an important role in complex diseases.[222,466,467]

In conclusion, The TNFSF13B protein may be of profound importance to reproductive success in women,[459] and may serve as a potential factor bridging the angiogenic and non-

cytotoxic response machineries at the maternal-fetal interface.[468] The finding of the genetic association between the *TNFSF13B* SNP and maternal preeclampsia susceptibility in two independent populations implicates this gene as a novel preeclampsia susceptibility gene, and supports the hypothesis that this gene may be of profound importance for a variety of processes essential to a successful human pregnancy. The fact that *TNFSF13B* resides within a region previously shown to be in linkage with preeclampsia susceptibility strengthens the association result.

# 5 DISCUSSION

## *5.1 Methodological considerations*

In the fall of 2005, when most of the genotyping in the Norwegian case/control cohort presented in this thesis was performed, the iPLEX Gold assay from Sequenom and the GoldenGate assay from Illumina were not yet launched. However, Applied Biosystems' SNPlex assay was launched January 2004, and was therefore chosen. Our experience with the SNPlex assay is that it is sensitive, but not as robust as the high-throughput iPLEX Gold and GoldenGate genotyping assays launched in late 2005 and early 2006, respectively.

Due to the high sensitivity of the SNPlex assay and the varying genotyping success rates (≤82%, sometimes considered less than satisfying), we decided to replicate SNPs that showed nominal and borderline association with preeclampsia with the SNPlex technology using a different technology. Sequenom's MassARRAY technology was available at the Centre for Integrative Genetics (CIGENE), a Norwegian genotyping service institution. With the iPLEX Gold genotyping assay we obtained very high genotyping success rates (≥98.5%).

To genotype samples in the Aust/NZ family cohort Illumina's GoldenGate assay was used. This assay is robust and gives high genotyping success rates. Both the iPLEX Gold and GoldenGate assays seem to be preferred to the SNPlex assay nowadays. In addition, both the Illumina and Sequenom platforms can be used for additional approaches such as gene expression analysis, methylation analysis and copy number variation analysis. Another advantage of the GoldenGate assay is that it allows a higher level of multiplexing than the SNPlex and iPLEX Gold assays. It should also be mentioned that Illumina is one of the two major vendors of high-throughput genome-wide SNP genotyping technology.

In conclusion, if I were to perform high-through put genotyping today, I would not have chosen the SNPlex assay. I would rather have chosen the GoldenGate or iPLEX Gold assay depending on the number of SNPs I were to genotype.

## 5.2 Study design

Study design is perhaps the single most important issue in the planning of any genetic study. When planning a genetic association study, one must consider four major components: 1) the disease or trait to be studied i.e. the phenotype, 2) the study groups, 3) the genetic markers to be typed (genotype), and 4) the analytic method to test for association between genotype and phenotype data. The feasibility, necessary sample size, statistical power of a study and statistical analysis of genetic association data depend critically on the study design. Accordingly, advice from statistical geneticists is most helpful before the study begins. The use of the latest "highly promising" new method – or new software implementation of a current method – cannot rescue a poorly designed study.

### 5.2.1 Phenotype

Definition of the phenotype is crucial in designing any genetic study,[469] especially an association study that intends to detect disease predisposing genes. Accurate phenotype definition (i.e. one that has high specificity) is necessary to minimize the number of genes that may affect risk of disease, and thus to increase the odds of finding these genes.[470] Definitions of phenotype in complex diseases are problematic due to issues of heterogeneity, gene-environment interactions, and gene-gene interactions.[471] Complex diseases may have diverging clinical manifestations, different biological paths leading to disease diagnosis, differences in severity of symptoms, symptoms that may deteriorate over time, and

differences in disease penetrance may all contribute to disease heterogeneity.[470,472,473] Metabolic syndrome (abdominal obesity, high serum triglycerides, low HDL cholesterol, high BP, high fasting blood glucose) and CHD (myocardial infarction, atherosclerosis, and sudden cardiac death) are two common examples of complex diseases that are clinically multifactorial and where each element may have its own genetic influence. Clinical heterogeneity, meaning variation in clinical characteristics such as asymptomatic versus symptomatic individuals, diagnostic classification schemes, age of onset, severity, family history, diagnosis being almost any combination of symptoms, also displays in most diseases of interest.[470] In cases where there are no biochemical markers or tests for the disease of interest, the phenotype may be defined using subjective assessments. Either through the use of diagnostic criteria, or by satisfying a predetermined number of symptoms can lead to imprecision and heterogeneity in the phenotype.[474] One can minimize heterogeneity by focusing solely on those satisfying certain criteria on the list. However, this may affect sample size and reduce statistical power.

Diagnostic error (misclassification) can substantially reduce the power to detect an association between a trait and marker locus.[475,476] Misclassification due to poorly defined phenotypes can make it impossible to detect a true association or to identify spurious associations stemming from biased classification of the phenotype or confounding of the phenotype.[477] Heterogeneity of phenotype and its underlying component can also lead to misclassification of phenotype.[477] Further, dichotomizing a continuous phenotype also leads to a loss of power. One can reduce the potential of misclassification by using standard phenotype definitions, medical records or consensus committee agreement for a valid diagnosis.[477] Validity refers to knowing what is being measured and how accurately it is being measured.[478] However, most phenotypes can be defined by more than one instrument. Use of

a variety of methods to assess and measure phenotypes, often referred to as measurement errors, adds variability in phenotype definition, and thus, contributes to variability to any genetic study.[474]

Preeclampsia in our Norwegian HUNT case/control cohort was defined according to the criteria given by National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy.[40] These diagnostic criteria are similar to the criteria in the 1998 guidelines from The Norwegian Society of Gynecology and Obstetrics (NGF) (BP≥140/90 mmHg and ≥0.3 g/L in a 24 hour urine sample or ≥1+ on a dipstick reading after 20 weeks of gestation).[61] National (Norwegian) guidelines for the preeclampsia diagnosis for clinicians responsible for taking care of pregnant women were not established until 1995. From 1967-1998, preeclampsia in the MBRN databank was registered by ICD-8 codes, whereas ICD-10 codes were used from 1998. The registration in MBRN is based on the information in the scheme filled out by midwifes/clinicians at the Delivery ward. This implies that the diagnosis of preeclampsia may have been used based on the criteria recommended by MBRN or alternatively, based on the midwife's/clinicians' own interpretation of the clinical manifestations presented. This may represent a problem with regards to the phenotypic consistency of the preeclamptic women included in MBRN since both definition and diagnostic criteria for preeclampsia have changed over time, also in Norway. Definition has changed with the revisions of the ICD codes, while diagnostic criteria have changed with the guidelines given by NGF, at least for the last 14 years. In 1995 NGF defined preeclampsia as BP ≥140/90 mmHg or an increase of DBP ≥15 mmHg compared with mean BP values measured before 20 weeks of gestation and proteinuria with ≥0.3 g/L in a 24 hour urine sample or ≥1+ on a dipstick reading after 20 weeks of gestation,[57] whereas the criteria used today are more restrictive; BP ≥140/90 mmHg combined with proteinuria ≥0.3 g/L in a 24

hour urine sample or a ≥1+ on a dipstick reading measured on at least two occasions with a 4-6 hour interval.[63] The todays NGF guidelines also specify mild and severe preeclampsia. Since there were no written national guidelines before 1995, it is difficult to evaluate changes in diagnostic considerations that might have taken place in Norwegian hospitals from 1967 (when MBRN was established) to 1995.

The importance of valid diagnoses when performing genetic studies has received increasing attention among researchers the last years. In accordance with this, we have started to validate the diagnosis of preeclampsia by using the hospital records for the women registered as cases in our HUNT cohort. The preliminary results show that approximately 90% of these women fulfill the diagnostic criteria (given by MBRN; BP≥140/90 mmHg and≥0.3 g/L in a 24 hour urine sample or ≥1+ on a dipstick reading after 20 weeks of gestation) used for preeclampsia in the presented work. In a different study we found that the majority of the cases that were not confirmed had increased BP, but they turned out to have no documentation of proteinuria in their hospital records (Vestrheim et al., *in preparation*). We assume that this is also the case in our HUNT case/control cohort. There is a possibility that the genetic associations observed in the present work would have been stronger if the genotypes of the approximately 10% that did not fulfill our diagnostic criteria were removed from the analysis. It remains to have a closer look at this when the validation process is finished.

After the genetic analyses were performed, we have also had a closer look at our control group to study if individuals included in the control group had suffered from conditions potentially disturbing the genetic comparisons between cases and controls. The control group has in the published papers presented in this thesis been described as normal healthy pregnant women. In fact, the control group was selected from the MBRN database as non-

preeclamptic, implying that they were not necessarily completely normal. By looking closer to the information in the MBRN database, we have found that some women in the control group had suffered from viral or bacterial infections during pregnancy (n=40), others had experienced pregnancy related complications such as hemorrhage, anemia (n=98) or hyperemesis (n=144), while six women had diabetes mellitus. Therefore, the controls have been described as non-preeclamptic women in this thesis. However, we assume that the mentioned conditions, with the exception of diabetes mellitus, are not related to the genetics of preeclampsia.

### *5.2.2 Study groups*

Selection of a population is an important issue when planning a genetic association study. Genetic homogeneous populations are preferred to populations with high heterogeneity, since heterogeneity of genetic backgrounds among individuals in association studies may lead to false positive or false negative results. It is also important to ensure that the cases and controls are drawn from the same population, in order to avoid population stratification. Population stratification or population structure is the presence of a systematic difference in allele frequencies between subpopulations, such as cases and controls, in a population due to an overrepresentation of an ethnic group among individuals in one of the groups and underrepresentation among individuals in the other group. In the scenario of population stratification a significant *p*-value obtained is likely to be a reflection of the allele frequency differences between the two groups rather than a true association with disease. To avoid population stratification one should measure association in well-mixed, outbred populations. Matching by geographical region and by any markers of ethnic origin (in the design or the analysis, or both) can reduce population stratification.[192]

Sample size is a key determinant of quality in an association study.[479] Calculating the actual sample size needed to find a true genotype-phenotype association under the study constraints, i.e. the power of a study, is an important part of the study design. How many subjects one needs for a well-powered analysis depends on the allele frequency of the genetic marker, the genotype relative risk (the allelic odds ratio), and the significance level ($\alpha$-level). In addition, one should consider the inheritance model (dominant, recessive or additive) and the measure of the overall disease risk in the population.

The preeclamptic cases and the controls in the work presented in this thesis were retrospectively identified from a ethnically homogeneous population, with less than 3% non-Caucasians, regarded as a representative of Norway with regard to geography, economy, industry, age distribution, morbidity, and mortality. Among the approximately 65,000 participating in the HUNT2 study we were able to identify 1,139 women registered with at least one preeclamptic pregnancy in the MBRN with a biological sample available from the HUNT biobank. A group of over 1,000 preeclamptic cases is considered rather large in case/control studies of candidate genes. Other published case/control studies from Great Britain[227] and Finland[480] included 657 and 248 preeclamtic cases, respectively. Due to the ethnic homogeneity and large sample size we consider our Norwegian case/control cohort well suited for genetic epidemiologic studies. Another advantage Norwegian researchers have is the access to data from good quality health registers, such as the MBRN.

**Subgroups of preeclampsia.** In our efforts to identify subsets of preeclamptic women classified cases according to clinical characteristics related to the syndrome; i.e. the parity of women developing preeclampsia (first pregnancy or subsequent pregnancies) was registered and if preeclampsia developed in one of more pregnancies and if preeclamptic symptoms

developed early (<34 weeks of gestation) or late (close to or at term) in pregnancy. Among our cases, 1003 women were registered with only one preeclamptic pregnancy (606 in their first pregnancy and 397 in a later pregnancy), whereas 136 women had experienced preeclampsia in more than one pregnancy (recurrent preeclampsia). Classification of cases by mild or severe and by early or late onset preeclampsia was not done, since this was only possible for the cases who delivered after 1998 when MBRN changed their routines and introduced a new registration form for deliveries. But, it turned out that the subgroups based on these criteria were too small to have the statistical power required for genetic comparisons. However, the research value of subgroups of significant size should not be underestimated. Preeclampsia is a condition likely to be a result of heterogeneous causes of both maternal and placental/fetal derivation, potentially involving several separate pathophysiological pathways. There is also a discrepancy in maternal outcome related to having preeclampsia as a multipara, as a recurrent event and by early onset of the disease. It has been shown that women experiencing early onset preeclampsia is more likely to have preeclampsia in a subsequent pregnancy.[481] Recurrent and early onset preeclampsia are associated with a higher risk of developing CVD later in life than experiencing this disease in first pregnancy at term.[19,20,482] These observations suggest that maternal constitutional factors are extensively involved in this subtype(s) of preeclampsia and that immune maladaption is not the solely causal factor. The recurrent and early onset preeclampsia are likely to be strongly associated with inflammation, oxidative stress and genetic susceptibility, whereas risk of preeclampsia among primiparas is more likely to represent the immune maladaption hypothesis. In conclusion, one should try to select the cases that seem most appropriate based on what the focus of the study is.

### *5.2.3 Genotype*

Genotyping involves errors, although to a much lesser degree than phenotype measurement. There will never be a perfect system for genotyping, or for detecting and correcting genotyping errors. Even with the best methods, some assays will be unreliable. Genotyping errors can be generated by different causes, there are errors linked to the DNA sequence itself, errors that are due to low quality or quantity of the DNA, biochemical artifacts and human factors. A mutation close to a marker can generate a genotyping error if this flanking sequence is involved in the marker-detection process. Genotyping errors are also promoted by low DNA quantity and/or quality. Extreme dilution of or degradation of the DNA results in a low number of target DNA molecules, favoring allelic dropouts (i.e. only one of the two alleles present at a heterozygous locus is amplified), false alleles (allele-like artifact generated by PCR) and increased risk of contamination.[483] An example of a biochemical artifact potentially generating genotyping error is the "+A artifact" which is a result of the Taq polymerase adding a non-template nucleotide (usually adenine (A)) to the 3' end of the newly synthesized strand at the end of the elongation step of a PCR.[484,485] This creates an artifactual band/peak on a gel/trace readout, respectively. The relative proportions of the true fragment and the +A artifactual fragment are very sensitive to the sequence of the 5′ end of the primer used in the genotyping assay, but also to PCR conditions and to the long elongation times that promote the +A artifact. In such a context, this biochemical artifact represents an important cause of genotyping error. Human factors have been identified as the main cause of genotyping errors in the few studies designed to analyze the precise causes of genotyping error.[486] In one study on microsatellites human factors (scoring, data input, sample mix-up, pipetting error or contamination) were responsible for about 93% of the errors.[487] Scoring errors might be an important issue in both manual and automated/semi-automated scoring of fluorescence profiles.[488,489] Allele calling has also been identified as a potential problem in

SNP studies.[490] However, the risk of human scoring error strongly depends on the quality of the data. When an estimated genotype is not the true genotype it is called a genotype misclassification error. The misclassification rate of genotypes depends on the type of marker (microsatellite or SNP), the specific marker being typed, the quality of the DNA sample and the protocol and experience of the laboratory staff among other factors. Misclassification rates of SNPs are much lower than of microsatellites and occur in less than 1% of genotypes with the current technology.[491] It may be problematic to type rare variants since these are difficult and sometimes impossible to distinguish from genotype errors and mutations in population studies.

Random genotyping error reduces the power to detect true case/control differences,[492,493] by each 1% rise in genotyping error an increase of the sample size by 2–8% might be required to maintain constant type I and type II error rates.[492] Although random genotyping errors have an effect on power, systematic genotyping errors are of greater concern. In case/control studies DNA samples may be processed in batches due to a large number of samples and that storage and subsequently genotyping of different sample sets are performed on separate plates. Cases and controls should be ascertained and genotyped together in order to ensure that differences in genotypes are not due to variation in processing the samples and variations between batches. It is also important that quality controls are in place for accurate genotype calling.

Until now only a few studies have dealt with the genotyping error issue related to association studies. Thus, the effect of genotyping errors remains largely unknown in this field.[483,488,494,495] Lack of studies and complexity of the problems are possible explanations for the delay in establishing strict standards to promote data quality in association studies.

However, researchers have become aware about the occurrence of genotyping errors and their potential effect, thus it is likely that increasing attention will be paid to these difficulties when designing experimental protocols and publishing results.

In our SNPlex genotyping we used 384 well plates, some with only cases and some with only controls, but there were plates also mixed with case and control samples. However, in the genotyping processing there were always both plates with cases and controls being treated at the same time. It is admitted that handling cases and controls on separate plates might have introduced errors, thus in our replication with the iPLEX Gold assay we ensured that both cases and controls were present on each plate. Also in the TaqMan genotyping (*paper III*), both cases and controls were present on each plate.

With the SNPlex genotyping assay, we observed that samples with low DNA concentrations (that had to be concentrated prior to genotyping processing) showed poorer genotyping results than those with high start concentration. To minimize the effect of poor genotype clustering, sample wells of poor quality were first identified and subsequently removed prior to genotyping clustering and statistical analysis.

### *5.2.4 Analytical methods*

Study design and analysis methods are tied to each other, and the type of data to collect (family or population-based) predetermine what analytical method to test for association between genotype and phenotype data that can be used (linkage or association). There are many different analytical methods available, however the researchers must consider which one is best in their study situation.

### *5.2.5 Summary*

The inconsistency of results in preeclampsia association studies is likely due to several causes:

1) there is no universal definition of preeclampsia and inclusion criteria are often inconsistent in terms of underlying chronic conditions, parity and ethnicity

2) the maternal genotype and the fetal (paternal) genotype is likely to have a predisposing effect,

3) association studies with small sample sizes often miss small-to-moderate effects because of lack of adequate statistical power and observed effects may be overestimated. It has been postulated that the main reason for the poor success in complex diseases with any approach is the lack of attention to experimental design.[352] Genotyping errors are also a possible source of bias in association studies.

Due to the lack of replication of initial findings in association studies, a strict phenotype definition is important. However, there is mounting evidence that natural selection has shaped a considerable part of the differentiation observed between different ethnic subsets. Differential frequency of the trait-causing genetic variants and/or interactions with varying environments in different populations makes it difficult to detect uniform effects in all populations. If this is a major feature in our genomic evolution then it will be even more important to examine and compare a variety of different ethnic groups in future genetic studies in order to reveal the genetic basis of preeclampsia. This will also be the case for other common complex diseases or traits.

## 5.3 Interpretation of results

Perturbations within vascular, immunological and inflammatory pathways constitute important physiological mechanisms in preeclampsia pathogenesis. All the genes reported associated with preeclampsia in the papers presented in this thesis pertain to one or more of these pathways, and it seems like most of them are pleiotropic. Abnormal placental development, predisposing maternal constitutional factors (such as diabetes, hypertension, obesity, and hyperlipidemia), oxidative stress, immune maladaption and genetic susceptibility are categories of suggested underlying causes of preeclampsia (reviewed in [64-66]).

### 5.3.1 Selenoprotein S (SEPS1)

Selenoproteins play crucial roles in regulating ROS and redox status in a wide variety of tissues and cell types, including those involved in innate and adaptive immune responses.[496-498] Thus selenoproteins such as SEPS1, obviously pertain to both the immunologist's and vascularist's views. Given the known association of SEPS1 with inflammation, we investigated the effect of a known functional genetic polymorphism in *SEPS1* on the risk of preeclampsia (*paper I*). A significant association was found between this polymorphism and increased risk of preeclampsia. This finding supports the hypothesis of Curran et al. suggesting that SEPS1 might be involved in the pathogenesis of many common complex diseases influenced by inflammation. Replications in independent population cohorts with large sample size are needed to clarify the role of the *SEPS1* -105G>A promoter polymorphism on the risk of preeclampsia.

### *5.3.2 Activin receptor type II A (ACVR2A)*

In *paper II* we report association between genetic polymorphisms within the activin receptor *ACVR2A* and preeclampsia. Activin receptors modulate signals for the multifunctional cytokine activin A, which is expressed in a wide range of tissues and cells. Endothelial cells, smooth muscle cells and macrophages in vascular tissue have been shown to express activin receptors,[390] and activin A has an established role in endothelial functioning and vascular homeostasis. Several studies suggest that activin A is involved in pathogenesis of diseases influenced by inflammation.[387-389] It has been proposed that activin A is an element of the early innate response to danger and that it is a defensive or regulatory component of the innate immune response.[499] In light of the pleiotropic effects of activin A, pertaining to both the vascular and immune systems, *ACVR2A* is a plausible candidate gene for preeclampsia from both the vascularist's and the immunologist's point of view. Altered ACVR2A binding may result in endothelial dysfunction triggering systemic inflammation, hypertension, proteinuria and edema. In addition, altered expression of ACVR2A and binding to activin A may result in abnormal development of placenta.[311,312]

Recently, the Australian research group has comprehensively re-sequenced the entire coding region of the *ACVR2A* gene and the conserved non-coding sequences.[321] This resulted in identification of 9 novel SNPs, which were genotyped together with 36 additional *ACVR2A* SNPs in 480 individuals from 74 Aust/NZ preeclampsia families. Associations between five *ACVR2A* polymorphisms, three being novel, and preeclampsia were observed ($p<0.03$). However, after correction for multiple hypothesis testing, none of these associations reached significance ($p>0.05$). Based on this data it remains unclear what role, if any, *ACVR2A* polymorphisms play in preeclampsia risk, at least in the Aust/NZ families. It remains a possibility that the observed association with preeclampsia in the Norwegian cohort is due to

LD between *ACVR2A* SNPs and a functional variant close to this gene. LD patterns for the SNPs tested in the two studies are shown in Figure 9. The inconsistent results may also be due to allelic heterogeneity between the Aust/NZ and Norwegian populations, or alternatively, the sample size of the Australian study may be too small. In conclusion, replication in sufficiently powered studies is needed to further clarify the role of *ACVR2A* in preeclampsia risk.

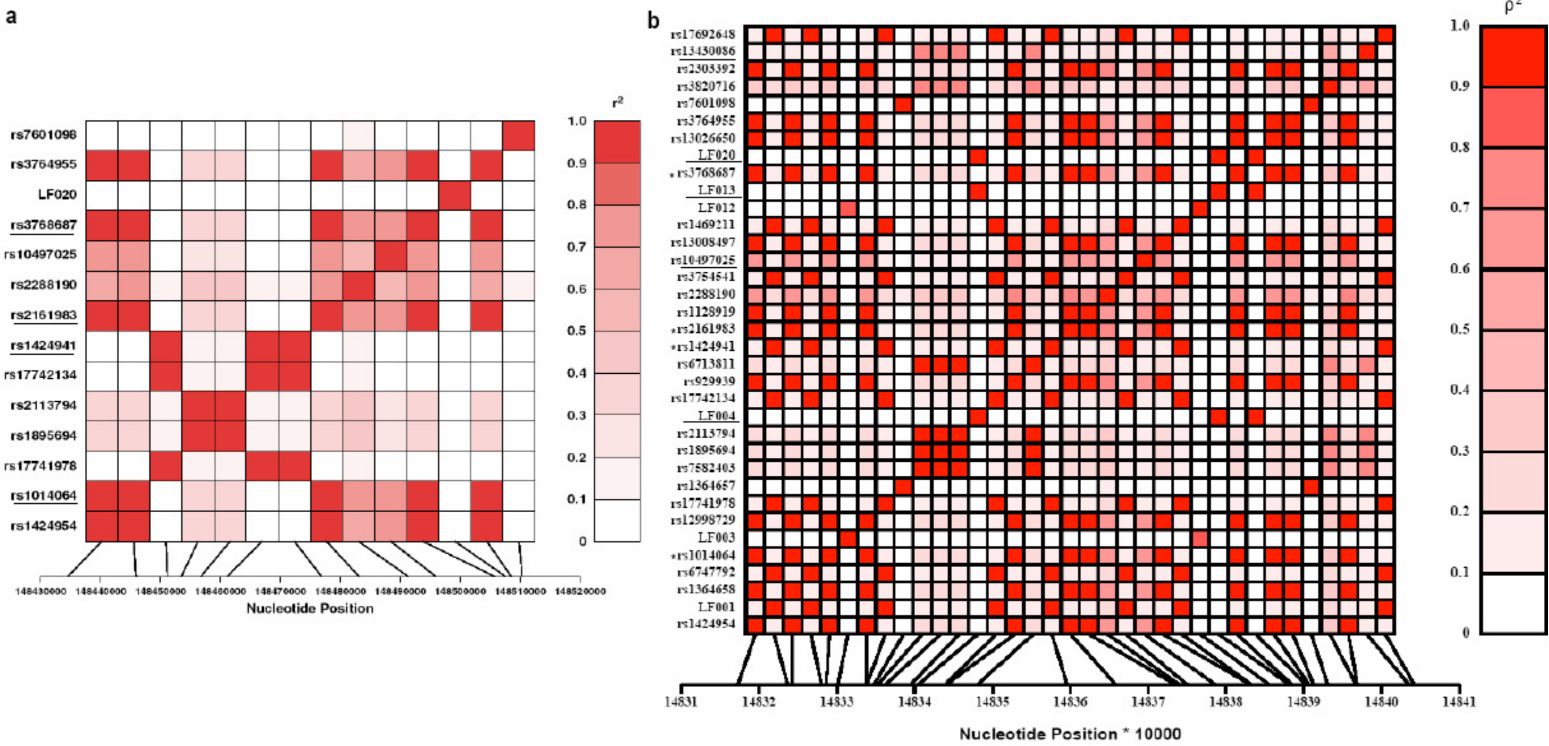


**Figure 9. The pattern of LD in ACVR2A.** The metric to the right of the plots ($r^2$ or $\rho^2$) indicates the strength of the correlation among genotypes at each SNP locus with 0.0 indicating no correlation (i.e. LD) and 1.0 indicating a perfect correlation (i.e. complete LD). **(a)** The pattern of LD in ACVR2A in the HUNT case/control cohort. The SNPs rs1424941, rs1014064, rs2161983 and rs3768687 (underlined in (a), and marked with * in (b)) showed significant association with preeclampsia.[322] **(b)** The pattern of LD in ACVR2A in the Aust/NZ family cohort. The SNPs rs10497025, rs13430086, LF004, LF013 and LF020 (underlined) showed significant association with preeclampsia before correcting for multiple testing.[321]

### *5.3.3 Catechol-O-methyltransferase (COMT)*

As a protein involved in BP regulation and oxidative stress, via its metabolite 2-ME, COMT represents the vascularist's view. We have shown that the *COMT* Val158Met polymorphism is not associated with preeclampsia risk (*paper III*), but we cannot *exclude* that this enzyme plays a role in the development of preeclampsia. It has been reported that the Val158Met polymorphism alone cannot account for the observed variation in COMT activity. A different SNP (rs4818) in the haploblock has been suggested to account for a greater variation in COMT activity compared to the Val158Met polymporphism.[288] However, the fact that the COMT locus has not been reported as a preeclampsia QTL further disagrees with the hypothesis of Kanasaki et al.. In order to clarify the role of *COMT* as a genetic risk factor for preeclampsia development, additional COMT polymorphisms have to be investigated in a large number of cases and controls.

### *5.3.4 Endoplasmic reticulum aminopeptidase 2 (ERAP2)*

With the key role *ERAP2* has in BP regulation via its involvement in RAAS, this enzyme obviously represents the vascularist's view. In addition, aminopeptidases, such as *ERAP1* and *ERAP2*, have important roles in protein maturation and protein stability in addition to cellular maintenance, growth, development, and defense mechanisms.[429] It is likely that ERAP2 plays a role in immunological pathways based on it's highly conserved extracellular domain with *ERAP1* and proposed divergence from a common ancestral gene.[427] Thus, ERAP2 may also fit in with the immunologist's view.

In *paper IV* we report significant association between *ERAP2* SNPs and preeclampsia risk in two independent populations; the Norwegian and the Aust/NZ. However, the associated SNPs seem to be population specific (Figure 10).

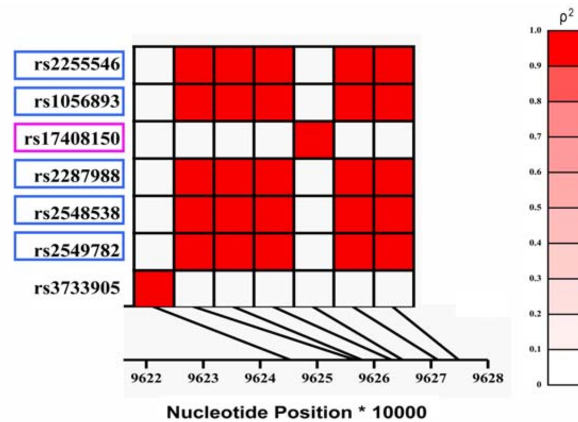


**Figure 10. The pattern of LD in ERAP2.** The metric to the right of the plot ($\rho^2$) indicates the strength of the correlation among genotypes at each SNP locus with 0.0 indicating no correlation (i.e. LD) and 1.0 indicating a perfect correlation (i.e. complete LD). The plot shows the pattern of LD in ERAP2 in both the HUNT case/control and Aust/NZ family cohorts. The SNP rs17408150 (pink box) showed significant association with preeclampsia in the HUNT case/control cohort, whereas the SNPs rs2255546, rs105 6893, rs2287988, rs2548538 and rs2549782 (blue boxes) showed significant association with preeclampsia in the Aust/NZ family cohort.

An alternative explanation for the divergent SNP associations in the two populations might be that we have observed a false positive association(s). On the basis of the physical proximity between *ERAP2*, *ERAP1* and *LNPEP* it is possible that there are other functional genetic variations in *ERAP1* or *LNPEP* that are in LD with the significant *ERAP2* SNP associations. Deep re-sequencing of all three genes followed by further association analyses in the Australian family and Norwegian case/control cohorts will be required to resolve this. Independent replication in other populations is also encouraged in order to clarify the role of *ERAP2* on preeclampsia risk.

### *5.3.5 Tumor necrosis factor ligand superfamily, member 13B (TNFSF13B)*

Our observed association between *TNFSF13B* and preeclampsia (*paper V*) suggests that the influence of the maternal immune system is significant. The observation appears consistent with the theory that maternal-fetal immune maladaption is central in the pathogenesis of the preeclamptic syndrome. A potential role of TNFSF13B as an angiogenic factor has also been suggested,[459] in that case it potentially also represents the vascularist's view. Furthermore, TNFSF13B has recently been suggested a role in implantation, cell differentiation and placental development/function[459,460] via its expression by maternal decidual stromal cells and trophoblasts.

Although we observed association between preeclampsia and an identical *TNFSF13B* SNP in two independent populations, we cannot rule out the possibility that there exists other as yet unidentified rare sequence variant(s) in LD with the associated SNP that are the actual causal mutations. Deep re-sequencing in, and around, the *TNFSF13B* gene in our preeclampsia cohorts will be required to enumerate and prioritize all variation prior to formal functional evaluation. Very recently, we have in close collaboration with the Australian researchers performed deep re-sequencing, and are currently planning further association analyses in both the Aust/NZ family and Norwegian case/control cohorts. We also encourage replication in other independent populations.

Finally, due to the observation of trophoblasts expressing TNFSF13B it would be interesting to look into how fetal *TNFSF13B* genotype, alone or in combination with maternal genotype, influences preeclampsia development.

## 5.4 *Inflammation, preeclampsia and other common complex diseases*

Inflammation has a role in and often precedes the onset of many common complex diseases, including cancer, atherosclerosis, diabetes and Alzheimer disease. It has been suggested that genetic factors have a key role in inflammation, and the search for genes and genetic variants of relevance is a strong ongoing research activity. Normal pregnancy is characterized by a mild, systemic inflammatory response, but the inflammatory manifestations are much more vigorous in preeclampsia with endothelial dysfunction and activation, together with increased levels of oxidative stress markers and pro-inflammatory cytokines.[500] Accordingly, it has been hypothesized that any factor that would increase the maternal inflammatory response to pregnancy would predispose to preeclampsia.[75] Abnormal maternal inflammatory responses seem to favor development of preeclampsia; i.e. increased preeclampsia risk has been reported in women with inflammatory cytokine[267] or cytokine receptor[276] gene polymorphisms. In accordance with this, we have observed increased frequency of other gene variants involved in inflammation, i.e. the *SEPS1*[501] and the *ACVR2A*[322] genes in HUNT2 preeclampsia cases.

SEPS1 is strongly suggested to be involved in ER stress,[279] which is likely to induce systemic inflammation.[502] ER stress has been associated with a wide range of diseases including stroke, cardiac disease, diabetes, cancer, neurodegeneration, bipolar disorders, muscle degeneration and others.[503] Some of these diseases are also associated with preeclampsia, e.g. CVD and diabetes. The number of studies investigating the effect of *SEPS1* polymorphisms on the risk of different diseases influenced by inflammation is increasing. A recent study of the effect of *SEPS1* SNPs on the risk of cardiovascular disease (including both coronary heart disease (CHD) and ischemic stroke (IS)) in Finnish cohorts is supportive. This study showed that genetic polymorphisms in *SEPS1* is likely to increase the risk for coronary heart disease and

ischemic stroke, particularly in females.[504] Suggestive associations of *SEPS1* SNPs were also seen with the known cardiovascular risk factors body mass index (BMI) and waist-hip ratio.[504] Interestingly, secreted SEPS1 has been associated with very low density lipoprotein (LDL) fraction of the serum suggesting a role also in the lipid metabolism.[356] Thus, *SEPS1* might be a genetic link between preeclampsia and CVD and diabetes, since BMI, waist-hip ratio and abnormal lipid profiles are associated with increased risk of all these conditions.

Other studies of *SEPS1* polymorphisms on the risk of variety of inflammatory diseases have also recently been reported, however with inconsistent results (Table 4).

**Table 4: Studies performed to date (April 2009) on *SEPS1* polymorphisms and a variety of diseases influenced by inflammation.**

| Publication | Disease | Sample size | SNPs | Association (+/-) | Population |
|---|---|---|---|---|---|
| Alanne et al. 2007[504] | **Cardiovascular**<br>CHD<br>IS | <br>999<br>1,223 | rs8025174<br>rs28665122<br>rs4965814<br>rs4965373<br>rs9874<br>rs7178239 | +[a]<br>÷<br>÷<br>÷<br>÷<br>+[b] | Finnish |
| Hyrenbach et al. 2007[505] | **Cerebrovascular**<br>IS, cervical artery dissection<br>IS, non-cervical artery dissection | <br>260<br>295 | rs28665122 | ÷ | German and Italian |
| Seiderer et al. 2007[506] | **IBD** | 359 | rs28665122 | ÷ | German |
| Moses et al. 2008[501] | **Preeclampsia** | 1,139 | rs28665122 | + | Norwegian |
| Martinez et al. 2008[507] | **Autoimmune inflammatory**<br>RA<br>IBD<br>T1D | 592<br>674<br>311 | rs11327127<br>rs28665122<br>rs4965814<br>rs12917258<br>rs4965373<br>rs2101171 | ÷<br>÷<br>÷<br>÷<br>÷<br>÷ | Spanish |
| Shibata et al. 2009[508] | Gastric cancer | 268 | rs28665122 | + | Japanese |

a – this SNP was associated with increased risk of CHD in females only.

b – this SNP was associated with increased risk of IS in females, and in females combined with males.

No association with the *SEPS1* -105G>A promoter polymorphism on the risk of cerebrovascular disease (including IS because of cervical artery dissection and IS of non-cervical artery dissection origin) has been observed, but the sample size of the groups studied were small.[505] Lack of association with *SEPS1* polymorphisms on the risk of autoimmune inflammatory diseases, such as rheumatoid arthritis (RA), inflammatory bowel diseases (IBD) (including both Crohn's disease and ulcerative colitis) and type 1 diabetes (T1D), was also recently reported,[506,507] but as for the cerebrovascular disease study, the sample sizes were small. The most recent study of the *SEPS1* -105G>A promoter polymorphism reports influence on susceptibility to gastric cancer in a Japanese population.[508] This study is also a small one, and further studies with a greater number of cases will be needed to fully address the importance of these *SEPS1* genetic polymorphisms on the risk for inflammatory diseases and gastric cancer.

As a key receptor for activin A, a critical mediator of inflammation and immunity, ACVR2A is likely to be of importance in inflammatory diseases. Our observation of association between *ACVR2A* SNPs and preeclampsia confirms this suggestion. To my knowledge there are no association studies published on *ACVR2A* SNPs and diseases associated with preeclampsia, such as cardiovascular diseases. However, aberrant gene expression (mRNA) of *ACVR2A* has been observed in unstable angina patients.[386] And it has been hypothesized that dysregulated expression of activin A and its receptors could contribute to atherogenesis and plaque destabilization.[386] Association studies of *ACVR2A* SNPs in large samples of common complex diseases influenced by inflammation are needed in order to clarify the role of this gene on the risk of these diseases.

It is possible that common genetic predisposition may underlie preeclampsia and diseases associated with preeclampsia, such as CVD. Thus, it is likely that new knowledge gained about genes/genetic mechanisms important in preeclampsia also will provide insight relevant for understanding the development of cardiovascular diseases in women.

# 6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Taken together, the results presented in this thesis confirm that preeclampsia is a complex genetic disease, involving different biological processes previously ascribed a role in the pathogenesis of this disease; inflammation, immune maladaption, abnormal placental development. The papers presented in this thesis show that maternal genes may contribute to the predisposition to all these processes. In this regard it is not surprising that simple clinical indicators are ineffective in identifying preeclamptic women, since the condition is likely to be a result of heterogeneous causes of both maternal and placental/fetal derivation, potentially involving several separate pathophysiological pathways. There is also a discrepancy in maternal outcome related to having preeclampsia as a multipara, as a recurrent event and by early-onset of the disease. This realization has led to the formulation of a new conceptual framework suggesting that a combination of markers (biochemical and/or biophysical) may be required to conduct comprehensive risk assessment for the syndrome.

To further elaborate the genetic contribution to development of preeclampsia one should focus on different preeclampsia phenotypes. Although we are able to classify our HUNT2 preeclampsia cases in different preeclampsia subgroups, we need to increase the sample sizes before performing association studies of significance. Increasing the sample sizes can be done by adding preeclampsia cases from the Norwegian Mother and Child Cohort Study (MoBa).[509,510] The MoBa cohort is an ongoing pregnancy study (started in 2001), with the aim of comprising 100,000 pregnancies. The purpose of the MoBa study is to find causes of diseases among mothers and children. A highly prioritized focus of attention for the MoBa cohort is genetic factors, as well as the potential interplay between genes and exposure (environmental factors). With the prevalence of preeclampsia among Norwegian pregnant

women,[511] approximately 2,550 preeclamptic pregnancies will be found among the 90,000 included at present in the MoBa cohort.

Investigators studying complex disorders are increasingly turning to integrated approaches, which must include an appreciation of underlying biological processes, but can also utilize the power of genome-wide screening, RNA microarray technology, proteomics and bioinformatics to generate novel hypotheses. One by one these resources may generate hundreds of candidate genes. Combining two or more of the resources seem to improve the yield in reducing the number of candidate genes and in enabling us to focus on the most likely candidate genes.[512,513] Figure 11 gives an overview of resources being available to my research group and ongoing or planned studies, and how these can be combined to improve the understanding of genetic susceptibility of developing preeclampsia.

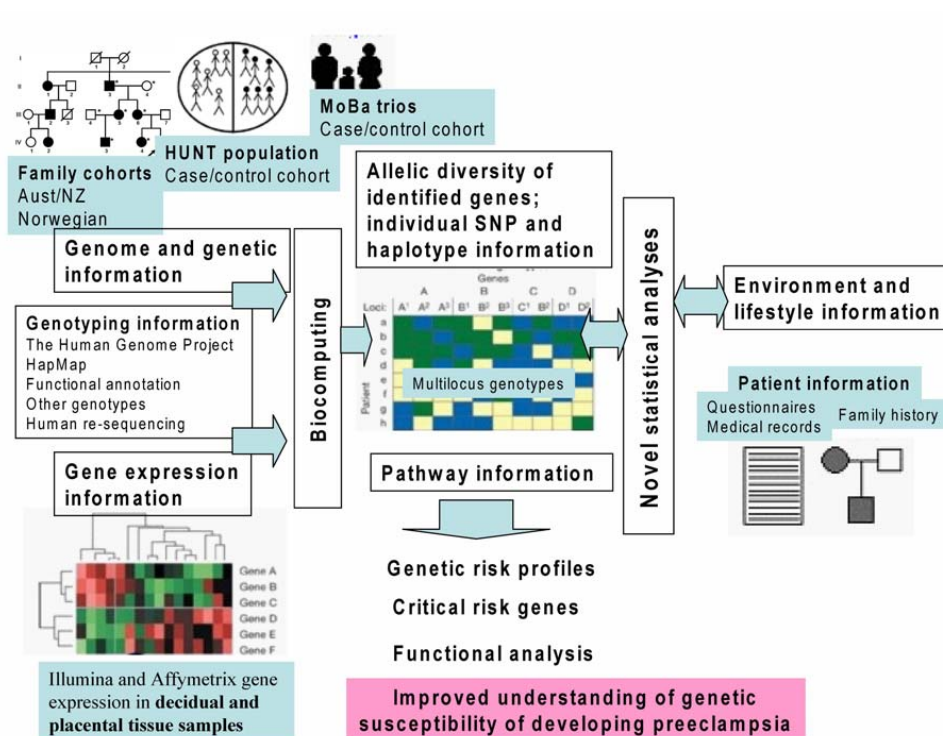


**Figure 11. Integrated approach.** Genetic and genome information can be provided by performing genotyping (both genome-wide and candidate approaches) in the Aust/NZ preeclampsia family cohort, the HUNT case/control cohort and in the MoBa. A Norwegian preeclampsia family biobank is also being established. Gene expression studies in placental and decidual tissues can provide information to help prioritize candidate genes and to identify interesting pathways and networks of genes. Environmental and lifestyle information together with phenotypic information can be provided from questionnaires and medical records. Combining all this information can improve our understanding of the genetic susceptibility of developing preeclampsia.

Due to dramatic increases in high-throughput sequencing for detecting sequence variants, as well as microarray technologies for gene identification, pathway analysis, and mapping of *cis* and *trans* regulatory elements the rate of gene discovery is expected to accelerate. These resources will greatly facilitate the identification of genes underlying the hundreds of QTLs mapped for complex diseases and phenotypes. Thus, many of the bottlenecks associated with traditional mapping and cloning approaches might be overcome. Although the capacity and costs of the technology are no longer significant bottlenecks, the availability of large

populations with consistent and well-characterized descriptions of clinical traits, or multiple relevant phenotypes, are challenges to overcome. Large efforts have been spent and are underway to develop large population-based biobanks. Over 100 biobanks with a sample size of more than 10,000 subjects have been completed or are currently being conducted. (P3G Observatory: http://www.p3gobservatory.org/) Some examples of large-scale cohort biobanks aiming to investigate the genetic basis of multiple important diseases are the Icelandic biobank deCODE, The UK Biobank, the CARTaGENE in Canada, the Estonian Genome Project and the Kadoorie Study of Chronic Disease in China. The HUNT biobank is also an example of large-scale population-based cohort biobank. There are also disease-specific biobanks, which often contain tissues relevant for the disease of interest in addition to genetic material. In preeclampsia research, birth cohorts (biobanks) are valuable, such as The Norwegian Mother and Child Cohort Study (MoBa) and the Danish National Birth Cohort.

The most widely used contemporary approach to relate genetic variation to phenotypic diversity, GWAS,[514] is an investigation of genetic variations across the entire genome aiming to identify genetic associations with traits, or the presence or absence of a disease or condition. The GWAS approach is hypothesis-free and represents an unbiased yet fairly comprehensive option that can be attempted even in the absence of convincing evidence regarding the function or location of the causal genes.[515] Results of these studies have identified several common variants across the genome influencing common complex traits over the past 2 years and have rapidly and substantially increased the understanding of the diverse molecular pathways underlying specific human diseases (published GWAS catalogue can be viewed at http://www.genome.gov/gwastudies/). Although there has been an increasing enthusiasm about GWAS and many believe that these studies are the answer to many scientific needs, there are also those who feel that these studies are fatally flawed

endeavors that are draining research funding. It has become clear that, GWA studies have several limitations; 1) the causal variant is rarely revealed, and there is great difficulty to identify the functional basis of the link between a genomic interval and a given complex trait, 2) SNP associations identified in one population are often not transferable to other populations, 3) they do not capture information about rare variants and have limited statistical power to detect small gene-gene and gene-environment interactions. Despite the limitations of GWA studies, there is no doubt that this approach may identify novel candidate pathways and genes involved in complex diseases. The knowledge gained has improved the understanding of GWA studies, and there has been some useful discussions on design, implementation, best practice and interpretation of such studies.[514] In particular, the difficulties of replication has been an issue since independent replication has been considered the gold standard for validating GWAS findings.[516] It should be admitted that this is still an evolving field, and it is likely that the challenges that remain will be solved. In my opinion it is just a matter of time. In addition, one should bear in mind that GWAS is a study at the DNA level, and further studies at DNA, RNA and protein level might be needed to work out how the gene(s) contribute to the disease or trait. It has recently been suggested that because of the introduction of novel high-throughput, low-cost sequencing methods, sequencing and genotyping will soon converge. Finally, the functional relevance of identified variant(s) should be confirmed by *in vivo* or *in vitro* studies.

To my knowledge there are yet no published GWAS on preeclampsia. However, we and other groups are currently planning such studies. In collaboration with Australian, American and British investigators we have applied to National Institute of Health (NIH) for funding of GWAS in Norwegian preeclamptic women and control women, including the validated cases in our HUNT case/control cohort and cases identified in the MoBa. In addition, a Norwegian

preeclampsia family biobank is under establishment. Approximately 150 families with increased incidence of preeclampsia have been identified. Thus, this family biobank has the potential to become one of the largest in the preeclampsia field. And in addition to collecting blood for DNA analyses, we also collect blood for RNA and protein analyses.

With the increased knowledge about genetics underlying complex diseases, it is interesting that one gene or genetic variant may be associated with multiple seemingly distinct diseases. This has led to the concept of the "diseaseome",[517] which maps a network of how different genes and pathways connect to various diseases. Figure 12a[517] shows a disease gene network where all known disease genes in the human genome, by May 2007, were included. A human disease network giving a global view of all known genetic disorders, by May 2007, has also been constructed (Figure 12b)[517]. However, these maps are far from complete, and with the explosion of all the novel genes or genomic loci identified during the last 2 years, these maps need to be updated.

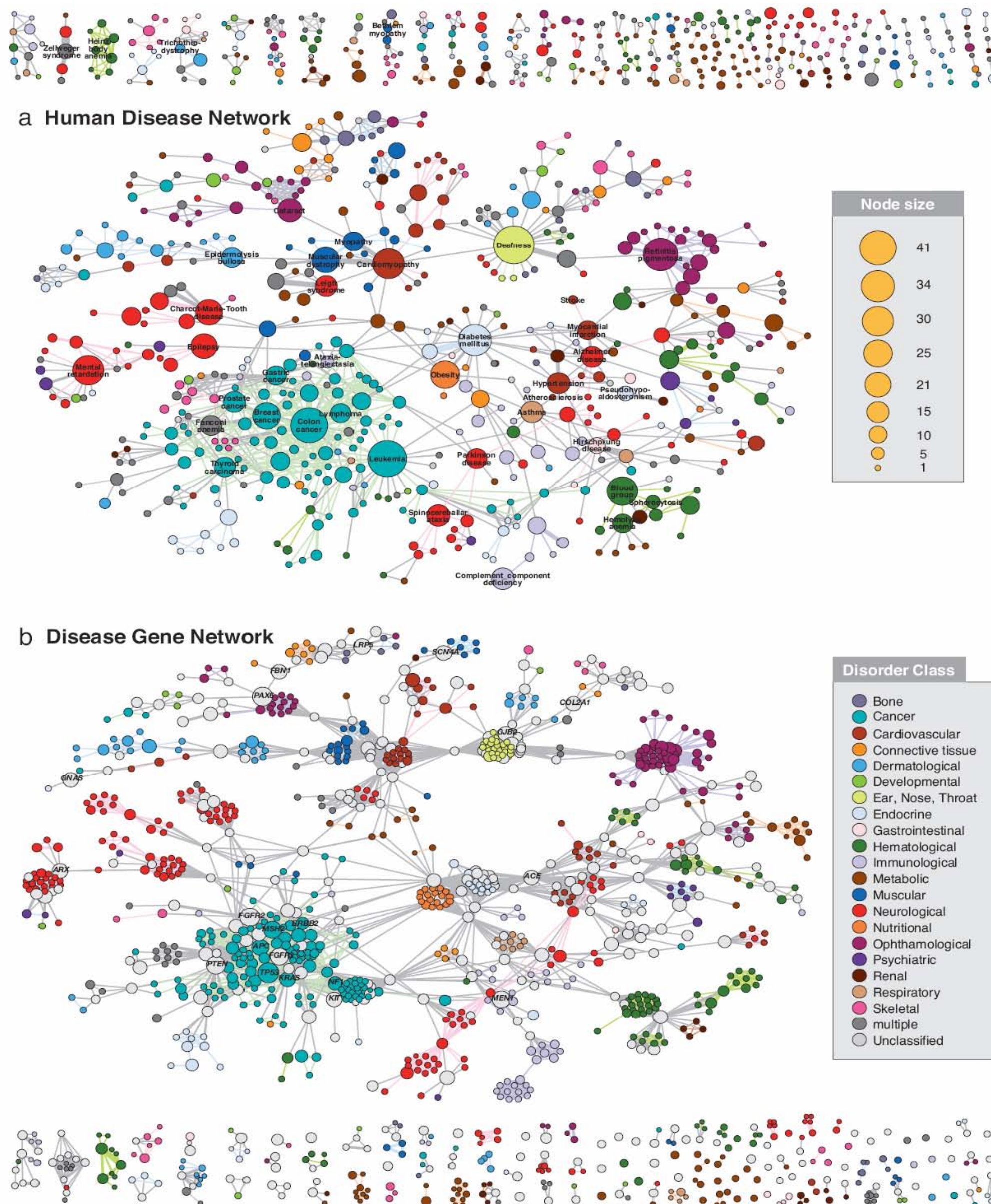


**Figure 12. (a)** In the Human disease network, each node corresponds to a distinct disorder (colored based on the disorder class to which it belongs). The size of each node is proportional to the number of genes participating in the corresponding disorder. **(b)** In the Disease gene network each node is a gene, with two genes being connected if they are implicated in the same disorder. The size of each node is proportional to the number of disorders in which the gene is implicated.[517]

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# I

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II

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III

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Molecular Human Reproduction

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# Evaluation of *COMT* as a maternal pre-eclampsia candidate susceptibility gene, assessed by genotyping of the *Val158Met* polymorphism and by transcriptional profiling of decidual tissue

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1 **RUNNING TITLE**

2 ***COMT* and pre-eclampsia**

3

4 **TITLE**

5 **Evaluation of *COMT* as a maternal pre-eclampsia candidate susceptibility gene,**

6 **assessed by genotyping of the *Val158Met* polymorphism and by transcriptional**

7 **profiling of decidual tissue**

8

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23 **ABSTRACT**

24 The suspected aetiology of pre-eclampsia is complex, with susceptibility being
25 attributable to multiple environmental factors and a large genetic component.
26 Although many candidate genes for pre-eclampsia have been suggested and studied,
27 the specific causative genes still remain to be identified. Catechol-*O*-
28 methyltransferase (COMT) is an enzyme involved in catecholamine and estrogen
29 degradation, and has recently been ascribed a role in the development of pre-
30 eclampsia. In this present study we have examined the *COMT* gene, by genotyping the
31 functional *Val158Met* polymorphism in a large Norwegian case/control cohort
32 ($n_{cases}$=1,134, $n_{controls}$=2,263). In addition, the expression of the *COMT* gene in
33 decidual tissues from pre-eclamptic (n=37) and normotensive (n=57) pregnancies was
34 compared. No association between the *Val158Met* polymorphism and pre-eclampsia
35 ($p>0.33$) was observed, and no difference in decidual *COMT* mRNA expression levels
36 was observed between pre-eclamptic and normotensive women ($p>0.7$). The Met-
37 allele of the *Val158Met* polymorphism is associated with a three- to four-fold
38 reduction in COMT activity, and thus, the observations obtained in the current study
39 do not support the suggested role of *COMT* as a maternal genetic risk factor for pre-
40 eclampsia.

41

42 **KEY WORDS**

43 Pre-eclampsia/ catechol-*O*-methyltransferase/ *COMT*/ *Val158Met*/ rs4680

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44 **INTRODUCTION**

45 The pregnancy-associated complication pre-eclampsia is a leading cause of maternal
46 and fetal morbidity and mortality. Approximately 3% of all pregnant women in the
47 Western World are affected by pre-eclampsia (Saftlas *et al.*, 1990), and in severe
48 cases the only effective treatment is delivery, irrespective of gestational age. The
49 classical clinical manifestations of pre-eclampsia are elevated blood pressure and
50 proteinuria. The etiology is complex and like other common complex disorders both
51 genetic and environmental factors influence the risk of developing the disease.
52 Genetic factors are suggested to be responsible for more than 50% of the liability to
53 pre-eclampsia (Moses *et al.*, 2006, Salonen Ros *et al.*, 2000), and several candidate
54 genes have been studied. However, the results are inconsistent and specific causative
55 genes involved in pre-eclampsia still remain to be identified.
56
57 A recent study put forward that deficiency in catechol-*O*-methyltransferase (COMT)
58 is associated with pre-eclampsia (Kanasaki *et al.*, 2008). COMT is a key enzyme in
59 the degradation of both catecholamines and estrogens (Creveling, 2003). High- and
60 low-activity variants of COMT, due to single base changes, have been discovered
61 (Diatchenko *et al.*, 2005). One polymorphism with functional implications is a non-
62 synonymous G to A base change (rs4680; NM_000754.2), the *COMT Val158Met*
63 polymorphism. The Met-allele of this polymorphism is associated with a three- to
64 four-fold decrease in COMT enzyme activity (Lotta *et al.*, 1995), and several clinical
65 conditions such as pain perception (Diatchenko *et al.*, 2005, Zubieta *et al.*, 2003),
66 psychiatric disorders (Azzam and Mathews, 2003, Prasad *et al.*, 2008, Woo *et al.*,
67 2002), hypertension (Annerbrink *et al.*, 2008, Hagen *et al.*, 2007, Happonen *et al.*,

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68 2006) and heart disease (Eriksson *et al.*, 2004, Hagen *et al.*, 2007, Voutilainen *et al.*,
69 2007) have been reported to be associated with this single base change.
70
71 Inspired by Kanasaki *et al.*'s hypothesis that COMT deficiency is associated with pre-
72 eclampsia we examined the potential role of the functional *COMT Val158Met*
73 polymorphism in a large Norwegian case/control cohort. In addition, since the
74 maternal-fetal interface is a likely site for the abnormal mechanisms in the
75 pathogenesis of pre-eclampsia (Robertson *et al.*, 1985) *COMT* gene expression in
76 decidual tissues from pre-eclamptic and normal pregnancies was also investigated.
77

## 78 MATERIALS AND METHODS

### 79 The HUNT population

80 All women subjected to genotyping were retrospectively identified from the second
81 Nord-Trøndelag Health Study (HUNT2) (Holmen *et al.*, 2003). Pre-eclampsia was
82 defined as onset of persistent hypertension (exceeding 140/90 mmHg), in combination
83 with proteinuria (exceeding 300 mg/l per day) after 20 weeks gestation. Women with
84 pre-eclamptic and non-preeclamptic singleton pregnancies in the HUNT cohort were
85 identified by linking the HUNT database to the Medical Birth Registry of Norway
86 (MBRN) (Moses *et al.*, 2008). The inhabitants of Nord-Trøndelag county are well
87 suited for genetic studies due to ethnic homogeneity (<3% non-Caucasians) (Holmen
88 *et al.*, 2003, Holmen *et al.*, 2004). The HUNT population is described in detail
89 elsewhere (Moses *et al.*, 2008).
90

### 91 Collection of decidual samples

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92 Human decidua basalis tissue was collected from Norwegian women, 13 with pre-
93 eclampsia, 24 with both pre-eclampsia and small for gestational age (SGA) neonates
94 and 57 with normal pregnancies. Mean gestational age for cases was 32±4 weeks, and
95 39±1 weeks for controls. The tissue was obtained by vacuum aspiration of the
96 placental bed during cesarean section (Harsem *et al.*, 2004, Staff *et al.*, 1999) at St.
97 Olavs University Hospital and Haukeland University Hospital, Norway. Pre-
98 eclampsia was defined as in the HUNT population, and SGA neonates were defined
99 as fetuses with a birth weight below the $10^{th}$ percentile (Haram and Gjelland, 2007).
100
101 **SNP genotyping**
102 DNA for genotyping was extracted from blood samples stored in the HUNT biobank,
103 as described elsewhere (Moses *et al.*, 2008). An Applied Biosystems' TaqMan
104 genotyping assay (Applied Biosystems, Foster City, U.S.A) was selected to genotype
105 the *Val158Met* (rs4680) SNP using 5 ng of genomic DNA from each of the 1,134 pre-
106 eclamptic women and 2,263 control samples. This assay was performed on an
107 Applied Biosystems 7900HT Fast Real-Time PCR System and sample genotypes
108 were interrogated using the integrated 7900HT system data analysis software.
109
110 **Decidual transcriptional profiling**
111 Total RNA was extracted from decidual tissues by a standard Trizol protocol
112 (Invitrogen, Carlsbad, U.S.A.), purified with Qiagen's RNeasy Mini Kit (Qiagen,
113 Hilden, Germany) and evaluated on the Agilent Bioanalyzer 2100 (Agilent
114 Technologies, Waldbronn, Germany). Anti-sense RNA (aRNA) was synthesized,
115 purified with the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin,
116 U.S.A.) and hybridized onto Illumina`s HumanWG-6 v2.0 Expression BeadChip®

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117 (Illumina, San Diego, U.S.A.). Hybridized aRNA samples were scanned on the
118 Illumina BeadStation 500GX BeadArray reader (Illumina, San Diego, U.S.A.) using
119 Illumina BeadScan image data acquisition software (version 3.5.49). Illumina's
120 BeadStudio Gene Expression software module (version 3.2.7) was used to subtract
121 background noise signals and generate an output file for statistical analysis.
122

123 **Statistical Analysis**

124 Statistical analyses of genotyping data were carried out in SPSS version 15 using a
125 Pearson's $\chi^2$ statistic. The decidual transcriptional profiles were pre-processed and
126 analyzed in the SOLAR statistical program (Almasy and Blangero, 1998) as
127 previously described in Göring *et al.* (Goring *et al.*, 2007). To evaluate the magnitude
128 of differential expression for the *COMT* transcripts we measured the displacement of
129 the mean transcript levels between case and control groups by regressing on the case
130 group.
131

132 **Ethics**

133 The study was approved by the Regional Committee for Medical Research Ethics, the
134 National Data Inspectorate and The Directorate of Health and Social Welfare in
135 Norway. Ethical approval for total RNA processing and decidua expression analysis
136 was obtained from the Institutional Review Board at The University of Texas Health
137 Science Center in San Antonio.
138

139 **RESULTS**

140 ***COMT* Val158Met genotyping and association analysis**

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141 Of the 1,134 pre-eclamptic cases and 2,263 controls, 1,100 cases (97.0%) and 2,188
142 controls (96.7%) were successfully genotyped. The *Val158Met* genotypes were
143 analyzed using both a dominant (GA+AA versus GG genotype frequency) and an
144 additive genetic model (G versus A allele frequency). We observed no association
145 between the *COMT Val158Met* polymorphism and pre-eclampsia in our Norwegian
146 cohort (dominant model p=0.93; additive model p=0.33) (Table 1).

147

148 ***COMT* transcriptional profiling in decidual tissue**

149 The two *COMT* mRNA transcripts (NM_007310.1 and NM_000754.2) represented on
150 each expression array were significantly expressed in decidual tissue. However,
151 decidual expression levels for neither of the two *COMT* transcripts differed between
152 the case group (pre-eclampsia and pre-eclampsia+SGA) and the healthy controls
153 (p>0.7).

154

155 **DISCUSSION**

156 *COMT* has recently been suggested to represent a novel pre-eclampsia susceptibility
157 gene (Kanasaki *et al.*, 2008). Kanasaki *et al.* based their conclusion on the observation
158 that administration of 2-methoxyestradiol (2-ME), a natural metabolite of COMT, to
159 pregnant *Comt-/-* mice ameliorated pre-eclampsia-like symptoms. However, the
160 relevance of this mouse model for pre-eclampsia in humans has been a matter of
161 discussion (Banerjee *et al.*, 2009), and based on this the present study was undertaken
162 to evaluate *COMT* as a candidate susceptibility gene in women. We observed no
163 association between the functional *COMT Val158Met* polymorphism and pre-
164 eclampsia (Table 1). In addition, no differential expression of *COMT* transcripts in the
165 decidual tissues from pre-eclamptic women and controls was found. In accordance

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166 with our findings, *COMT* is not located in any of the regions previously shown to be
167 in significant linkage with pre-eclampsia susceptibility, contradicting the suggested
168 role of this gene as a maternal risk factor for pre-eclampsia (Arngrimsson *et al.*, 1999,
169 Fitzpatrick *et al.*, 2004, Johnson *et al.*, 2007, Lachmeijer *et al.*, 2001, Laivuori *et al.*,
170 2003, Moses *et al.*, 2000, Oudejans *et al.*, 2004). On the other hand, studies have
171 reported association between *COMT* single nucleotide polymorphisms, e.g.
172 *Val158Met*, and blood pressure in humans (Annerbrink *et al.*, 2008, Hagen *et al.*,
173 2007, Happonen *et al.*, 2006, Kamide *et al.*, 2007), but such an association has not
174 been found in pregnancy-induced hypertension (Sun *et al.*, 2004), suggesting that
175 mechanisms underlying hypertension in the pregnant and non-pregnant state may
176 differ.
177
178 The mouse model for pre-eclampsia used by Kanasaki *et al.* provides a link between
179 redox-regulated signaling and human pregnancy pathology (Kanasaki *et al.*, 2008).
180 The COMT metabolite 2-ME acts like a pro-oxidant and has direct involvement in
181 redox-regulated signaling (Banerjee *et al.*, 2009). Moreover, 2-ME degrades hypoxia
182 inducible factor-1 alpha (HIF-1α) (Mooberry, 2003, Rimon *et al.*, 2008), and since
183 HIF-1α is essential in angiogenesis this activity may inhibit angiogenesis. Lack of 2-
184 ME, as in the *Comt-/-* mice, may result in stable HIF-1α and thus, increased oxidative
185 stress and vascular pathology. Due to absence of 2-ME in *Comt-/-* mice, the relevance
186 of this model for pre-eclampsia in humans has been questioned (Banerjee *et al.*,
187 2009). Taken together with the additional potential role of HIF-1α in inflammation
188 (Cramer *et al.*, 2003) and lipopolysaccharide-induced sepsis (Peyssonnaux *et al.*,
189 2007) it has been suggested that stable HIF-1α alone at late pregnancy could elicit the
190 pre-eclampsia-like phenotypes in *Comt-/-* mice (Banerjee *et al.*, 2009). Finally, it

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191 should be addressed that blood pressure regulation apparently differs in these mice
192 and humans. An association between *COMT* and blood pressure in non-pregnant
193 women has been revealed (Hagen *et al.*, 2007). In contrast, blood pressure did not
194 differ between non-pregnant *Comt-/-* and wild type (*Comt+/+*) mice.
195
196 The COMT enzyme is reported to be active in both placenta (Barnea *et al.*, 1988) and
197 decidua (Casey and MacDonald, 1983). In pre-eclamptic women reduced placental
198 COMT protein expression at term was observed in one study (Kanasaki *et al.*, 2008),
199 and reduced placental COMT activity was detected in a second (Barnea *et al.*, 1988).
200 However, both studies are small, 6 cases/6 controls and 10 cases/20 controls,
201 respectively. Levels of 2-ME vary throughout gestation, with extremely low levels
202 early in pregnancy and increasing levels towards term (Berg *et al.*, 1983, Sugawara *et*
203 *al.*, 2005). In one study reduced levels of circulating 2-ME was detected in pre-
204 eclamptic pregnancies (8 cases/13 controls) (Kanasaki *et al.*, 2008). When our *COMT*
205 transcriptional profiling results in decidua basalis tissue, from a relatively large
206 number of pre-eclamptic and control women (37 and 57, respectively), are
207 inconsistent with the existing observations, we can only speculate about the cause of
208 these divergent observations. Possibilities include, different function of COMT in
209 placenta, which is of fetal origin, and decidua basalis, representing the maternal side,
210 the mRNA level does not correlate with protein levels, or the case groups of the
211 previous studies (Kanasaki *et al.*, 2008, Barnea *et al.*, 1988) are simply too small with
212 insufficient statistical power.
213
214 A few observations in humans suggest that placental COMT protein
215 expression/activity is altered in pre-eclampsia, but our COMT gene expression in

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216 decidua basalis tissue does not. Our highly powered case/control cohort study does
217 not support the hypothesis that an association between COMT/2-ME deficiency and
218 pre-eclampsia is due to variations in maternal *COMT* genotype. However, since only
219 one single polymorphism has been evaluated in the present study it may be too early
220 to draw final conclusions about the role of this enzyme in development of pre-
221 eclampsia.
222
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231
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238
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382 **Table I: Distribution of *COMT Val158Met* genotypes and alleles in preeclamptic cases and controls. G; guanine, A; adenine.**

| | Genotypes | | | | Alleles | |
|---|---|---|---|---|---|---|
| | ***GG*** | ***GA*** | ***AA*** | ***GA* plus *AA*** | ***G (Val)*** | ***A (Met)*** |
| Preeclamptic cases (n=1,100) | 209 (0.1900) | 521 (0.4736) | 370 (0.3364) | 891 (0.8100)[a] | 939 (0.4268) | 1,261 (0.5732)[b] |
| Controls (n=2,188) | 413 (0.1888) | 1,097 (0.5014) | 678 (0.3099) | 1,775 (0.8112) | 1,923 (0.4394) | 2,453 (0.5606) |

383

384 Frequencies are shown in parentheses.

385 [a] Not significantly different from the value for the control group when compared with the frequency of the *GG* genotype using Pearson's $\chi^2$

386 analysis in a 2 x 2 contingency table ($\chi^2$=0.01, p=0.93).

387 [b] Not significantly different from the value for the control group when compared with the frequency of the *G* allele using Pearson's $\chi^2$ analysis in

388 a 2 x 2 contingency table ($\chi^2$=0.95, p=0.33).

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# IV

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V

**Identification of *TNFSF13B* as a genetic risk factor for preeclampsia: replication of association in Australian and Norwegian populations.**

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**Running title:** Genetic association between *TNFSF13B* and preeclampsia.

**Key words:** preeclampsia, genetic association, chromosome 13q, TNFSF13B, BAFF.

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## Abstract

**Preeclampsia susceptibility demonstrates a complex pattern of inheritance. Epidemiological data suggest that both maternal and fetal genetic factors contribute to the risk of developing preeclampsia. Maternal susceptibility has previously been linked to chromosomes 2q, 5q and 13q in an Australian/New Zealand (Aust/NZ) familial cohort. In this present study, we aimed to investigate positional candidate SNP variations at the 13q locus in an extended set of Aust/NZ preeclampsia families and in a large Norwegian population-based case/control cohort (1 139 preeclampsia cases, 2 269 controls). Known coding and/or validated SNPs within several positional candidate genes were selected for genotyping in the two cohorts. Nominal significant evidence of association for an identical SNP in the tumor necrosis factor ligand superfamily member 13B (*TNFSF13B*) gene was detected in both the Aust/NZ and Norwegian cohorts (rs16972199; p=0.015 and p=0.017, respectively). Subsequently, a combined cohort association analysis was performed providing greater evidence for association with preeclampsia susceptibility (p=0.002). This combined cohort association result remained significant after correcting for the total experiment (p=0.047). TNFSF13B influences the immune response and has previously been ascribed an important role in the development of preeclampsia by affecting the normal maternal-fetal immune adaptation. This physiological evidence emphasizes the importance of our genetic findings whereby we propose *TNFSF13B* as a novel maternal preeclampsia susceptibility gene.**

## Introduction

Preeclampsia is a pregnancy-associated complication with substantial maternal and fetal morbidity and mortality. The incidence of preeclampsia is estimated to range from 2-5%,[1] although this rate is higher in developing countries[2] and amongst certain ethnic groups.[3] A complete understanding of the etiology and pathogenesis of the preeclampsia syndrome remains elusive. Whilst the clinical gestational hypertensive and proteinuric manifestations usually present themselves after 20 weeks gestation, the mechanisms underlying these manifestations are likely to occur at a much earlier gestational age. This dichotomy of pathophysiological events has therefore been proposed to represent a two-stage process, with poor placental perfusion (stage 1) leading to the maternal responses evoked with varying degrees of clinical manifestations (stage 2).[4] This model has recently been modified,[5] based on the hypothesis that abnormal implantation/placentation occurs before abnormal vascular remodeling of spiral arteries.[6] In addition, it is suggested that maternal constitutional factors (genetic, behavioral or environmental) may play a more extensive role than previously thought and that several factors, which may differ in different women, may serve as linkers between the two stages.[5]

An intriguing aspect of normal pregnancy is the maternal host's "acceptance" of paternal antigens derived from the fetal allograft, which presents a genetically distinct set of human leukocyte antigens (HLAs; HLA-C, -E and -G). In a preeclamptic pregnancy however, this "acceptance" is thought to be perturbed in a manner akin to the immunological dynamics as seen in organ graft rejection [7]. The proposed maternal-fetal immune maladaption has been ascribed to a disturbed interaction between maternal immune cells (natural killer (NK) cells) and invading trophoblasts. At the

epidemiological level, immune maladaptation may be involved due to an increased risk of preeclampsia seen in primiparous women,[8] women receiving donor insemination,[9-11] women having only a short period of sexual cohabitation with their partners before conception and in multiparous women after changing partners (primipaternity)[12-14].

It is well documented that preeclampsia is a complex disorder with both genetic and environmental factors attributing to a susceptibility continuum for developing the disease.[15] However, like the majority of other common complex diseases, the mode of preeclampsia inheritance is unclear. Positional cloning efforts have been initiated in attempts to identify the genetic risk factors. By examining the probability of co-segregating loci within a familial cohort, several loci most likely to harbor maternal susceptibility genes have been identified (reviewed in [16-18]). Linkage studies in an Australian/New Zealand (Aust/NZ) familial cohort initially identified a maternal preeclampsia susceptibility locus to chromosome 2q.[19,20] The Aust/NZ genome-wide linkage scan data set has been re-analyzed under the assumption that the underlying liability of preeclampsia is inherently quantitative, implying that any preeclampsia susceptibility gene will represent a quantitative trait locus (QTL). Such an approach allows a more refined variance-components-based procedure utilizing a biological threshold model for the preeclampsia phenotype. Application of this efficient genetic linkage analysis method to the original Aust/NZ genome-wide linkage scan data set [19,20] resolved and the strengthened the chromosome 2 linkage signal to 2q22.[21] In addition, two novel maternal preeclampsia susceptibility QTLs to chromosomes 5q and 13q were revealed.[22]

In our efforts to identify the susceptibility gene(s) at the 2q22 QTL, we have utilized an extended Aust/NZ familial cohort and an independent retrospective Norwegian case/control cohort. Association to the activin A receptor, type IIA (*ACVR2A*) gene on 2q22 was found.[23,24] We now report on our efforts to identify the susceptibility gene(s) at the 13q QTL. Several plausible genes were interrogated with a genetic association identified for a concordant SNP in the tumor necrosis factor (ligand) superfamily 13B (*TNFSF13B*) gene and preeclampsia susceptibility. The independent confirmation of a *TNFSF13B* SNP is a novel finding and a possible role for this gene in preeclampsia pathogenesis is discussed.

## Materials and method

### Australian study population

The Aust/NZ familial cohort consists of the original set of 34 (26 Australian and eight New Zealand) families that we have previously used to localize the 2q, 5q and 13q preeclampsia susceptibility QTLs[19-22] and an additional 40 (Australian) preeclampsia families that we have subsequently ascertained and recently described.[23] The original 34 families are herein called "The 34 Family Cohort", the entire familial sample is herein called "The 74 Family Cohort" and all family members are of Caucasian origin.

### Norwegian study population

All women in the Norwegian cohort were retrospectively identified from Nord-Trøndelag County in Norway as part of a large multipurpose health survey conducted during 1995-1997 (the HUNT2 study). More than 65 000 inhabitants participated. The people living in Nord-Trøndelag County are considered representative of the Norwegian

population, and are well suited for genetic studies because of ethnic homogeneity (<3% non-Caucasians).[25,26] The study population is described in detail elsewhere.[27]

**Preeclampsia diagnosis**

Preeclampsia diagnosis in the Australian and Norwegian cohorts was based on the development of hypertension and an elevation of protein levels in the urine during pregnancy. Multiple blood pressure readings and a quantitative (24 hour urine sample) or qualitative (random proteinuria dipstick reading) test were performed.

Australia/New Zealand

Preeclampsia diagnosis was performed by qualified clinicians, using criteria set by the Australasian Society for the Study of Hypertension in Pregnancy.[28,29] Pregnant women were considered preeclamptic if they, on at least two occasions six or more hours apart, had (1) a rise from baseline systolic blood pressure (SBP) of at least 25mmHg and/or a rise from baseline diastolic blood pressure (DBP) of at least 15mmHg; or (2) SBP ≥140mmHg, and/or DBP ≥90mmHg. Additionally, proteinuric levels were >0.3 g/L in a 24 hour specimen or at least a +2 proteinuria dipstick reading from a random urine collection. Women who satisfied these criteria and experienced convulsions or unconsciousness in their perinatal period were diagnosed as having eclampsia. Women with pre-existing hypertension or other medical conditions known to predispose for preeclampsia (e.g., renal disease, diabetes, twin pregnancies or fetal chromosomal abnormalities) were excluded. Under these criteria, The 74 Family Cohort (n=480) included 140 women coded as affected (20 with eclampsia, 120 with preeclampsia) and 90 women coded as unaffected (normotensive and non-proteinuric).

Norway

Preeclampsia in the Norwegian case/control cohort was defined using the criteria given by National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy.[30] Preeclampsia was defined as the onset of persistent hypertension (blood pressure ≥140/90 mmHg), in combination with proteinuria (≥0.3 g/L in a 24 hour urine sample or ≥1+ on urine dipstick) after the 20th week of gestation. Preeclamptic women and women who had normal pregnancies in the HUNT cohort were identified by linking the HUNT database to the database at the Medical Birth Registry of Norway (MBRN) described in more detail elsewhere.[27] Clinical information of all deliveries in Norway has been sent to MBRN since 1967. We identified 1 139 women registered with preeclampsia (cases) and 2 269 women with a history of normal pregnancies as controls, with blood samples available at the HUNT biobank.

**Positional candidate SNP selection**

Publicly available sequence SNPs were selected from the NCBI SNP database (*Homo sapiens* dbSNP build 125, http://www.ncbi.nlm.nih.gov/SNP/).

**SNP genotyping in the Aust/NZ cohort**

Extraction of genomic DNA from peripheral blood samples has been previously described.[20] SNP typing in The 74 Family Cohort was performed with Illumina's GoldenGate® SNP Genotyping Assay (Illumina Inc., San Diego, CA). The design of two allele specific oligos and one locus specific oligo in conjunction with a universal set of amplification primers applied to a bead array substrate makes the GoldenGate assay

highly robust and specific in a large multiplex reaction. A custom SNP pool was designed using Illumina's Assay Design Tool and sample genotyping was performed using Illumina's 8×12 Sentrix Array Matrix (SAM). Each SAM was imaged on the Illumina BeadStation 500GX BeadArray Reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). SNP genotype clustering and individual sample genotype calls were interrogated using the Illumina BeadStudio software, Genotyping Module (version 2.3.41).

**SNP genotyping in the Norwegian cohort**

DNA for genotyping was extracted from peripheral blood samples stored in the HUNT biobank as described elsewhere.[24,27] The Applied Biosystems SNPlex™ Genotyping System (Applied Biosystems, Foster City, CA) was employed for SNP genotyping. All samples were electrophoretically separated on a 3730 DNA Genetic Analyzer (Applied Biosystems), and automated allele calls and genotype clustering of each individual sample was performed using Applied Biosystems' GeneMapper® Software (version 4.0) as previously described.[24,27]

**Statistical methods**

SNP allele frequency estimation

We used the statistical genetics analysis program SOLAR to estimate SNP allele frequencies by using maximum likelihood techniques that account for pedigree structure.[31] Tests for deviations from Hardy-Weinberg equilibrium (HWE) were also performed in SOLAR.

Intra- and inter-genic SNP linkage disequilibrium estimates

Estimates of pairwise linkage disequilibria parameters amongst intra-genic SNPs were used in a basic correlation method to assess all disequilibria jointly in SOLAR. In this approach, SNP genotypes are scored as -1, 0 and 1 (for the AA, AB and BB genotypes, respectively) and the correlations among these data vectors are calculated to give an unbiased estimate of the squared LD correlation, rho ($\rho$).

SNP association analysis

The SNP association analyses were conducted using SOLAR's QTLD procedure.[32] This procedure performs a test for population stratification and two commonly used association tests: the quantitative transmission disequilibrium test (QTDT),[33] and the measured genotype test.[34] The QTDT procedure as described by Abecasis *et al.*[33] is not limited to the scoring of allele transmission from parents to offspring but extends further to assess the entire pedigree structure. The scoring of allele transmission can be performed for quantitative or qualitative traits and it has been modified in SOLAR to work with discrete traits using a threshold model.[35] The QTDT statistic is used to report association results for the Aust/NZ familial cohort. The measured genotype analysis uses a standard threshold model assuming an underlying normal distribution of liability. The threshold model and its assumptions are near identical to those used in standard logistic regression but benefits from the ease of interpretation with regard to genetic effects. The measured genotype test of associations can assess the extent of genotypic mean differences (or the liability or risk scale) between case and control singletons assuming a model of additive gene action.[34] The measured genotype test is used to report statistical results for the Norwegian case/control cohort. To accommodate for

multiple hypothesis testing, we used the approach of Li and Ji[36] to determine the effective number of independent SNPs (and tests). This algorithmic approach has been implemented into SOLAR and it evaluates the strength of correlation amongst the observed genotypes at each SNP locus within a gene.

Combined cohort analysis

To analyze concordant results in both the Aust/NZ and Norwegian cohorts we used Fisher's combined probability test.[37] Fisher's combined probability test uses the independent p-values ($p_i$) from $y$ tests to calculate the test statistic $\chi^2_{2y} = -2\sum_{i=1}^{y}\ln(p_i)$ with 2$y$ degrees of freedom for the combined cohorts. With this approach we evaluate the same null hypothesis; positional candidate SNPs are not associated with preeclampsia genetic susceptibility, in both cohorts.

**Ethics**

Australia

Ethical approval for the recruitment of Aust/NZ preeclampsia family members was granted by the Royal Women's Hospital Research and Ethics Committees, Melbourne, Australia. Written informed consent was obtained from study participants prior to them being phlebotomized. Ethical approval for the quantitative genetic analysis of The 34 Family Cohort plus molecular genetic investigation across the 13q QTL in The 74 Family Cohort was obtained from The University of Texas Health Science Center at San Antonio, Institutional Review Board.

Norway

Prior approval to use the Norwegian case/control cohort for genetic studies was obtained by the Regional Committee for Medical Research Ethics, Norway and approved by the National Data Inspectorate and The Directorate of Health and Social Welfare. Ethical approval for genotyping and statistical analysis of the Norwegian case/control cohort was obtained from The University of Texas Health Science Center at San Antonio, Institutional Review Board.

## Results

### Positional Candidate SNP Selection

We interrogated the 3-LOD drop (99% confidence) interval at the Australian 13q QTL to prioritize positional candidate genes for further study. The interrogated region extended from D13S158 (98.96 cM) to D13S285 (123.78 cM). Based on relevance to current and emerging concepts of the pathophysiology of preeclampsia, the tumor necrosis factor (ligand) superfamily 13B (*TNFSF13B*), ephrin-B2 (*EFNB2*) and the coagulation factor VII (*F7*) and X (*F10*) genes were selected for further study. The *TNFSF13B* and *EFNB2* genes were selected because of their emerging role in placental development and function.[38-43] The *F7* and *F10* genes were considered to be plausible biological candidates given the known relationship between maternal thrombophilias and an elevated risk of developing preeclampsia.[44]

From the four prioritized genes we selected 24 validated sequence SNPs from NCBI's SNP database (*Homo sapiens* dbSNP build 125) for genotyping in the Aust/NZ and Norwegian cohorts (Table 1). Coding SNPs representing variants likely to be functional and SNPs in gene regulatory regions (i.e. the proximal promoter and the 5' and 3'

untranslated regions), were given high priority. In order to provide sufficient coverage across the *TNFSF13B* gene we also selected validated intronic SNPs at ~6 kb intervals.

**SNP genotyping and association analysis in the Aust/NZ cohort**

All 24 SNPs were successfully designed into an Illumina custom SNP pool (Table 1). In the Aust/NZ cohort one SNP failed the genotyping assay and 11 SNPs were non-polymorphic (Table 1). In order to effectively analyze SNP genotype data, these and an additional SNP (with very low copy number of the minor allele, n<5) were subsequently omitted from all statistical analyses. For the 11 remaining SNPs genotyped in the Aust/NZ cohort we observed a very high individual sample genotyping success rate (≥98.3%), and all SNPs conformed to Hardy-Weinberg expectations (HWE) (p>0.05). Based on the prior assumption that the underlying liability of preeclampsia is inherently quantitative, and therefore any preeclampsia gene represents a QTL,[21,22] we have independently analyzed the four 13q QTL candidate genes.

The *TNFSF13B* gene presented a nominal association for the rs16972199 SNP with preeclampsia (p=0.015) in the Aust/NZ cohort (Table 1). The evaluation of intra-genic SNP dependence in this gene for the Aust/NZ cohort reports an adjusted p-value of 0.013 based on the six genotyped SNPs effectively representing 3.7 independent tests (Figure 1a). None of the tested SNPs in the remaining three genes (*EFNB2*, *F7* and *F10*) were associated with preeclampsia in the Aust/NZ cohort (p>0.05) (Table 1).

**SNP genotyping and association analysis in the Norwegian cohort**

In the Norwegian cohort two SNPs failed the Applied Biosystems SNPlex assay design, one SNP could not be successfully genotyped and 10 SNPs were non-polymorphic (Table 2). In order to effectively analyze SNP genotype data, these and an additional two SNPs (with very low copy number of the minor allele, n<5) were subsequently omitted from all statistical analyses (Table 1). Of the remaining nine SNPs genotyped in the Norwegian cohort we observed a moderate individual sample genotyping success rate (mean=77%; range 60.3-82.1%) and all SNPs conformed to HWE (p>0.05).

In the Norwegian cohort the *TNFSF13B* gene also presented a nominal association for rs16972199 (p=0.017) with preeclampsia (Table 1). The evaluation of intra-genic SNP dependence in the *TNFSF13B* gene for the Norwegian cohort revealed an adjusted p-value of 0.015 based on the five genotyped SNPs effectively representing 3.3 independent tests (Figure 1b). None of the tested SNPs in the remaining three genes (*EFNB2*, *F7* and *F10*) were associated with preeclampsia in the Norwegian cohort (p>0.05) (Table 1).

***TNFSF13B* SNP association confirmed in the combined Aust/NZ and Norwegian cohorts**

We used a Fisher's combined probability test[37] to analyze the combined rs16972199 association data from the Aust/NZ and Norwegian cohorts. This analysis generated a significant association with preeclampsia susceptibility ($\chi^2$=16.569; p=0.002). To correct our combined rs16972199 p-value at an experiment-wide level we first independently generated experiment-wide p-values for the Aust/NZ and Norwegian cohorts. In the Aust/NZ cohort we used SOLAR[31] to simulate a quantitative trait with a

heritability of 0.51[21] and assigned affection status to a fixed number of affected women with the highest simulated trait values. The number of affected women was determined from The 74 Family Cohort (n=140 women with preeclampsia). The QTDT was repeated 10 000 times to our simulated trait resulting in an experiment-wide p-value of 0.1009 for rs16972199 in the Aust/NZ cohort. Permutation analyses (n=10 000) for rs16972199 in the Norwegian cohort generated an experiment-wide p-value of 0.0815. The combined, experiment-wide analysis of rs16972199 in the Aust/NZ and Norwegian cohorts confirms a significant association of this SNP with preeclampsia genetic susceptibility ($\chi^2$=9.601; p=0.047).

## Discussion

The elucidation of genetic risk for developing preeclampsia is a major challenge in obstetric medicine. More than 50 candidate genes have been considered, with the majority of studies being underpowered and providing weak associations that fail to replicate in different population samples.[18,45] We have adopted the positional cloning approach, initially performing linkage analysis in affected families to localize the chromosomal regions most likely to harbor genetic risk factors. Having identified susceptibility loci on chromosomes 2q, 5q and 13q we are now in a position to focus our efforts on plausible positional candidate genes. In this current study, significant association between a single SNP (rs16972199) within the *TNFSF13B* gene and preeclampsia susceptibility was found in independent population samples from Australian/New Zealand and Norway. The *TNFSF13B* gene belongs to the TNF superfamily of genes that play important roles in the immune response. TNFSF13B plays an important role in adaptive immune responses,[46,47] potentially regulating the

function of innate immune cells.[48] Recent reports indicate that both placental trophoblast cells and maternal decidual cells express TNFSF13B.[40,49] The observed association between *TNFSF13B* and preeclampsia in this study suggests that the influence of the maternal immune system is significant and may be consistent with the theory that maternal-fetal immune maladaption is central in the pathogenesis of the syndrome.

TNFSF13B, also known as BAFF, BLYS, TALL-1, zTNF4, THANK, CD257, TNFSF20 and DTL, is a member of the TNF superfamily. In general, ligands and receptors of the TNF superfamily are involved in cell signaling pathways within basic processes such as development/differentiation, cell survival/death and host defense.[50] A vital role has been ascribed to TNFSF13B in the adaptive immune system (B-lymphocyte stimulator), but recently, it has been documented that TNFSF13B also regulates function of innate immune cells.[48] More specific, TNFSF13B stimulates monocyte survival and induces activation and differentiation into macrophage-like cells.[48] It is well known that NK cells have a major role in human reproduction.[51] NK cells are suggested to provide benefit by secreting a number of cytokines, chemokines and angiogenic factors rather than to exert a cytotoxic activity,[52] and the interaction between decidual NK cells and the allogenic extravillous trophoblast (EVT) cells has been suggested to contribute to the depth of EVT cell invasion during implantation/placentation.[53] An inadequate, shallow EVT cell invasion is a precursor to insufficient decidual spiral artery remodeling leading to ischemic, oxidative stress responses because of reduced placental perfusion and by this, the pathogenesis of preeclampsia may be initiated. NK cell activity has been shown to be elevated by

TNFSF13B in mice,[54,55] but so far, it is not known if or how the TNFSF13B protein influences the human NK cell response *in vivo*.

During implantation, the accumulation of NK cells in decidua is probably based on recruitment from maternal circulation.[56] Maternal decidual stromal cells (DSCs) are apparently central in this NK cell recruitment.[56] DSCs constitute the main cellular component of the decidua, and they are involved in a number of different immune functions that are important for the immunological cross-talk between the mother and the fetus, with consequences for pregnancy outcome.[57-61] Recently, DSCs have been shown to express TNFSF13B mRNA and protein,[49] implying that our finding of an association between *TNFSF13B* and preeclampsia may reflect an abnormal immunological function of DSCs at the maternal-fetal interface.

The establishment of an intrauterine immune privileged site for the fetus is essential for successful pregnancy and the invading fetal trophoblasts have been assigned a major role in this process.[62] Among the factors expressed by trophoblasts known to confer immune privilege are the death-inducing members of the TNF superfamily ligands.[38,63-67] The non-apoptosis-inducing TNFSF13B has also been suggested to play a role in cell differentiation and placental development/function.[38-40] Both TNFSF13B gene and protein are expressed by cytotrophoblasts.[40,49] A potential role in angiogenesis has also been suggested based on the observation that TNFSF13B is detected in blood vessel endothelium of normal pregnant women, being absent in abortion patients.[49] Angiogenesis is essential for normal placental development, and abnormal angiogenesis with insufficient remodeling of spiral arteries is a hallmark of preeclampsia.

Furthermore, in normal early pregnancy, a prominent expression of TNFSF13B has been detected in trophoblasts and deciduas, with decreased levels in tissues from women with recurrent spontaneous abortions.[49] It has been suggested that trophoblast TNFSF13B may navigate maternal leukocytes toward a maternal immune response, being beneficial for the fetus, instead of a harmful one.[49] If so, fetal TNFSF13B alone or in combination with maternal genotype, may influence preeclampsia development. However, addressing this question was outside the scope of the present investigation.

The *TNFSF13B* rs16972199 SNP showing association with preeclampsia in this study is a rare intronic SNP for which there is no obvious functional consequence. The functional evaluation of SNPs in putative gene regulatory regions (including intronic sequences) is particularly challenging and becoming more important as the pace of discovery in complex disease genetics increases.[68] Recent examples include association between intronic SNPs and breast cancer,[69,70] systemic lupus erythematosus (SLE),[71] lumbal-disc herniation (LDH),[72] Crohn's disease,[73] schizophrenia,[74] asthma[75] and dyslipidemia[76]. At this stage however, we cannot rule out the possibility that there exists other as yet unidentified rare sequence variant(s) in linkage disequilibrium (LD) with the rs16972199 SNP that are the actual causal mutations. Deep re-sequencing in and around the *TNFSF13B* gene in our preeclampsia cohorts will be required to enumerate and prioritize all variation prior to formal functional evaluation.

In conclusion, the finding of genetic association between the *TNFSF13B* SNP and maternal preeclampsia susceptibility in two independent populations strongly implicates this gene as a novel preeclampsia susceptibility gene, and supports the hypothesis that

this gene may be of profound importance for a variety of processes essential to a successful human pregnancy. Moreover, TNFSF13B might be a potential factor bridging the angiogenic and non-cytotoxic response machineries at the maternal-fetal interface.

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## Titles and legends to figures

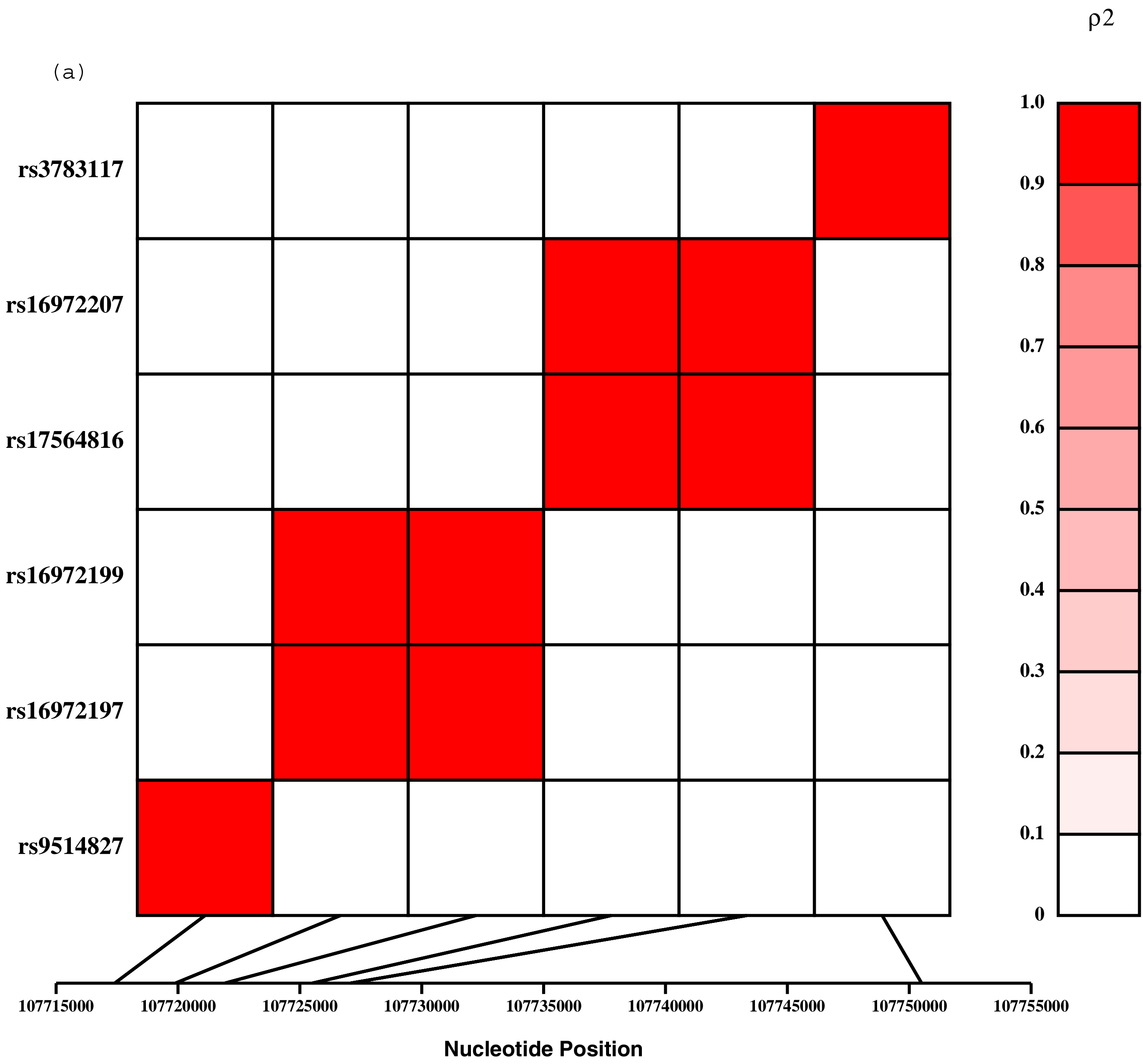
**Figure 1. The pattern of linkage disequilibrium (LD) for successfully genotyped *TNFSF13B* SNPs in (a) the Aust/NZ family cohort and (b) the Norwegian case/control cohort. LD is measured by the squared value of the pairwise correlation amongst intra-genic genotypes ($\rho^2$) and the strength of correlation is depicted in the colored bar to the right of the LD plot. The intensity of red colour in a block increases with the strength of SNP allele correlation from white (0) indicating no correlation (i.e. LD) to red (1.0) indicating a perfect correlation (i.e. complete LD).**

**Table 1. Chromosome 13q positional candidate gene-based SNP association analyses in Australian/New Zealand and Norwegian preeclampsia cohorts.**

| Gene | SNP | Locn | Function | Aust/NZ cohort | | | Norwegian cohort | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **GoldenGate genotyping** | | | **SNPlex genotyping** | | |
| | | | | **Allele (frequency)** | | **QTDT P** | **Allele (frequency)** | | **MG P** |
| *EFNB2* | rs1046903 | 105941953 | 3'UTR | Excluded[1] | | | Excluded[1] | | |
| | rs9520087 | 105942321 | 3'UTR | A (0.418) | G (0.583) | NS | A (0.384) | G (0.616) | NS |
| | rs16968757 | 105942587 | 3'UTR | Failed genotyping | | | Failed assay design | | |
| | rs7995379 | 105943464 | Synonymous | NP | | | NP | | |
| | rs1046755 | 105963058 | Missense | NP | | | NP | | |
| *TNFSF13B* | rs9514827 | 107717404 | Proximal promoter | A (0.670) | G (0.330) | 0.608 | A (0.700) | G (0.300) | 0.630 |
| | rs16972197 | 107719892 | Proximal promoter | C (0.009) | G (0.991) | 0.159 | C (0.002) | G (0.998) | 0.262 |
| | **rs16972199** | **107721919** | **Intronic** | **A (0.993)** | **G (0.007)** | **0.015** | **A (0.993)** | **G (0.008)** | **0.017** |
| | rs17564816 | 107725504 | Intronic | A (0.163) | G (0.837) | 0.332 | A (0.189) | G (0.811) | 0.570 |
| | rs16972207 | 107727067 | Intronic | C (0.161) | G (0.839) | 0.473 | Failed assay design | | |
| | rs7993556 | 107733797 | Intronic | NP | | | Excluded[a] | | |
| | rs3783117 | 107750508 | Intronic | C (0.983) | G (0.017) | 0.168 | C (0.981) | G (0.019) | 0.687 |
| *F7* | rs3093238 | 112809203 | Synonymous | NP | | | NP | | |
| | rs6042 | 112818069 | Synonymous | A (0.100) | G (0.900) | NS | A (0.096) | G (0.904) | NS |
| | rs6045 | 112820806 | Missense | NP | | | NP | | |
| | rs6046 | 112821160 | Missense | A (0.088) | G (0.913) | NS | A (0.088) | G (0.912) | NS |
| | rs3093251 | 112821996 | 3'UTR | NP | | | NP | | |
| | rs3093254 | 112822314 | 3'UTR | NP | | | NP | | |
| *F10* | rs5963 | 112825189 | Missense | NP | | | NP | | |
| | rs5961 | 112831786 | Missense | NP | | | NP | | |
| | rs3211772 | 112843317 | Missense | NP | | | NP | | |
| | rs3211783 | 112846237 | Missense | A (0.005) | G (0.995) | NS | Failed genotyping | | |
| | rs5960 | 112849738 | Synonymous | A (0.869) | G (0.131) | NS | A (0.881) | G (0.120) | NS |
| | rs1803564 | 112851652 | Synonymous | NP | | | NP | | |

Abbreviations: Locn, base pair chromosome location; QTDT P, quantitative transmission disequilibrium test p-value (uncorrected); MG P, measured genotype test p-value (uncorrected); NP, non-polymorphic; NS, not significant ($p_r>0.05$).

[1] SNP excluded from statistical analysis (observed minor allele count <5).


(a)
rs3783117
rs16972207
rs17564816
rs16972199
rs16972197
rs9514827
ρ2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
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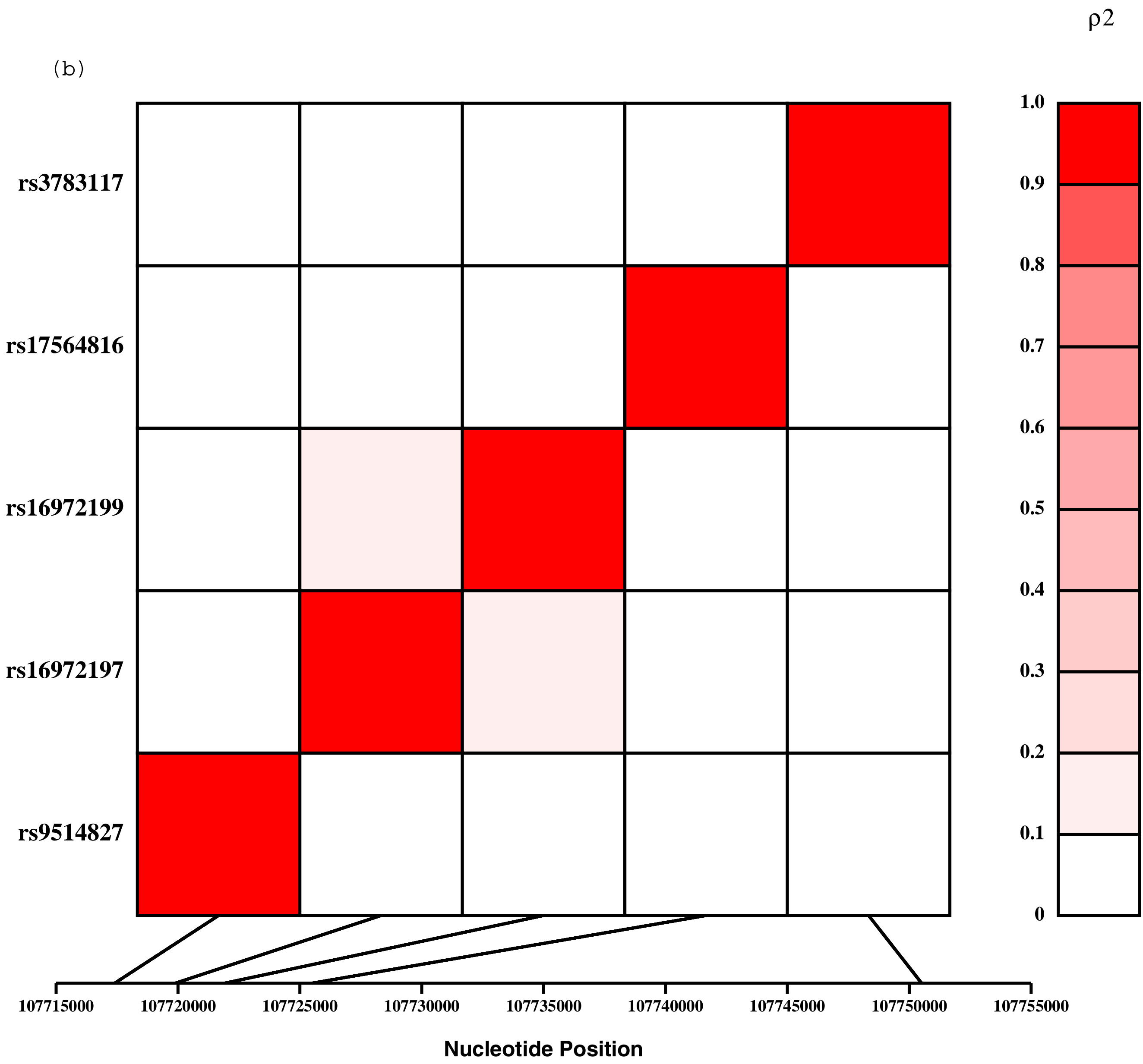
107720000
107725000
107730000
107735000
107740000
107745000
107750000
107755000
Nucleotide Position


(b)
rs3783117
rs17564816
rs16972199
rs16972197
rs9514827
ρ2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
107715000
107720000
107725000
107730000
107735000
107740000
107745000
107750000
107755000
Nucleotide Position

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124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.

134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

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141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.

163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS

193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN

218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES

245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT

273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS
280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OF PATHOLOGICAL GAMBLING IN NORWAY
288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
290. Jakob Nakling: RESULTS AND CONSEQUENCES OF ROUTINE ULTRASOUND SCREENING IN PREGNANCY – A GEOGRAPHIC BASED POPULATION STUDY
291. Anne Engum: DEPRESSION AND ANXIETY – THEIR RELATIONS TO THYROID DYSFUNCTION AND DIABETES IN A LARGE EPIDEMIOLOGICAL STUDY
292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
295. Gunilla Klensmeden Fosse: MENTAL HEALTH OF PSYCHIATRIC OUTPATIENTS BULLIED IN CHILDHOOD
296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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298. Haakon R. Skogseth: INVASIVE PROPERTIES OF CANCER – A TREATMENT TARGET ? IN VITRO STUDIES IN HUMAN PROSTATE CANCER CELL LINES
299. Janniche Hammer: GLUTAMATE METABOLISM AND CYCLING IN MESIAL TEMPORAL LOBE EPILEPSY
300. May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
302. Siri Malm: LEFT VENTRICULAR SYSTOLIC FUNCTION AND MYOCARDIAL PERFUSION ASSESSED BY CONTRAST ECHOCARDIOGRAPHY
303. Valentina Maria do Rosario Cabral Iversen: MENTAL HEALTH AND PSYCHOLOGICAL ADAPTATION OF CLINICAL AND NON-CLINICAL MIGRANT GROUPS
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