Summarizing discussion

Pre-eclampsia and the HELLP syndrome are pregnancy associated diseases and are the major cause of maternal and fetal morbidity and mortality. The clinical symptoms of these syndromes do not present until 20 weeks of gestation onward in the mother; however, the origin can be found in the first trimester placenta. Evidence from various studies show that defective spiral artery remodeling with impaired trophoblast invasion is central in the origin of pre-eclampsia and HELLP [1]. As it is furthermore established that pre-eclampsia is a multifactorial disease [2], the aim of this thesis was to identify potential links between different factors so far identified and shown to have (SNP-driven) altered functions in early onset pre-eclampsia and HELLP that may provide additional links between genes expressed in the fetus (placenta) and mother (decidua).

The factors studied in this thesis are *STOX1, STOX2, ACVR2A*, *NODAL* and *LINC-HELLP*, that are expressed in the maternal decidua and/or the fetal placenta. These factors are originally identified in human genetic screens and a mouse model in different studies and by different groups, and are related to patho-physiological features of pre-eclampsia or the HELLP syndrome. The main goal of this thesis was to identify the signaling crosstalk between decidual and placental expression of these factors and how they would interact in a causative pathway leading to reduced trophoblast invasion and subsequent spiral artery remodeling leading to pre-eclampsia and HELLP.

In this chapter our main findings are summarized and discussed. Furthermore, directions for future research are provided. The findings and hypothesized disease mechanisms which are presented in this thesis are also summarized in **figure 1**.

NODAL

Chapter 2 describes how NODAL, a secreted signaling protein from the TGFβ-super family, is involved in the pathogenesis of pre-eclampsia due to affected expression in the decidua. Previously, it was found that maternal decidua-specific Nodal knockout mice show intrauterine growth restriction (IUGR) and preterm birth [3]. Secondly, the chromosomal location of *NODAL* is in the same linkage area as the Dutch pre-eclampsia susceptibility gene *STOX1*. As the *STOX1* linkage was originally identified in children (two sisters) being born from a pre-eclamptic pregnancy as well as suffering from pre-eclampsia themselves, the linkage could in part be caused by *NODAL*, which made it highly interesting to study the potential maternal-fetal interaction between *STOX1* and *NODAL*. In **chapter 2** it was shown that in pre-eclampsia families when the *STOX1*

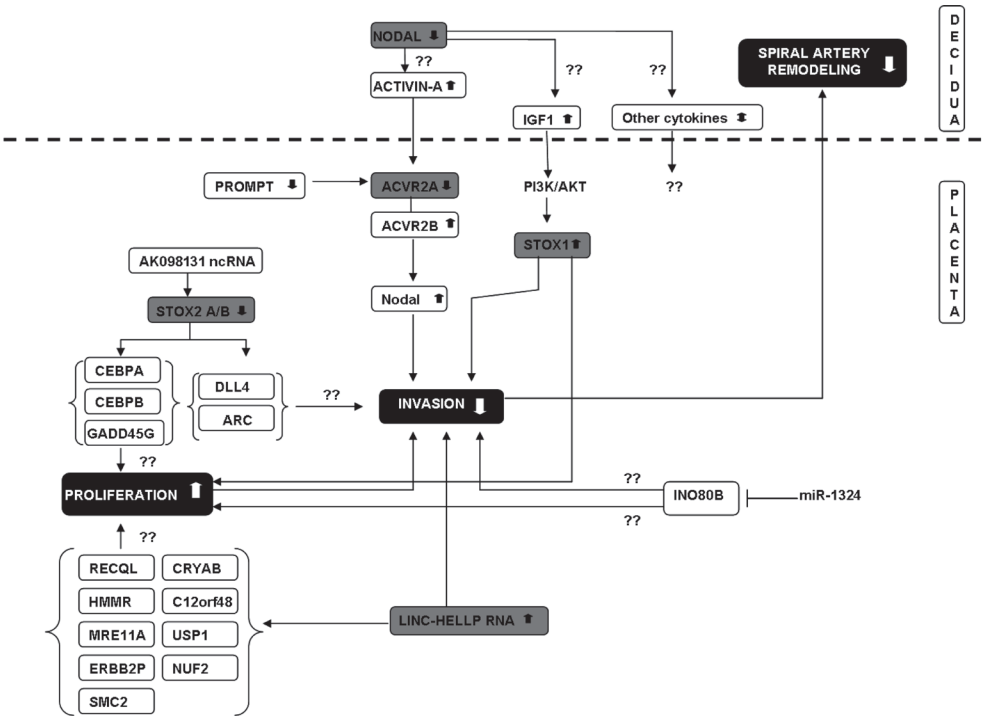

Figure 1. Summarizing model of the molecular pathogenic pathways currently identified in pre-eclampsia/HELLP. The dashed line differentiates between the maternal (decidua) and fetal (placenta) side. Gene names in grey boxes represent genes studied in this thesis, black boxes represent feto-maternal processes. Genes between brackets represent downstream effector genes that could alter the cellular processes. Up and down arrows represent up- or downregulated gene regulation.

susceptibility allele was expressed by the children born from pre-eclamptic pregnancies, the pre-eclamptic mothers themselves all carried the *NODAL* H165R SNP, which causes a 50% reduced activity [4]. Surprisingly, in decidua-specific Nodal knockout mice the fetal placenta showed up-regulation of *STOX1* and *NODAL* expression. We identified Nodal to be expressed in glandular epithelial and decidual stromal cells of human first trimester decidua, and we showed that Nodal knockdown in the maternal decidua gives upregulation of *NODAL* and *STOX1* mRNA expression in fetal extravillous trophoblast cells, potentially via upregulation of Activin-A in the maternal decidua. As both Activin-A and Nodal have been implicated in pre-eclampsia, being increased in serum of pre-eclamptic women [5] and upregulated in pre-eclamptic placentas [6] respectively, this interaction at the maternal-fetal interface might play a substantial role in the development of pre-eclampsia.

ACVR2A

In **Chapter 3** another pre-eclampsia susceptibility gene, *ACVR2A*, coding for Activin receptor type II, was investigated. This receptor is able to bind Activin-A, the ligand identified in **chapter 2** to be upregulated upon Nodal knockdown in decidua. A polymorphism (rs1424954 (A>G)) located at 1770 bp upstream of the transcription start site of *ACVR2A*, i.e. within or near the promoter region, has previously been shown to be significantly associated with pre-eclampsia [7-10]. The effects of this variant on *ACVR2A* expression and its function in the Activin-A signaling pathway were studied in extravillous trophoblasts. We showed that the *ACVR2A* promoter susceptibility variant causes downregulation of *ACVR2A* expression. Secondly, we provided evidence for transcription of a so-called PROMPT (PROMoter-uPstream-Transcript) in the opposite direction of *ACVR2A*. This PROMPT contains the polymorphism, and is downregulated when the susceptibility allele is carried. This implicates that this PROMPT either shares the same promoter as *ACVR2A* or is a non-coding RNA that is able to enhance *ACVR2A* transcription in cis. Finally, when the effects of the susceptibility variant were mimicked by knockdown of *ACVR2A*, physiologic concentrations of Activin-A caused a reduction in *NODAL* mRNA expression in extravillous trophoblasts, indicative of a protective effect as reduction in *NODAL* expression is associated with an increase in trophoblast invasion [11]. However, at pathologic levels of Activin-A, as found in pre-eclampsia [5], this effect was no longer seen, and potentially caused by a lack of downregulation of *ACVR2B,* the only other known receptor for Activin-A [12]. These combined data suggest a double hit phenomenon in which the first hit, the promoter variant, together with the second hit, pathological levels of Activin-A, lead to high levels of *NODAL*, associated with reduced trophoblast invasion and as observed in pre-eclamptic placentas [13].

STOX2

Chapter 4 describes about *STOX2* a paralogous to *STOX1* located in syntenic loci on chromosome 4q in the Finnish population. It is found to be differentially expressed in the decidua of preeclamptic women [14]. Further focus of this chapter was on the identified nuclear sense lncRNA transcript called AK098131 generated from within the intron 3 of *STOX2* locus containing a paternal insertion mutation (CT insertion). Through experimental downregulation, targeted qRT-PCR and whole genome RNA sequencing, intronic AK098131 lncRNA is found to regulate and affect alternative splicing of *STOX2* gene in placental trophoblast cells. Furthermore, downregulation led to increase of *CHD5* a chromodomain helicaes DNA-binding protein at the mRNA level, at the protein level, it led to inclusion and upregulation of short alternative

exon(IIIb) in multiple alternative transcripts causing to deletion of conserved domain of 63 amino acids in the C-terminal of *STOX2* at the protein level. Finally and phenotypically, as like other PE genes [15,16], *STOX2* also showed its effect on essential process of trophoblast differentiation where trophoblast cell cycle exit accompanies the transition from proliferative to invasive cells by regulating genes like *CEBPA, DLL4, GADD45G, STK4-AS1, ARC* and *GNN* through lncRNA transcribed from within *STOX2* gene.

LINC-HELLP

In **Chapter 5**, chromosomal linkage was found on chromosome 12q23 to be associated with the HELLP-syndrome (Hemolysis, Elevated Liver enzymes, Low Platelets) in Dutch families and this region contained a long non-coding RNA (205kb) transcript called LINC-HELLP RNA. Through in situ hybridization expression was detected in extravillous trophoblasts of the first trimester placenta. The effects of siRNA-mediated knockdown of this *LINC-HELLP* RNA were investigated by whole genome RNA sequencing followed by pathway analysis. This showed a function of *LINC-HELLP* in the cell cycle, with a reduced activity of the G2/M phase and increased activity of G1/S phase. Furthermore, blocking the regions containing the mutation sites identified in the HELLP families by using morpholino oligos showed upregulation of the *LINC-HELLP* RNA, while the invasion capacity of trophoblast cells was reduced. Together, this indicated that *LINC-HELLP* negatively affects the differentiation of trophoblasts from a proliferative to an invasive phenotype.

Future perspectives

Role of decidual cytokines in the pre-eclampsia molecular pathway

In **Chapter 2,** the human TGF-β/BMP signaling pathway PCR array on decidua and the T-HESC decidual stromal cell line in combination with knockdown of Nodal showed affected expression levels of different cytokines that can bind to different receptor classes: BMP2, GDF5, NBL1 and NOG that bind to BMP receptors; INHBA, INHBB, GDF3 and FST that bind to Activin receptors; LTBP4 that binds to TGF-beta receptors; and finally IGF1 and IGFBP3 that bind to IGF1 receptors. These have been previously identified in other studies to affect placental trophoblast invasion [17-22]. Considering the differential expression of the above cytokines, in the future we would like to investigate how these cytokines could function as an intermediate step in affecting the factors studied in this thesis. Most interesting would be Insulin growth factor 1 (*IGF1*) of

which it is known that upon binding to its receptor it activates the PI3K-Akt pathway. As *STOX1* activity has previously been shown to be regulated through phosphorylation by AKT [23], this might reveal how Nodal knockdown in decidua is able to upregulate *STOX1* in trophoblasts.

Role of PROMPT in the pre-eclampsia molecular pathway

In **Chapter 3**, we concluded that the PROMPT transcript located within the promoter region of *ACVR2A* might regulate *ACVR2A* transcription. To address the exact role and mechanism of action it has on the molecular pathways affected in pre-eclampsia, studies through RNAi-mediated knock-down or forced overexpression of PROMPT followed by RNA-Seq analysis in SGHPL5 trophoblast cells, would not only reveal the exact link between PROMPT and *ACVR2A*, but can also identify if and how PROMPT expression can give rise to differentially expressed genes related to the phenotypic end-effects associated with pre-eclampsia, i.e. proliferation and invasion of trophoblast cells. Considering the extensive effects of different Activin-A concentrations (physiologic vs. pathologic) on the molecular pathways downstream of ACVR2A (**chapter 3**), it is further advisable to assess how the Activin-A concentration levels regulate the expression of *ACVR2A* in combination with down-regulation or forced overexpression of PROMPT. These studies would further clarify the possible mechanism by which PROMPT participates in transcriptional regulation of *ACVR2A*.

Role of *STOX2* in the pre-eclampsia molecular pathway

In **Chapter 4,** a couple of genes have been identified to be downstream effector genes of *STOX2.* These are known to be involved in either cell proliferation (*CEBPA* and *GADD45G*) [13] or cell invasion (*DLL4* and *ARC*). Further focus of this chapter will be on the second paternal mutation identified between exon 1 and 2 covering around ~12kb and qualifying to act as a super-enhancer region in the Finnish pre-eclamptic population. Molecular activities of cis-versus-trans acting effects due to down and upregulation of lncRNA remains to be explored. However, the phenotypic end-effects of the *STOX2* isoforms themselves have not yet been studied in detail, which would provide additional information regarding the function of *STOX2* in the first trimester placenta.

INO80B in the pre-eclampsia molecular pathway

INO80B contains a novel winged helix domain similar as found in *STOX1* and is recently shown to be another, placental-expressed susceptibility gene for pre-eclampsia [16]. Like *STOX1* [15] and *LINC-HELLP*, *INO80B* also possess a gene defect in this gene which for INO80B is located at the 3'-UTR interacting with miR-1324. This interaction mediates silencing of *INO80B*, being lost upon binding with the pre-eclampsia susceptibility allele, that might reflect its action towards cell cycle exit and/or a defect

in trophoblast invasion as *INO80B* is part of the chromatin remodeling complex. In this regard, it is noteworthy to investigate the involvement of *INO80B* in trophoblast proliferation and invasion to investigate if this gene is also part of the pathway shared between the genes studied in this thesis, i.e. a conserved mechanism related to cell cycle exit/trophoblast invasion.

Ex vivo trophoblast invasion and spiral artery remodeling

This thesis provides more fundamental insights into the causative pathways leading to pre-eclampsia, however, studies within this thesis have predominantly been performed *in vitro* using cell lines. So, it would be highly informative to investigate what combinations of factors are necessary to cause changes in phenotype as seen in pre-eclampsia and HELLP, i.e. reduced trophoblast invasion and spiral artery remodeling. For this, a human first trimester placental explant model can be used. By using conditioned media obtained from different deciduas in combination with explants of different placentas changes in placental outgrowth of extravillous trophoblasts can be measured over a certain time period. By also studying the decidua and placental tissues used regarding their genotype and/or expression levels of the factors studied, models can be obtained predicting which combination of factors would lead to worsening of the phenotype. These experimental models can be even extended further by directly studying spiral artery remodeling by performing placenta–decidua co-cultures [24,25].

Biomarkers for pre-eclampsia/HELLP

The ultimate goal of all research performed on the origin of early-onset pre-eclampsia and HELLP is to identify a biomarker for pre-symptomatic diagnosis of these diseases, allowing an accurate prediction during the first trimester and permitting a window of opportunity for truly effective treatment that may help in complete recovery or reduce the severity. Although the past decade has brought an extensive increase in understanding of the pathogenesis of the disease, there still lies a quest for new biomarkers. To guarantee the usability in diagnosis, the ideal biomarker should be measurable without invasive procedures, i.e. in blood, permitting diagnosis before symptoms occur and of course be highly sensitive and specific. It may also be required to combine one or more biomarkers in a biomarker panel to increase the precision and sensitivity.

Combinations of factors as described in this thesis as well as other factors so far identified or yet to be identified by other groups via genetic screens or otherwise might fulfill these criteria. Factors that are of special interest but not described in this thesis are firstly Corin; found to show reduced uterine levels in pre-eclamptic patients, while Corin gene muta-

tions were identified in pre-eclamptic patients leading to decreased Corin activity [26,27]. Secondly of interest is an immunological fetal-maternal genetic interaction; *HLA-C* is a polymorphic gene expressed by the invasive extravillous trophoblasts and a ligand for killer immunoglobulin-like receptors (*KIR*s) that are expressed on the maternal uterine natural killer cells (uNK). *KIR*s have two different alleles, A and B. It has been found that mothers that have the AA *KIR* genotype while the fetus has *HLA-C* belonging to the *HLA-C2* group have an increased risk of developing pre-eclampsia [28-30].
In order to test the usability of the factors described in this thesis as well as the factors described above or identified in the future as biomarker or as part of a biomarker panel large biobanks are required obtained non-invasively, i.e. blood, and pre-symptomatic, i.e. first trimester.
From these blood samples cell-free DNA and RNA can be collected from plasma to investigate fetal and maternal genotypes and RNA expression levels. Recent studies showed cell free fetal DNA amounts to 10-20% of the total DNA amount present in maternal plasma [31,32], which makes it difficult to discriminate fetal DNA from the high background of maternal DNA. However, through a size fractionation approach cell-free fetal DNA fragments can be selected as it has been shown that fetal DNA fragments are of a smaller size than maternal cell-free DNA fragments [20]. It thereby permits the detection of otherwise masked fetal genetic loci such as short tandem repeats or single-nucleotide polymorphisms (SNPs)

Regarding fetal RNA expression levels, these will be more informative if maternal RNA expression of that particular factor is absent in maternal blood though few factors are expressed by both placenta and mother, but increased in affected pregnancies. It is also possible to isolate proteins from blood samples to investigate their expression levels. However, as can already be concluded from a couple of the factors studied in this thesis which are non-coding RNAs, protein analysis severely reduces the amount of potential biomarkers that could be obtained from maternal plasma compared to using the complete transcriptome as potential source.
Therefore, a genome wide data driven analysis approach, as used for maternal plasma DNA sequencing for trisomy detection [33-35], should be used to study the placental transcriptome. For this the cell free RNA source in the maternal circulation can be used and further analyzed by genome-wide RNA sequencing. This RNA originates from early syncytiotrophoblasts, in direct contact with maternal blood in the placenta and therefore the major contributors of cell free DNA and RNA as compared to extravillous trophoblasts [36,37]. This fact makes it less likely to successfully identify blood based

biomarkers that are based on factors identified in first trimester extravillous trophoblasts as studied in this thesis.
Next to large biobanks, it is also necessary to optimize techniques to be able to analyse the low levels of fetal RNA in maternal plasma. Recently, our lab is using an adapted library preparation approach for RNA sequencing, called nanoCAGE. This is a high resolution sequencing method to identify the 5' ends of the capped RNA transcripts that allow to analyze small sized samples with as little as 10ng of total RNA, depleted of ribosomal RNA. Library preparation of this method includes a two step process, a reverse transcription reaction of the cap of the messenger RNA to enrich 5'ends, followed by a stepwise semi-suppresive polymerase chain reaction to minimize the short PCR artifacts. This method helps to identify and increase the new 5' end capturing that are compartment specific unlike other methods that are focused on polyA priming. It also helps for the systemic analysis of genomic regions and transcription start sites that can be mapped, providing a view of how gene expression is regulated [38,39]. Another potential source of material for prenatal diagnosis would be the trophoblast cells from the distal endocervical canal [40]. Endocervical fetal cells obtained during early gestation are found to be mostly extravillous trophoblasts [41,42]. By using a technique named trophoblast retrieval and isolation from the cervix (TRIC) [43], intact extravillous trophoblast cells can be obtained which are more than 95% free of maternal cells and contain the complete fetal genome. In addition, it is expected that the protein and RNA expression patterns will directly correlate with gynaecological pathologies as these cells do represent the cells directly involved in the origin of early-onset pathologies, i.e. extravillous trophoblasts. To further investigate the usefulness of fetal cells isolated from the cervix in early non-invasive diagnostics large proteomic and transcriptomic studies will need to be performed. One potential drawback in using this source is the technique used to obtain these samples, i.e. a cervical swab, as this technique in some countries is not performed during pregnancy as there still is an ongoing debate regarding its potential to induce miscarriage.

To conclude, the current results in this thesis broaden our knowledge regarding the molecular mechanisms and interactions at the feto-maternal interface and thus provide further insights into the origin and disease pathogenesis of early onset pre-eclampsia and the HELLP syndrome. However, future studies as described in this discussion are clearly needed, not only to identify potential biomarkers, but also to translate the molecular pathways associated with pre-eclampsia and HELLP into optimized management and potentially even treatment thereby minimizing the long term effects on both mothers and children.