Recombinant human activated protein C as a therapy for pre-eclampsia

by

Gang Peng

Bachelor Degree in Clinical Medicine, Wuhan University, China, 2001
Master Degree in Oncology, Wuhan University, China, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in
THE FACULTY OF GRADUATE STUDIES
(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

August 2007

© Gang Peng, 2007
INTRODUCTION: Pre-eclampsia remains one of the most common causes of maternal mortality in the developed world, and we still have no known effective prophylaxis and proven modifiers. The recent successful clinical trial of recombinant human activated protein C (rhAPC) in the management of SIRS (systemic inflammatory response syndrome) has drawn attention to the possible use of this medicine for other conditions. Pre-eclampsia, has remarkable similarity to SIRS and may be such a condition.

METHODS: We established the safety and the efficacy of rhAPC in two scenarios: early-onset pre-eclampsia and severe postpartum pre-eclampsia. Twenty one women were enrolled in the trial; the rhAPC infusion ran at 24 μg/kg/h for up to 96h. For basic research, blood samples were drawn at 0.5 hr before rhAPC infusion, 4 hr, 28 hr, 52 hr and 76 hr of infusion, and 4 hr after infusion was stopped. The influence of rhAPC on haemostatic parameters were measured by Thromboelastography (R-time, etc.) and ELISA, the regulatory effects on the activities of circulating cytokines were detected by Luminex assay, the mRNA and protein levels of peripheral blood neutrophil components were tested by real-time RT-PCR and Western immunoblotting.

RESULTS: R-time (clotting time) in postnatal patients was significantly delayed by rhAPC (p =0.0454, 28hr: 29.7%; 52hr and 72hr: 60.9%). The infusion also resulted in significant decreases in post-natal F1+2 (4hr: 2.9%; 28hr: 22.4%; p =0.0218), PAI-1 (4hr: 23.6%; 28hr: 69.0%; p =0.0078), IL-6 (4hr: 18.8%; 28hr: 49.8%; p =0.0319) and IL-10 (4hr: 30.7%; 28hr:
36.6%; \(p = 0.0317\) concentration respectively. Moreover, rhAPC caused significant reduction in post-natal neutrophil TLR-2 \((p = 0.0002)\), TLR-4 \((p < 0.0001)\) and cryopyrin \((p < 0.0001)\) mRNA expression respectively. Significant up-regulation of caspase-3, at the same time, have been observed in antepartum patients \((p = 0.0323)\).

**CONCLUSIONS:** rhAPC exhibits anticoagulant effect. It may also stimulate fibrinolysis by forming a complex with or degrading PAI-1. Its therapeutic mechanism partly depends on its inhibitory effect on the deleterious Th1 type cytokines and pro-inflammatory cytokines. It may repress the activation of neutrophils in postnatal pre-eclampsia through TLR-2, -4 and cryopyrin signaling pathways and promote apoptosis of neutrophils via caspase-3.
# TABLE OF CONTENTS

Abstract ................................................................................................................................. ii

Table of Contents ................................................................................................................ iv

List of Tables ....................................................................................................................... viii

List of Figures .................................................................................................................... ix

List of Abbreviations ....................................................................................................... x

Acknowledgements ........................................................................................................ xiii

Chapter 1

Background ......................................................................................................................... 1

1.1 Pre-eclampsia ............................................................................................................... 2
   1.1.1 Historical background ......................................................................................... 2
   1.1.2 Epidemiology ......................................................................................................... 3
   1.1.3 Aetiology ............................................................................................................... 6
   1.1.4 Management ......................................................................................................... 12
      1.1.4.1 Prevention ..................................................................................................... 12
      1.1.4.2 Treatment .................................................................................................... 16

1.2 Haemostatic changes in pre-eclampsia ...................................................................... 19
   1.2.1 Pregnancy is related with changes in the maternal haemostatic system .......... 19
   1.2.2 Disturbance in the haemostatic system is a characteristic feature of pre-eclampsia 22

1.3 Pre-eclampsia and systemic inflammatory response syndrome (SIRS) .................... 25
   1.3.1 Maternal intravascular inflammation in normal pregnancy ............................... 25
   1.3.2 The similarity of pre-eclampsia and SIRS: clinical features and neutrophil activation ................................................................. 29
Chapter 2 Research plan

2.1 Overall Hypothesis

2.2 Aims

2.3 Subjects

2.3.1 Cohort 1 - Early-onset pre-eclampsia with dismal fetal prognosis

2.3.1.1 Inclusion criteria

2.3.1.2 Exclusion criteria

2.3.2 Cohort 2 - Postpartum pre-eclampsia

2.3.2.1 Inclusion criteria

2.3.2.2 Exclusion criteria

2.4 Infusion protocol

2.4.1 Starting rules

2.4.2 Infusion rules

2.4.3 Stopping rules

2.4.4 Restarting rules

2.5 Potential risks and management of adverse events

2.5.1 Obstetric haemorrhage

2.5.2 Teratogenicity and fetotoxicity

2.5.3 Other adverse events

2.5.4 Management of adverse events

2.6 Sample size and statistical analysis

2.7 Experimental plan of basic science

Chapter 3 Recombinant human activated protein C (rhAPC) may act as a potent modifier of haemostasis in women with pre-eclampsia

3.1 Hypothesis

3.2 Materials and methods

3.2.1 Study cohorts
3.2.2 Thrombelastography (TEG) ................................................................. 57
3.2.3 Enzyme-linked immunosorbent assays (ELISA) ............................... 61
3.2.4 Statistical analysis ......................................................................... 62

3.3 Results ................................................................................................. 63
3.3.1 Clinical characteristics of the study patients ................................. 64
3.3.2 TEG indices .................................................................................... 65
3.3.3 Haemostatic parameters measured by ELISA .................................. 68

3.4 Discussion ............................................................................................. 71
3.4.1 rhAPC influences TEG indices as an anticoagulant agent ............... 71
3.4.2 rhAPC influences plasma haemostatic parameters as an anticoagulant and fibrinolytic ...................................................... 72

Chapter 4 The effects of rhAPC on circulating cytokines in the women with severe pre-eclampsia ................................................................. 75

4.1 Hypothesis ............................................................................................ 76
4.2 Materials and methods ........................................................................ 76
4.2.1 Study cohorts .................................................................................. 76
4.2.2 Multiplexed fluorescent microsphere immunoassay (Luminex assay) ................................................................. 76
4.2.3 Statistical analysis ......................................................................... 77
4.3 Results .................................................................................................. 77
4.3.1 Clinical characteristics of the study patients .................................. 77
4.3.2 Maternal circulatory cytokines measured by Luminex assay .......... 78
4.4 Discussion ............................................................................................. 81
4.4.1 Th1-type cytokines ....................................................................... 82
4.4.2 Pro-inflammatory cytokines ............................................................ 84
4.4.3 Regulatory (anti-inflammatory) cytokines ..................................... 86
Chapter 5  The mechanisms of cell activation in peripheral blood neutrophils during pre-eclampsia and the effects of rhAPC on the neutrophil activation

5.1 Hypothesis

5.2 Materials and methods

5.2.1 Study cohorts

5.2.2 Separation of peripheral blood neutrophils

5.2.3 RNA isolation and reverse transcription

5.2.4 Quantitative Real-Time Reverse-Transcription PCR

5.2.5 Preparation of protein samples

5.2.6 SDS gel electrophoresis and immunoblotting techniques

5.2.7 Digital imaging

5.2.8 Statistical analysis

5.3 Results

5.3.1 Clinical characteristics of the study patients

5.3.2 Measurement of mRNA levels with real-time RT-PCR

5.3.3 Measurement of protein levels with Western blotting

5.4 Discussion

Chapter 6  Summary

6.1 Aims

6.2 Conclusions

6.2.1 The effects of rhAPC in women with postnatal pre-eclampsia

6.2.2 The effects of rhAPC in women with antenatal pre-eclampsia

6.3 Significance

6.4 Future directions

Bibliography
LIST OF TABLES

Table 1.1  SIRS criteria........................................................................................................ 30
Table 1.2  Pre-eclampsia and SIRS share clinical and laboratory features........ 32
Table 2.1  Number and percentage of subjects reporting adverse events for events occurring in >1% of subjects in phase 1/1B studies........... 52
Table 3.1  Demographic and base-line clinical characteristics of the study patients........................................................................................................ 64
Table 3.2  TEG indices in women with pre-eclampsia................................................. 66
Table 5.1  The sequences of the primer pairs used in RT-PCR......................... 93
LIST OF FIGURES

Figure 3.1  A schematic diagram of the TEG® analyzer technology (the source of the original figure) ................................................................. 58

Figure 3.2  A schematic diagram of the thromboelastography trace (the source of the original figure) ............................................................. 60

Figure 3.3  RhAPC delayed R-time and K-time, at the same time, reduced Angle in women with postnatal pre-eclampsia ..................................... 67

Figure 3.4  RhAPC decreased plasma TAT, F1+2 and PAI-1 levels in women with severe pre-eclampsia ......................................................... 70

Figure 4.1  RhAPC decreased serum TNF-α, IL-1, IL-2, IL-6 and IL-10 in postnatal severe pre-eclampsia ....................................................... 79

Figure 4.2  The effects of rhAPC on serum levels of TNF-α and IL-10 in women with antepartum pre-eclampsia ........................................ 80

Figure 5.1  RhAPC decreased neutrophils TLR-2, TLR-4 and cryopyrin mRNA in women with severe pre-eclampsia, while it increased caspase-3 expression ................................................................. 97

Figure 5.2  The effects of rhAPC on TLR-2, TLR-4 and caspase-3 protein expression of neutrophils in severe pre-eclampsia ...................... 99
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Antepartum</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>APH</td>
<td>Antepartum haemorrhage</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>ASA</td>
<td>Acetylsalicylic acid</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CHS</td>
<td>Canadian Hypertension Society</td>
</tr>
<tr>
<td>COSTART</td>
<td>Coding symbols for thesaurus of adverse reaction terms</td>
</tr>
<tr>
<td>CWHCBC</td>
<td>Children’s and Women’s Health Centre of British Columbia</td>
</tr>
<tr>
<td>DCHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep venous thrombosis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>EoPET</td>
<td>Early-onset pre-eclampsia</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>F1+2</td>
<td>Prothrombin fragment 1+2</td>
</tr>
<tr>
<td>FDP</td>
<td>Fibrinogen/fibrin degradation product</td>
</tr>
<tr>
<td>FFP</td>
<td>Fresh frozen plasma</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HELLP syndrome</td>
<td>Haemolysis, elevated liver enzymes and low platelets syndrome</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon -γ</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>INR</td>
<td>International normalized ratio</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>LAK</td>
<td>Lymphokine-activated killer</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>LRP</td>
<td>Leukocyte-rich plasma</td>
</tr>
<tr>
<td>MA</td>
<td>Maximum amplitude</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal intensive care unit</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NPC</td>
<td>Normal pregnancy control</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen activator inhibitor-2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Pre-eclampsia</td>
</tr>
<tr>
<td>PIENTERS database</td>
<td>Pre-eclampsia Integrated Estimate of Risk Score database</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes or neutrophils</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PP</td>
<td>Postpartum</td>
</tr>
<tr>
<td>PPH</td>
<td>Postpartum haemorrhage</td>
</tr>
<tr>
<td>PROWESS trail</td>
<td>Recombinant human protein C worldwide evaluation in severe sepsis trail</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>rhAPC</td>
<td>Recombinant human activated protein C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIR</td>
<td>Systemic inflammatory response</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>STBM</td>
<td>Syncytiotrophoblast microfragment</td>
</tr>
<tr>
<td>TAT</td>
<td>Thrombin-antithrombin complex</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell-1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell-2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>u-PA</td>
<td>Urinary plasminogen activator</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methyltetrahydrofolate reductase</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

It is a pleasure to thank the many people who made this thesis possible.

First of all, I would like to thank my Supervisor, Dr. Peter von Dadelszen, for many insightful conversations during these two years. I could not have imagined having a better advisor and mentor for my Master Degree of Science.

I would like to thank my thesis committee, Drs Moon, Douglas, Walley, Magee and Turvey, for their inspiration, encouragement and advice.

At BC Women's Hospital, I would like to thank Pia Ganz and Claudette Hildebrand for recruiting patients in this study. I am very grateful to Fang Xie for sharing her data with me. Moreover, these experiments could not have been done without the direction and help of Yuxiang Hu, our lab manager; I owe her a debt of gratitude.

This research was supported in part by the Canadian Institutes of Health Research (CIHR) and the Michael Smith Foundation for Health Research Grant (awarded to Dr. von Dadelszen).
Chapter 1

Background
1.1 Pre-eclampsia

Pre-eclampsia is a placenta-dependent disorder with both local and systemic anomalies, and was once characterized by the classic triad of hypertension, proteinuria and oedema. However, oedema has been eliminated as a requirement for diagnosis (1, 2), since it is a common finding in normal pregnancy and approximately one third of eclamptic women never demonstrate the presence of oedema (3). At present, the term pre-eclampsia primarily refers to the new onset of hypertension (>140/90 mmHg) and proteinuria after 20 wk of gestation in previously normotensive, non-proteinuric women (4). Eclampsia is defined as seizure activity in a patient with the presentation described above, and the term comes from the Greek word for lightning. Despite intensive research, pre-eclampsia still accounts for significant morbidity and mortality for either mother or baby or both, complicating 5-7% of all pregnancies and exposing them to a 3- to 25-fold increased risk of severe obstetric complications (5-7).

1.1.1 Historical background

The earliest possible references to eclampsia are alleged to appear in ancient Egyptian, Indian and Chinese writings. Bernhart once quoted the works from Kahun papyrus dating from about 2200 BC (8), he also wrote that the Indian Atharva-Veda of old but unknown dates described an amulet to ward off convulsions in childbirth (8). Bernhart suggested that the ancient Chinese recognized eclampsia, moreover, the source of this suggestion was Wang Dui Me, whose work was translated into German by Lo (9). The work was originally published in AD 1832, and was thought to be free of any Western influence.
The first convincing allusion, however, according to Leon C. Chesley, is found in the pre-Hippocratic Coan Prognosis of the ancient Greeks (10). From Scott and Jenkins' review on the aetiology of pre-eclampsia (11), we learn that Hippocrates noted that headaches, convulsions, and drowsiness were ominous signs associated with pregnancy and that Varandaeus in 1619 used the original Greek term ‘eclampsia’ based upon the flashing lights often complained of before a seizure in pregnant women. Clonic spasms in association with pregnancy were described by Pew in 1694. In 1772, De la Motte recognized that prompt delivery of pregnant women with convulsions favoured their recovery (12). Rayer's landmark contribution (1839-1841) provided evidence for renal involvement with the observation of protein in the urine of pregnant, oedematous women. In 1843, Lever reported that eclamptics had albuminuria, and concluded that disappearance of proteinuria after delivery of the child was evidence that eclampsia was different from Bright's disease (13). At the same time, since hypertension and sometimes excessive oedema were also present for considerable time before fits occurred, the syndrome of ‘pre-eclampsia’ was identified. Then, great success in the employment of the intraspinal magnesium sulfate for tetanu in 1906 soon resulted in the evaluation intravenous magnesium for eclampsia (14).

1.1.2 Epidemiology

The disease has a bimodal frequency, being more common in young primiparous and older multiparous women and the overall incidence of pre-eclampsia is 5% to 7% among the general population (15). Nevertheless, there is disparity in reported rates of pre-eclampsia
across studies. This variation in reported rates results at least partly from the inconsistency in diagnostic criteria of the disorder, and also may be due to the geographic, social, economic and racial differences.

There are several risk factors identified for the development of pre-eclampsia, including socio-demographical factors (extremes of reproductive age, socio-economic status, ethnic group), pregnancy factors (primigravida, previous pre-eclampsia), genetic factors or personal medical history (obesity, chronic renal disease, chronic hypertension, diabetes mellitus, thrombophilia).

**Nulliparity**

Epidemiological studies have shown that pre-eclampsia happens more frequently in nulliparous than multiparous women, and multiparous women who change partners also have an increased incidence in a subsequent pregnancy (16). A previous normal pregnancy is associated with a considerably lowered incidence of pre-eclampsia; even a previous abortion provides some protection in this respect. This fact, in combination with the observation that a change in partner is a risk factor for subsequent pre-eclampsia, has led to the belief that immunologic incongruity and subsequent tolerance play a role in pre-eclampsia (17, 18).

**Ethnicity**

Ethnicity is associated with elevated risk in many studies (19). The results of a prospective study once showed that severe pre-eclampsia was more common among women
of African American and Hispanic origins from the clinic service as compared to non-pre-eclamptic controls (20). Nevertheless, other surveys have not confirmed this risk (21). Whether pre-eclampsia is more frequent in black women is still ambiguous. The higher frequency of essential hypertension in black women makes pre-eclampsia more prevalent in multiparous but probably not in primiparous black women (22).

Age

Women under 20 or over 35 years-of-age are considered to be linked with increased incidence. The independent risk of pre-eclampsia in younger women has not been conclusively determined; it is possible that the reported higher incidences for the younger patient may be due to the likelihood that the younger woman is primiparous. For the higher age cohort, there is ample proof of risk associated with the increased occurrence of hypertension in older women (23).

Obesity

Eskenazi and colleagues have pointed out that a high pre-pregnancy body mass index significantly increased the risk of pre-eclampsia (24). In general, there is even a six-fold increase in the likelihood of subsequent development of pre-eclampsia in obese women (25). In the investigation of risk factors for severe pre-eclampsia, Stone reported severe obesity (prepregnancy body mass index greater than 32.3 kg/m$^2$ or pre-pregnancy weight in excess of 84 kg) led to a higher risk of 4.9 and 5.1 in primiparous and multiparous women, respectively (20).
Family history of pre-eclampsia

Epidemiological evidence indicates that pre-eclampsia has a familial prevalence. In the 1980s, Chesley found the incidence of pre-eclampsia was 37% in sisters, and 26% in daughters of eclamptic women (26). In a population-based study, the incidence of pre-eclampsia in primiparous women who had a sister with pre-eclampsia was 20% (27). The role of a paternal contribution to the pathogenesis of pre-eclampsia was suggested by examination of a large Utah database. Men who were themselves the product of a pre-eclamptic pregnancy were 2.1 times more likely than control to have a partner with pre-eclampsia (28). Although the genetic susceptibility to pre-eclampsia is known from these data, the exact mode of inheritance has not yet been determined.

Preexisting medical conditions

Personal history of underlying essential hypertension, diabetes mellitus, renal disease, antiphospholipid syndrome, insulin resistance, collagen vascular diseases, hydatidiform mole, pre-eclampsia in previous pregnancy and twin pregnancies all increase the risk of pre-eclampsia (25, 29). In a study to evaluate predictors of pre-eclampsia, it has been shown that women with a past history of hypertension were nearly twice as likely to develop pre-eclampsia (24).

1.1.3 Aetiology

A concise description of normal placental development is essential before recognizing what is aberrant about placentation in pre-eclampsia. The term 'placenta' originates from
the Latin word for 'flat cake'. The human placenta is the interface between the mother and fetus in the uterus, and it is the major life support organ for the developing fetus, serving as the site of maternal-fetal exchange of oxygen, carbon dioxide, and nutrition, while protecting against immunological rejection. Additionally, the placenta secretes various hormones and prostaglandins that affect physiological variations during pregnancy. The placenta develops primarily from trophoblasts, which are invasive, eroding, and metastasizing cells and are the working ends of the placenta. They come from the blastocyst wall, are formed during the first stage of pregnancy and are the first cells to differentiate from the fertilized egg. They cover all villi and also surround the membranes, the chorion laeve. Traditionally, there are two types of trophoblastic cells: the cytotrophoblast is the inner layer of mononuclear cells and the syncytiotrophoblast is the outer layer of multinuclear cells (30). Trophoblast invasion is a key process during human placentation.

The aetiology of pre-eclampsia has not yet been clearly established. Classically, the pathogenesis of pre-eclampsia is considered a two-stage process resulting from the interaction between the placental disease and maternal responses. Placental hypoperfusion, secondary to inadequate trophoblast invasion (caused by abnormal biology of the trophoblast, or increased cytotoxicity of maternal immunocompetent cells, or a combination of both) and artery remodelling at the feto-maternal junction, may be the underlying cause. Placental hypoxia/ischaemia (the first step of the pathogenesis) is associated with release of products into the maternal circulation, and maternal responses evoked may result in endothelial activation (the second step). Immune responses appear to underlie the placental disease,
whereas maternal susceptibility for endothelial activation, hence development of pre-eclampsia, seems to be determined by genetic factors.

This paradigm probably describes the majority of early-onset pre-eclampsia (that arising prior to 34 weeks’ gestation) and some late-onset disease. However, it must be recognized that, at term, pre-eclampsia is more often associated with fetal macrosomia and/or multiple pregnancies (29-32) – under these circumstances, it is likely that an adequately formed placenta is not able to meet fetal demands, and that relative fetoplacental hypoxia ensues triggering the maternal syndrome that defines the disease (33).

The classical paradigm of poor placentation is very complex and cannot be attributed to any one single cause, nonetheless, just as described above, there are three main factors believed to be involved in the development of pre-eclampsia: 1) genetic susceptibility, 2) immune maladaptation, 3) placental ischaemia and oxidative stress (34-36).

Genetic susceptibility

From the biological viewpoint it is apparent that the pre-eclampsia phenotype includes more than hypertension and proteinuria only. Because a family history of pre-eclampsia increases the risk of pre-eclampsia, numerous investigations during the past decade have evaluated a possible link between pre-eclampsia and specific genetic mutations or thrombophilia (37, 38). Despite intensive efforts to identify the underlying susceptibility genes of pre-eclampsia and considerable progress in unraveling the network of contributing
factors, the genetic basis of the disorder remain poorly understood, similar to most diseases of complex inheritance patterns.

The following genetic mutations have been investigated with respect to pre-eclampsia: the factor V Leiden G1691A mutation, (causes resistance to activated protein C), the angiotensinogen T235 allele, the genetic variants in methyltetrahydrofolate reductase (MTHFR), the prothrombin gene, the presence of anticardiolipin antibodies, and inherited deficiencies in protein C, protein S and antithrombin III have been performed in different populations, as have other genes involved in blood pressure, thrombophilia, vascular volume control, lipid metabolism, oxidative stress, and endothelial dysfunction (37-45). Unfortunately, the findings of these studies have been highly inconsistent, because it is likely that no one major gene determines pre-eclampsia risk and most genetic studies of pre-eclampsia to date have concentrated on the maternal genotype alone. It is necessary to better evaluate the role of fetal genotypes and possible maternal-fetal genotypic interactions at multiple loci.

Immune maladaptation

For the following reasons epidemiological studies strongly suggest that immune maladaptation is involved in the aetiology of pre-eclampsia. 1. The risk of pre-eclampsia is decreased after the first pregnancy (16). 2. The protective effect of multiparity, however, is lost with change of partner. 3. Exposure to sperm offers at least a partial protection against the risks of pre-eclampsia (17, 18). 4. Similar to altered paternity, artificial donor
insemination and oocyte donation cause a substantial increase in pre-eclampsia (46).

Although pre-eclampsia is manifested late in pregnancy, the onset is during the early stages of gestation. During normal pregnancy, trophoblasts interact in the decidua with the unique uterine natural killer (NK) cells, modifying their cytokine secretion profile, regulating the expression of matrix metalloproteinases and adhesion molecules. Immune maladaptation, especially an excessive maternal inflammatory response, may result in the defective decidual endovascular trophoblast invasion, and eventually lead to a chain of events including abnormal spiral artery remodelling, endothelial cell dysfunction, hypoxia, thrombosis and infarction of the placenta (47). The following infarction of placenta results in increasing amounts of cytokines/placental fragments into the maternal circulation and inappropriate endothelial cells activation. Currently, the most common and immediate goal in the treatment of pre-eclampsia is to alleviate symptoms such as hypertension, whereas attempts to convert aberrant or exaggerated immune reactions may be a future possibility (48-50).

Placental ischaemia and oxidative stress

Pre-eclampsia occurs regularly in the presence of the placenta or a hydatidiform mole and remits dramatically post-partum after the delivery of the placenta. At least two theories have been advanced to link placental ischaemia to the aberrant endothelial metabolism, or endothelial pathologic lesions.
Firstly, the syncytiotrophoblast microfragments (STBM) is the placental surface that is in direct contact with maternal blood and it is well known that higher levels of STBMs have been measured in the peripheral circulation and uterine veins of women with pre-eclampsia. The STBM degradation complexes have endothelium disrupting and inhibition activities (51, 52). On the other hand, it has been postulated that oxidative stress resulting from placental ischaemia is the fundamental abnormality leading to endothelial damage in pre-eclampsia (52, 53). A number of pathologic conditions including battle field injuries, skin burns, sepsis haemorrhagic shock, and decreased organ perfusion, result in the release of reactive oxygen species (ROS) in the circulation. Epithelial tissues are prime targets for these species. So diminished placental perfusion, combined with maternal alterations in lipid metabolism or maternal predisposition such as antioxidant deficiency, might promote oxidative stress that the mother has no ability to block, thereby leading to endothelial cell dysfunction (52, 53). Generalized endothelial dysfunction may be responsible for most of the clinical aspects of the maternal syndrome in pre-eclampsia (49, 50), although some aspects of the disease relate directly to activated peripheral blood leukocytes (33).

Oxidative stress is caused by an imbalance between oxidants and antioxidants and characterized by peroxidants predominating over antioxidants. Oxidative stress is presumed to be the critical final step in causing the endothelial cell dysfunction. According to the oxidative stress hypothesis, oxidative stress is a postulated linking factor between placental ischaemia and the known endothelial dysfunction, which leads to the clinical manifestations of this disease (54-57). The abnormality possibly has its genesis via components, such as
ROS, released from the shallowly implanted and poorly perfused placenta. Short-lived ROS can react with lipids resulting in the formation of stable lipid peroxidation products that are potentially highly injurious to cell structures. It has been proposed that maternal dyslipidaemia may also play a prominent role in the production of oxidative stress and pathogenesis of pre-eclampsia. Hypertriglyceridaemia, increased apolipoprotein B concentrations, decreased high density lipoprotein (HDL) cholesterol and the presence of small dense lipoprotein (LDL) particles may be involved in the development of this disorder (58, 59). At the same time, it was hoped that supplementing women with antioxidants during pregnancy may help to counteract oxidative stress and thereby prevent or delay the onset of pre-eclampsia (60).

1.1.4 Management

1.1.4.1 Prevention

Numerous clinical trials were conducted to evaluate the effectiveness of various methods to prevent or reduce the incidence of pre-eclampsia; nevertheless, there currently are no well-established measures for preventing pre-eclampsia. Several recent examples will be discussed below.

Calcium supplementation

Recent studies have emphasized the possible role of general nutritional deficiency or imbalance of several specific elements in the aetiology of the disease, the obtained results are contradictory and further study is necessary. Low calcium intake has been associated with
pre-eclampsia and calcium metabolism may be disturbed in women with this disorder. Clinical observations have shown that supplementation with calcium can significantly reduce the frequency of pre-eclampsia, especially in populations with a low dietary calcium intake (61,62). Although the occurrence of pre-eclampsia is reduced, perinatal outcome is unaffected (61).

Magnesium and Zinc supplementation

It is well known that magnesium sulfate can be used in obstetrics to prevent severe pre-eclampsia from becoming eclampsia or to stop the convulsions of eclampsia. This relationship led to the speculation that magnesium deficiency could play an important role in the pathogenesis of pre-eclampsia, particularly in regulating the tonus of arterioles and veins. Many pre-eclamptic women, especially those from disadvantaged backgrounds, commonly have dietary intakes of magnesium below recommended levels. Supplemental magnesium treatment during pregnancy may be able to decrease the risk of pre-eclampsia, and increase birth weight (63). Deficiency of the essential trace element zinc is also thought to be associated with certain pregnancy complications, one of which is pre-eclampsia, although there is not enough reliable scientific evidence to show that zinc supplementation during pregnancy is effective in preventing illness (64).
Antiplatelet agents

Pre-eclampsia is associated with deficient intravascular production of prostacyclin, a vasodilator, and excessive production of thromboxane, a platelet-derived vasoconstrictor and stimulant of platelet aggregation. These observations led to the assumption that administration of antiplatelet agents may prevent pre-eclampsia. Aspirin has been the mainstay antiplatelet agent for prevention of pre-eclampsia. A great number of small phase II studies showed that the women who received antiplatelet agents were less likely to develop pre-eclampsia; however, larger trials had a relatively minor effect (21, 65, 66). Recently, a meta-analysis based on 31 randomized trials of primary prevention, detected that antiplatelet agents, particularly aspirin, during pregnancy are associated with moderate but consistent reductions in the relative risk for pre-eclampsia, preterm births before 34 weeks' gestation, and pregnancy with serious adverse outcome (67).

Fish oil

Fish oil, which is rich in n-3 polyunsaturated fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DCHA)), has been shown to interfere with prostaglandin metabolism resulting in a decrease in the thromboxane (TX) and prostacyclin ratio. The effect of fish oil on blood pressure has often been assumed to due to this mechanism. A recent, double-blind, multi-center clinical trial compared fish oil with placebo in women at high risk due to prior preterm delivery, intrauterine growth restriction (IUGR), or pre-eclampsia (68). No significant evidence was found that fish oils could prevent pre-eclampsia.
Antioxidants

As noted earlier, there is ample evidence that increased levels of peroxidation, a hallmark of excessive attack, are found in the pre-eclamptic placenta. These increased levels could arise from increased production of oxidants, but may also signify a decrease in antioxidant activities. In fact, oxygen free radicals, including superoxide anions, are shown too elevated in the pre-eclamptic placenta, while the activities of the antioxidants superoxide dismutase, glutathione peroxidase and vitamin E are all reduced. Therefore, the use of antioxidants has been suggested. A research group leaded by Chappell recently reported a randomized controlled trial of prophylactic vitamin C and E supplementation in a group of 283 pregnant women at increased risk of pre-eclampsia, as defined by abnormal uterine artery Doppler waveform or by past history of the disease (69). The trial was designed to test the effect of antioxidants on biomedical markers of disease. Plasminogen activator inhibitor (PAI)-1 is considered to be an activation marker in endothelial cells, and PAI-2 is a marker of placental function. The PAI-1/PAI-2 ratio decreases in normal pregnancy, but is high in pre-eclampsia owing to endothelial cell activation and placental insufficiency (70). In this trial, the ratio of PAI-1/PAI-2 was significantly decreased in the vitamin-treated group, though further studies are needed to fully elucidate the dosage, timing, and outcome. However, recent adequately powered randomized controlled trials (RCTs) have shown that antioxidants have no protective effects against the maternal syndrome of pre-eclampsia, and may have deleterious fetal effects (71-73).
1.1.4.2 Treatment

Because the cause of pre-eclampsia is not known, effective interventions have been elusive. The only specific, definitive treatment is delivery to prevent development of maternal or fetal complications from disease progression. This is always the option of choice for issues around maternal outcomes. The issue of preterm delivery is more complex. Prolongation of the pregnancy is favourable to the fetus (74) and preterm babies have increased adverse outcomes. The expectant objectives are to prolong pregnancy if possible, to allow fetal organ maturity while preventing progression to severe disease and eclampsia.

Term patients

Decisions are not complex in women with pre-eclampsia at term. Delivery is the obvious solution when pre-eclampsia is diagnosed at or beyond 38 weeks of pregnancy, regardless of the severity of the disease. If the patient is not in labour, labor should be induced to expedite delivery. Vaginal delivery is the goal in these patients.

Mild pre-eclampsia

Although there is certain agreement about the management of mild pre-eclampsia at term, medical care of mild pre-eclampsia remote from term is still contentious. Expectant management (observation) at home or in the hospital is sometimes used to manage complications of mild pre-eclamptic pregnancy. Expectant management at home requires decreased activity and careful checking and daily recording of fetal activity, maternal blood pressure, urine protein measurement and weight. Expectant management in the hospital
requires bed rest and includes more frequent laboratory electronic and monitoring of the mother and fetus.

There is disagreement regarding the benefits of antihypertensive medications in patients with mild pre-eclampsia. It may benefit the mother only and not the fetus. A review by Magee and colleagues on management of hypertension in pregnancy noted the following three perinatal effects of antihypertensive use: 1) respiratory distress syndrome was reduced without a concomitant change in prematurity; 2) more neonatal bradycardia in trials of β blockers; and 3) an enhanced trend towards the incidence of small-for-gestational-age infants (74). Therefore, there is really no perfect benefit to chronic antihypertensive therapy in the setting of mild pre-eclampsia (75, 76).

Severe pre-eclampsia

From 1950-1980 there was no reduction in the maternal mortality from hypertension in pregnancy. During the 1980s the first fall was seen in deaths from this condition. This correlates with an increase use of antihypertensive drugs by obstetricians in the management of hypertension in pregnancy. Cerebral haemorrhage and cerebral oedema are the most frequent causes of death in pre-eclampsia (77, 78), so the antihypertensive therapy may be associated with the apparent reduction of death from cerebrovascular accident. Although a blood pressure above 140/90 mmHg can affect both perinatal and maternal mortality, the most commonly used threshold for treatment is a sustained diastolic blood pressure of 110 mmHg or higher (79-81) and/or systolic of 160 mmHg or higher (82).
There are several antihypertensive drugs that are appropriate during pregnancy, such as methyldopa, hydralazine, β-blockers (labetalol or oxprenolol), and calcium channel blockers (nifedipine). Since administration of angiotensin-converting enzyme (ACE) inhibitors during the second and third trimesters can result in a number of fetal adverse effects, it should be avoided.

**Eclampsia**

Eclampsia is defined as the development of convulsions and/or coma unrelated to other cerebral conditions during pregnancy and the postpartum period in patients with signs and symptoms of pre-eclampsia (83). It now is a rare occurrence in hypertension in pregnancy, occurring in approximately 1/300 of all pregnant patients with hypertension (84, 85).

Parenteral magnesium sulfate (MgSO₄·7H₂O, USP) was first reported to inhibit the seizures of pre-eclampsia in 1906 (14). It does not cause any significant maternal or neonatal central nervous system depression when used properly and is still the drug of choice to treat and prevent convulsions around the world. Magnesium causes relaxation of smooth muscle by competing with calcium for entry into cells at the time of cellular depolarization (86). The exact mechanism of action of the magnesium ion in the control of eclamptic convulsions is unknown. However, it is speculated that it works as an anticonvulsant by vasodilating the smaller-diameter intracranial vessels distal to the middle cerebral artery and alleviating cerebral ischemia (87).
The efficacy of magnesium sulfate therapy in the prevention and control of eclamptic convulsions has been well documented over the past one hundred years. Recently, the collaborative eclampsia trial compared the outcomes of different anticonvulsants and found a significant depression in the incidence of recurrent convulsions in women treated with magnesium compared to both phenytoin and diazepam (88).

1.2 Haemostatic changes in pre-eclampsia

1.2.1 Pregnancy is related with changes in the maternal haemostatic system

Haemostasis is a fundamental mechanism for protecting an organism from loss of body fluids through the interaction of platelets, coagulation factors, fibrinolysis and the vessel wall. These components interact intimately and interlink at many stages with cross-over reactions and positive or negative feedback loops. Normal pregnancy is associated with profound changes in the coagulation and fibrinolytic systems resulting in a hypercoagulable state in Caucasians and Asians (89, 90). These changes are traditionally thought to represent an adaptive and preparatory mechanism for the haemostatic challenge of the delivery but result in an increase risk of thrombotic disorders (91).

Mild thrombocytopenia is associated with pregnancy, particularly near the end of the third trimester (92-96). However, the detailed mechanisms of how blood platelets reduce in this special period are still unanswered. Following delivery, the platelet count increases in
reaction to, and in compensation for, the consumption of platelets by the haemostatic challenge of delivery.

During healthy pregnancy there are physiological changes which lead to increases in the majority of coagulation factors (97, 98). Factor XIII, high-molecular weight kininogen and prekallikrein all increase in pregnancy; although reports on the latter have not been completely consistent, with some authors reporting no changes in prekallikrein levels. Factor XI levels fall gradually through pregnancy, reaching their lowest values at term, while factors IX appears to remain static or increase slightly. Both factor VIII coagulant activity and von Willebrand’s factor antigen increase progressively throughout pregnancy. Factor VII, the main component of the extrinsic coagulation, also increases in pregnancy. Factor X increases while factors II and V show no change. Fibrinogen increases substantially and progressively during pregnancy, yet factor XIII shows an initial increase but then falls to non-pregnant values in late gestation.

The endogenous inhibitors of coagulation are necessary to ensure that thrombin generation remains limited and localized. An important component is antithrombin, which acts as an inhibitor of thrombin (99). Antithrombin binds with thrombin to form the stable thrombin–antithrombin complex, thereby preventing the conversion of fibrinogen to fibrin by thrombin. In addition to these properties, antithrombin can also inactivate factors Xa, IXa and VIIa. Antithrombin III was initially thought to decrease in pregnancy; however, more recent studies have shown that levels remain stable during pregnancy (100, 101).
The protein C/protein S pathway is another specific feedback mechanism. This pathway becomes activated when thrombomodulin binds to thrombin to form a complex that, in turn, activates protein C to so-called activated protein C (APC) (102, 103). Protein S acts as a catalyst for activated protein C. Activated protein C inactivates both factor VIIIa and factor Va activity. Both protein C and protein S are hepatocyte products that depend on vitamin-K-dependent enzymes to γ-carboxylate their glutamine-rich plasma membrane-binding domains. Protein C and protein S have plasma half-lives of 6–8 and 42 hours, respectively. Circulating protein S exists in both free (40%) and bound (60%) forms. The complement 4b-binding protein serves as a carrier protein for protein S. Only free protein S complexes with APC; therefore, any condition (e.g. pregnancy, inflammation, surgical stress) that increases the complement 4b-binding protein will reduce protein S activity. Protein C levels appear to remain constant or increase slightly, while acquired APC resistance is found in over half of the normal pregnancies. Protein S normally exists in plasma in two forms: the functionally active free protein and protein S combined with C4b-binding protein which is functionally inactive. Levels of total and free protein S gradually decrease throughout pregnancy (104, 105).

Activation of coagulation mechanisms normally leads to a fibrinolytic response (106). Fibrinolysis is a physiologically protective and reparative mechanism and is necessary to remove fibrin from the vessel; therefore, it is the key to controlling both the consequences of any procoagulant response and vascular patency. Fibrinolytic activity appears to be
impaired during pregnancy, although it returns rapidly to normal following delivery (107, 108). This seems to be the result of placentally derived PAI-2 which is present in substantial quantities during pregnancy, and it has also been shown that the endothelial-derived PAI-1 increases (92). The two PAIs successfully depress fibrinolytic activity in pregnancy. Elevated levels of tissue plasminogen activator (t-PA), urinary plasminogen activator (u-PA) and plasminogen have also been reported (109). Despite the reduction in fibrinolytic activity, fibrinolysis cannot be totally shut down as fibrinogen/fibrin degradation products (FDPs) remain in the plasma and increase as pregnancy progresses (98).

Most of the changes in the haemostatic systems revert rapidly to normal following separation of the placenta. In particular the fibrinolytic system rapidly returns to its baseline state in keeping with the loss of the placental source of PAI-2. There is evidence of contact system activation and platelet consumption immediately following delivery, then the expansion in fibrinogen, factor VIII:C and platelet count a few days later (91, 110). The haemostatic system appears to return to the typically non-pregnant state by 4 weeks after delivery.

1.2.2 Disturbance in the haemostatic system is a characteristic feature of pre-eclampsia.

Pre-eclampsia is associated with a variety of abnormalities in blood coagulation and platelet function. In cases that progress to severe pre-eclampsia, striking disorders of the haemostatic mechanism develop, associated with disseminated intravascular coagulation (DIC) and bleeding. It is still not clear whether these abnormalities are of aetiological
importance in the hypertensive diseases of pregnancy or whether they represent an extreme form of the usual haemostatic response to profound vascular injury. However, it is plausible that the disorders of haemostasis, which are generally pro-coagulant, may contribute to the widespread thrombosis seen in severe cases of pre-eclampsia. A vicious circle may be set up in which activation of haemostasis by vascular or immunological means produces platelet and fibrin deposition leading to further vascular damage.

The majority of observers have seen a marked reduction in platelet count in pre-eclampsia, and the fall is related to clinical severity and fetal outcome (91, 111, 112). Although infrequently determined, the bleeding time has been prolonged in women with pre-eclampsia in comparison with normal pregnancy, probably as a consequence of the fall in platelet count. The lifespan of circulating blood platelets has been found to decline in patients with pre-eclampsia. The most sensitive indicators of whether platelets have been activated in the circulation or not are the plasma concentrations of the platelet proteins, β-thromboglobulin and platelet factor 4 (113). Investigators generally agree that plasma β-thromboglobulin is increased in severe pre-eclampsia. Levels of platelet factor 4 similarly rise in patients with pre-eclampsia, but the change from normal pregnancy is not as pronounced as those seen with plasma β-thromboglobulin (114-116). These data on platelet function in pre-eclampsia indicate that platelet activation occurs during the disease and that the degree of platelet involvement rises with increasing disease severity.

In general, results of standard coagulation tests from women with pre-eclampsia have not
been very informative. Some researchers found the prothrombin time (PT) was significantly shortened in women with pre-eclampsia compared with pregnant controls (117). Others have found no shortening of the PT, while in the most severe cases such as those with eclampsia and DIC, PTs are prolonged. The activated partial thromboplastin time (APTT) is usually normal, again increasing with DIC.

Many studies have looked at the levels of individual coagulation factors in pre-eclampsia. Most have found no difference in concentrations of coagulation factors X, XI, and XII in maternal blood comparing pre-eclamptic with normal pregnancy at the time of delivery. However, other researchers have found that concentrations of certain coagulation factors are increased in women with pre-eclampsia, particularly factor VIII, although sometimes the difference has not been large enough to be statistically significant (118). Other subtle abnormalities, such as the slightly elevated von Willebrand factor/factor VIII ratio, have also been observed in pre-eclampsia (119, 120). Levels of antithrombin activity are reduced in patients with severe pre-eclampsia, and results from several studies subsequently confirmed the reduction in antithrombin III concentration. As a result of increased thrombin generation, thrombin–antithrombin (TAT) complex and prothrombin fragment 1+2 (F1+2) levels increase in normal pregnancy, and even more so in pre-eclampsia (114, 121, 122).

Enhanced fibrinolysis, as assessed by increased levels of FDPs in serum or urine, has been observed in normal pregnancy and pre-eclampsia, although not consistently (122, 123). D-dimer levels in both pre-eclampsia and normal pregnancy were higher than observed in
non-pregnant women. Labouring women with pre-eclampsia had higher mean D-dimer levels than in normal pregnancy (106, 124). In most studies, plasma fibrinogen levels have been found to be slightly increased in women with pre-eclampsia (125). As mentioned above, the fibrinolytic system is activated by fibrin generation and is regulated by activators, t-PA and u-PA, and their inhibitors, PAI-1 and -2. Both t-PA and u-PA increase in parallel with PAI-1 in normal pregnancies and again more markedly in pre-eclampsia, probably as a compensatory mechanism in normal pregnancy and as a consequence of endothelial dysfunction in pre-eclampsia (126, 127). PAI-1 is considered as an acute phase protein (128), suggesting that there is up-regulated vascular endothelial synthesis of these proteins in this kind of abnormal condition. On the other hand, the decreased PAI-2 level in pre-eclampsia in Caucasians (129) and Asians (130, 131) would suggest a prognostic parameter for reduced placental function.

1.3 Pre-eclampsia and systemic inflammatory response syndrome (SIRS)

1.3.1 Maternal intravascular inflammation in normal pregnancy

The inflammatory response can be categorized into two main types: local inflammatory response and systemic inflammatory response (SIR).

The regional inflammatory reaction involves the blood vessels close to the injured tissue, the cells circulating within these vessels, and adjacent connective tissue. The early stages of this response produce several distinct signs: redness, swelling, heat, pain (or itching), and
loss of function, due to vasodilation of the blood vessels, then the invasion of leukocytes from the circulatory system into the interstitial fluid, towards the site of infection (132-134). In addition, there are many other factors involved in the development of local inflammation, such as the location, type and nature of injury, the cellular and humoral immune state, etc (132-134). The SIR differs markedly from the regional response, which is confined to specific organs or tissues. The former is dispersed throughout the whole body, involving a variety of cells and proteins within the blood vessels, even affecting inflammatory networks outside the circulation system. The major inflammatory cells are dendritic cells, NK cells and the phagocytes (132-134), but a SIR also involves endothelial cell, which become diffusely activated. Such cells can produce a diversity of pro-inflammatory cytokines, stimulate and/or be stimulated by leukocytes, present antigen to T cells after appropriate stimulation (135). Other tissue cells (hepatocytes and adipocytes), platelets, and a range of humoral elements are also having their unique roles (135). Therefore, the SIR contains complicated networks of cellular and biomolecular interactions, which are still incompletely understood.

The local inflammatory response occurs early in pregnancy and is a vital step of normal placentation. During placentation, lymphocytes of the innate immune system, especially NK cells, act as a prominent part of in the decidual inflammatory reaction (136). Decidual NK cells are a select population; they usually present in the endometrium of the luteal phase prior to conception and appear to modulate placentation by secreting cytokines that promote infiltration of the invasive trophoblast into the maternal spiral arteries (136-138).
The local inflammatory response cannot be ignored as it relates to placentation. However, here we will focus on systemic inflammatory responses that appear during the second half of pregnancy and in pre-eclampsia. During normal pregnancy, the mild inflammatory changes do not indicate a pathological condition. Pregnancy itself is a state of modest systemic inflammation, as pro-inflammatory cytokines appear to be involved in cellular events that establish and maintain pregnancy, although the detailed mechanisms have not yet been clarified (139, 140).

The total white cell count rises in pregnancy as a result of an increase in human polymorphonuclear leukocytes or neutrophils (PMNs). These white blood cells are essential to the innate immune response against bacterial pathogens and are a key part of the acute inflammatory response (141, 142). Neutrophils increase from the 45th day of pregnancy to reach a peak at 30 weeks and plateau during the third trimester. There is a further neutrophilia at the onset of labour, the total leukocyte count rising as high as $40 \times 10^9$/$L$ in uncomplicated pregnancies. The count returns to non-pregnant levels by six days post partum. There is also a tendency for the total leukocyte count to rise with increasing parity, but this is not significant. Normal pregnant women may have up to 3% myelocytes or metamyelocytes in their circulating blood.

While the numbers of granulocytes in the peripheral circulation increase during pregnancy, the metabolic activities of cells are also strengthened. Neutrophils are alerted
and have enhanced phagocytosis (143). Circulating neutrophil elastase concentrations are regarded as the indirect indices of neutrophil activation, and normally they are significantly elevated in pregnant women compared to non-pregnant women. This neutrophilia and the increase in metabolic activity may be regarded as the result of oestrogen stimulation (139, 144). Circulating monocytes also show an augmented activation status including monocytosis (145) and these cells are primed to produce the cytokine in normal human pregnancy (146). The leukocyte alkaline phosphatase score rises from the non-pregnant state to reach levels in the third trimester which are usually encountered only during major infections in the non-pregnant woman. The activity returns to the non-pregnant level by six weeks post partum but breast feeding may prolong the elevation (147). Hexose monophosphate activity, glucose oxidation and myeloperoxidase activity are also increased (148-150). In the late 1990s, flow cytometric analysis of pregnancy changes in the surface markers of fresh unstimulated leukocytes shows that activation extends to lymphocytes, as well as monocytes and granulocytes (151).

The inflammatory response in pregnancy encompasses not only activation of leukocytes (151), but also the kindling of the complement system, clotting system and circulating inflammatory cytokines, including interleukin (IL)-6, tumour necrosis factor (TNF)-α and its two circulating soluble receptors (152). IL-6 is secreted from activated macrophages, monocytes, T or B lymphocytes, endothelial and other types of cells. The target cells for IL-6 include T-cells, other leukocytes, and hepatocytes. The levels of plasma IL-6 are elevated in normal pregnant women (153). TNF-α has a transient half-life so its two soluble
receptors are commonly used as substitute markers to measure its circulating level. Using this technique it has been found that circulating TNF-α increased in pregnancy (154).

In summary, these findings show that normal pregnancy, especially in the third trimester, is associated with changes in peripheral blood leukocytes and circulating inflammatory cytokines. These indicate that pregnancy can be considered as a state of marked generalized inflammatory response, complete with evidence for an acute phase reaction and activation of multiple components of the inflammatory network, in some aspects comparable in degree to those once observed in cases requiring intensive care for sepsis.

1.3.2 The similarity of pre-eclampsia and SIRS: clinical features and neutrophil activation.

It is only recently that pre-eclampsia has been considered as arising from inflammatory processes that are an essential stage of normal pregnancy, although in a much less intense form. In other words, pre-eclampsia is not a separate island but the extreme end of a continuum of maternal systemic inflammatory responses caused by pregnancy itself. A similar inflammatory response occurs in pre-eclampsia, but it is associated with greater intensity and range than normal pregnancy (155). The inflammatory response combined with systemic endothelial dysfunction, which occurs in normal pregnancy, is even more marked in pre-eclampsia. The inflammatory response ranges from inflammatory markers, leukocyte surface antigen expression, to inflammatory changes in clotting system and endothelium (151, 155). These alterations not only affect endothelial cells and leukocytes,
but also many adjuvant elements, such as the complement system, clotting system or platelets, to various degrees.

This is reminiscent of SIRS (systemic inflammatory response syndrome). SIRS is a serious medical condition which is the development of a severe and inappropriate systemic inflammatory state of the whole body caused by a variety of severe clinical insults. This condition even persists following the removal of the inciting stimulus such as infection or trauma. Usually, SIRS is caused by sepsis and it is associated with dysregulation of the immune system (156).

SIRS can be diagnosed when three or more of the following conditions are present (Table 1.1)
Table 1.1 Systemic inflammatory response syndrome (SIRS) Criteria

I. **Temperature** >38°C or <36°C;

II. **Heart rate** >90 beats per minute;

III. **Respiratory rate** >20 breaths per minute, or PaCO₂ <32 mm Hg; or mechanical ventilation for an acute process

IV. **White blood cell count** >12,000/mm³, <4,000/mm³, or >10% immature neutrophils

1. SIRS = the systemic inflammatory response to a variety of severe clinical insults.
2. The response is manifested by three or more of the above four SIRS criteria. If the patient is on drugs that modify the heart rate, she must have two of the rest three conditions.

SIRS and pre-eclampsia share many clinical and laboratory characteristics (Table 1.2) (157). Both disorders persist following the removal of the initiating cause, that is, gram-negative sepsis (in SIRS) or the poor placenta (in pre-eclampsia), but do not develop in the same manner as in the presence of that respective initial agent. Moreover, disordered activation of the clotting cascade happens in these two syndromes, resulting in DIC, microvascular thrombosis and haemolysis. Hypertension-induced cerebral haemorrhage used to be the most common cause of pre-eclampsia-associated maternal death (158). Now, with effective antihypertensive therapy (74), women dying of pre-eclampsia most commonly die (40.5% of associated maternal deaths) of either hepatocellular necrosis or acute respiratory distress syndrome (ARDS) (159-161), similar to SIRS.
Table 1.2 Pre-eclampsia and SIRS share clinical and laboratory features (modified from von Dadelszen et al)

<table>
<thead>
<tr>
<th>Clinical finding</th>
<th>Laboratory finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disseminated intravascular coagulation</td>
<td>Endothelial function and inflammation</td>
</tr>
<tr>
<td>Microvascular thrombosis and haemolysis</td>
<td>↑ plasma TNF-α</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>↑ plasma IL-6*</td>
</tr>
<tr>
<td>Acute renal failure</td>
<td>↑ PAI-1; ↓ PAI-2</td>
</tr>
<tr>
<td>Hyperdynamic state</td>
<td>↑ thromboxane:prostacyclin ratio</td>
</tr>
<tr>
<td>End organ hypoperfusion</td>
<td>↑ endothelin</td>
</tr>
<tr>
<td>↑ vascular permeability</td>
<td>↑ caeruloplasmin</td>
</tr>
<tr>
<td>Hepatic necrosis</td>
<td>↑ α1-antitrypsin</td>
</tr>
<tr>
<td>Acute respiratory distress syndrome</td>
<td>Complement activation</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>↓ plasma transferrin</td>
</tr>
<tr>
<td>Differential response to initiating agent (placenta/trauma/infection)</td>
<td>Hypoalbuminemia</td>
</tr>
<tr>
<td>Persistence following removal of initiating agent</td>
<td>↓ activated protein C*</td>
</tr>
<tr>
<td></td>
<td>Neutrophil activation</td>
</tr>
<tr>
<td></td>
<td>Neutrophilia and delayed neutrophil apoptosis</td>
</tr>
<tr>
<td></td>
<td>↑ plasma neutrophil elastase*</td>
</tr>
<tr>
<td></td>
<td>↑ neutrophil CD11b expression</td>
</tr>
<tr>
<td></td>
<td>↑ basal intracellular ionized calcium</td>
</tr>
<tr>
<td></td>
<td>↑ oxidative stress</td>
</tr>
</tbody>
</table>

IL-6: interleukin-6; PAI: plasminogen activator inhibitor; TNF-α: tumour necrosis factor-α
↑: increased compared with normal;
↓: decreased compared with normal;
*: compared with normal pregnancy.
The understanding of pathogenesis of organ dysfunction in patients with SIRS suggests a central role for activated neutrophils. Similarly in pre-eclampsia, there is activation of neutrophils has also been shown, including increase plasma concentration of neutrophil elastase, increased oxidative stress, increased surface expression of CD11b, increased concentration of basal intracellular ionized calcium, and, in vitro, increased chemoattractant-induced neutrophil superoxide production. Neutrophilia is prominent in normal pregnancy, pre-eclampsia and SIRS. There is a gestational age-related delay in apoptosis that explains the neutrophilia of normal pregnancy. In normotensive IUGR, neutrophil apoptosis is also as delayed as in normal pregnancy. In contrast with these two conditions above, pre-eclampsia and SIRS are associated with both a relative severer neutrophilia and greater delays in PMNs apoptosis. Both hepatocellular necrosis and acute respiratory distress syndrome in pre-eclampsia and SIRS are characterized by neutrophil infiltration and are believed to be neutrophil mediated (162).

The comparison between pre-eclampsia and SIRS, however, may sometimes seem counterintuitive because pre-eclampsia is considered to be a hypertensive state with low maternal mortality (0.06%) and SIRS a hypotensive state with high mortality (30%). However, 21% of women with pre-eclampsia have no documented hypertension before their first eclamptic seizure (163), and early onset pre-eclampsia is associated with up to a 1% risk of maternal mortality (164) in women in their second to fourth decades of life. Similarly, in SIRS, 96% of patients do not require positive inotrop support, and in the absence of superimposed sepsis, mortality is 7% in patients predominately in their seventh and eighth
decades of life (165).

1.4 Recombinant human activated protein C (rhAPC)

Despite the considerations discussed above, very seldom have anti-inflammatory approaches been used to prevent and manage pre-eclampsia. One example is the use of high dose corticosteroids to lessen the HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome. Currently this is a focus of recent research. (166).

A randomized controlled trial of recombinant human activated protein C (rhAPC) has recently demonstrated very encouraging results in the treatment of SIRS with infection (septic SIRS) (167). This important benefit, in addition to the comprehensive similarity of SIRS and pre-eclampsia (155), supports the use of rhAPC as a valuable option for the management of pre-eclampsia.

Activated protein C (APC) is a potent modulator of both coagulation and inflammation. It is converted from its inactive precursor, protein C, by thrombin in complex with thrombomodulin, an endothelial cell-surface receptor (168). It has several different regulatory effects: anti-thrombotic, profibrinolytic, and anti-inflammatory. Firstly, APC inactivates two key cofactors, Va and VIIIa, which are responsible for the generation of thrombin from prothrombin, and thereby inhibits thrombosis. Secondly, APC promotes fibrinolytic activity by inhibiting PAI-1 activity. PAI-1 is the critical inhibitor of u-PA and
t-PA, which are the activators of plasminogen and hence fibrinolysis. Finally, *in vitro* data suggest that the reduction in the synthesis of several inflammatory cytokines by monocytes may partially explain its anti-inflammatory effect (169, 170). *In vivo*, APC infusion protects baboons from the lethal effects of *Escherichia coli*, and the levels of inflammatory cytokines rise when APC is inhibited (171), and it can reduce ischaemia-reperfusion injuries in other animal models (172, 173). Recently, Bartolome S and his colleagues also investigated the protective effects of APC against microvascular injury in a randomized prospective animal study (174); they found APC really has anti-inflammatory effects on microvascular physiology during systemic hypoxia, as evidenced by analysis of leukocyte adherence, emigration, and venular permeability.

Issues of maternal and fetal safety must be addressed before mounting a phase II clinical trial of rhAPC administration in women with severe pre-eclampsia. In general, the first trimester (embryonic stage) is more vulnerable than the second or third trimesters (fetal stage) (175), while pre-eclampsia was defined as increased diastolic blood pressure and proteinuria after 20 weeks' gestation, so APC could not be considered as a teratogenic agent from this viewpoint. However, potentially harmful effects on embryo or fetus should be still carefully taken into consideration.

The biological basis for the fetotoxicity may depend on the feto-placental transfer of the chemicals, and one critical factor affecting placental drug transfer is the physicochemical properties of the drug. One of the preferred agents for anticoagulation in pregnancy, low
molecular weight heparin (LMWH), has molecular weight (MW) of approximately 3KD (176), does not cross the placental barrier. Human APC is a serine protease of MW 56.2KD, so it is too large to be transferred across the placenta via passive diffusion into the fetal circulation. Although there is a theoretical possibility that transmission can occur through breaks in the placental barrier, no evidence for gross breaks at the human maternal-fetal interface in the literature with respect to IUGR or pre-eclampsia has been found (177-182). And in in vitro isolated placental cotyledon perfusion experiments, there is no evidence that protein C crosses or is degraded by the placenta (183).

Haemorrhagic events must be assessed as they relate to the maternal and fetal safety. The results of a phase II clinical trial on the use of lyophilized pasteurized APC for treatment of postpartum DIC associated with placental abruption suggested that APC was effective in correcting hypercoagulable state without any adverse effect, including postpartum haemorrhage (184). Evidence from a more recently multicentre phase III randomised controlled trial conducted by Simon Nadel et al suggested serious bleeding events were similar between the placebo and APC groups, and the overall safety performance is acceptable (except in children younger than 60 days), though they did not find any efficacy of rhAPC in children with severe sepsis (185). There is even a case report of its successful use in a severe septic trauma patient with intracranial haemorrhage (186). RhAPC should be administered by continuous intravenous infusion because it has a very short half-life of 15 mins in maternal circulation (170), so it is easy to remove the APC infusion from patient before a major haemorrhage occurs.
In conclusion, the use of rhAPC for pre-eclampsia has the potential to revolutionize treatments for this “toxaemia of pregnancy” which can seriously threaten the lives of both the mother and the fetus, and currently where there is no effective prophylactic agent and therapeutic strategy available.
Chapter 2

Research plan
2.1 Hypothesis

We postulated that recombinant human activated protein C (rhAPC) will safely modify the pathophysiology of pre-eclampsia, permitting the safe prolongation of pregnancies complicated by severe early-onset disease and the rapid resolution of severe postnatal disease, without a significant increase in bleeding complications.

2.2 Aims

The overall hypothesis will be examined by clinical observation, laboratory testing, and basic science investigations. This thesis is focussed on the basic science elements of the hypothesis, and tested our hypothesis by: 1) determining whether rhAPC may act as a potent modifier of coagulation (anticoagulant) in women with pre-eclampsia; 2) identifying the effects of rhAPC on temporal changes of pro-inflammatory cytokine activity in the serum of women with pre-eclampsia; 3) investigating the basic mechanisms of cell activation in peripheral blood neutrophils during pre-eclampsia.

2.3 Subjects

We are planning to establish the safety and the efficacy of rhAPC in two scenarios. One is early-onset pre-eclampsia where the fetal prognosis is dismal. The other is severe postpartum pre-eclampsia, as it is in the early puerperium that pre-eclampsia causes most maternal deaths (158-161, 187, 188). To be eligible, women will have severe pre-eclampsia, defined to reflect
its systemic nature, and not solely by hypertension and proteinuria (1, 189). Either one or both of these elements must be present to fulfill the entry criteria. Twenty women will be enrolled in each arm of the trial; the rhAPC infusion will run at 24 μg/kg/h for up to 96h. For basic research, blood samples have been drawn before the infusion begins, then 4h, 28h, 52h, 76h following the start of the rhAPC infusion, and 4h after the rhAPC ends.

The trial is ongoing, so the clinical elements and ongoing basic and laboratory science elements of the project remain incomplete.

2.3.1 Cohort 1 - Early-onset pre-eclampsia with dismal fetal prognosis

The target population consisted of women at <27wks' gestation with proteinuric gestational hypertension, and/or an estimated fetal weight <600g. Proteinuric gestational hypertension, which will be referred to as pre-eclampsia, is usually defined as maternal hypertension (BP＞140/90) presenting ≥20wks' gestation with >0.3g/day of proteinuria.

2.3.1.1 Inclusion criteria

The development of either proteinuria (≥0.3g/24h) and/or hypertension (≥140/90mmHg, measured twice, at least 4h apart) and dysfunction of one or more organs after 20 weeks' gestation. By organ system, these dysfunctions include the following:

i. Cardiovascular: heart rate >90bpm, severe systolic or diastolic hypertension (≥170mmHg or ≥110mmHg, respectively), mean arterial pressure ≤70mmHg for at least 1h despite adequate fluid resuscitation, systolic hypotension (sBP ≤90mmHg), or evidence of
cardiomyopathy (190-194).

ii. **Renal**: hyperuricaemia, oliguria (<10ml/h for 2 consecutive hours), >5g proteinuria/24h, or serum creatinine >2 times the upper limit of normal range.

iii. **Coagulation**: Presence of D-dimers, dysfibrinogenaemia, thrombocytopenia (30-80 x 10^9/L) or a 50% decrease in the platelet count from the highest value recorded over the previous three days, >10% immature neutrophils, microangiopathy (blood smear), prolonged PT or APTT, LDH >600IU/L, transfusion of >10U of any combination of blood products.

iv. **Central Nervous System**: eclampsia, cortical blindness, or Glasgow coma scale <13.

v. **Respiratory**: respiratory rate ≥20/min, >50% inspired O2 (maintain SaO2>90% (pulse oximetry)), or intubation, or, in the presence of an arterial line, PaCO2 ≤32mmHg, PaO2/FiO2 ≤250.

vi. **Hepatic**: transaminitis (either AST or ALT > twice upper limit of normal range), hypoalbuminaemia, hyperbilirubinaemia, hypoglycaemia.

vii. **Live intrauterine fetus(es)**, with an estimated fetal weight <600g by ultrasound, if gestational age >27⁰wks. If the estimated fetal weight is ≥600g, then the admission for severe pre-eclampsia must be <27⁰wks. For multiple pregnancies, all fetuses must meet the eligibility criteria.

### 2.3.1.2 Exclusion criteria

i. **Contraindication to prolonging pregnancy for maternal or fetal reasons.** Maternal reasons include: severe pre-eclampsia mandating delivery, even in the absence of either proteinuria or hypertension (e.g. intractable hypertension, severe headache, >5 beats clonus,
severe thrombocytopoenia (<30 $\times 10^9$/L), enzymatic/glucose/ultrasound evidence of liver failure or haematoma, acute renal failure, acute respiratory distress syndrome, or cardiomyopathy). Fetal reasons include: reversed end diastolic flow on umbilical arterial Doppler insonation and severe oligohydramnios.

ii. **Lethal or life-threatening fetal anomaly**, including aneuploidy, structural anomalies, partial hydatidiform mole, and congenital infection.

iii. **Current bleeding.** Evidence of hepatic haematoma complicating either pre-eclampsia or HELLP syndrome. Bleeding liver, spleen, retroperitoneal bleed, or pelvic fracture, or compartment syndrome.

iv. **Increased risk for bleeding.** 1. Congenital bleeding diathesis (e.g. von Willebrand's disease). 2. Clinical abruption within 48h. 3. Platelet count <30 $\times 10^9$/L. 4. Women with an epidural catheter or when it is anticipated that they will receive an epidural catheter during study drug infusion. In response to this concern, the infusion will be stopped with the onset of labour and/or 120min prior to any planned Caesarean section. 5. Any woman who has undergone major surgery (requires general, spinal or epidural anaesthesia (e.g. Caesarean section)) within last 12h, has active postoperative bleeding (other than normal lochia); or has surgery planned during the study drug infusion period. Again, the infusion stopped with the onset of labour and/or 120min prior to any planned Caesarean section (167). 6. PT/INR (International Normalized Ratio) >3.0 (APTT cannot be reliably used during the infusion as rhAPC may variably prolong the APTT). 7. Acetylsalicylic acid (ASA) >650mg/d or compounds containing >650mg/d within three days of study drug infusion (167). Doses of ASA >120mg/d are contraindicated in pregnancy due to fetal
concerns. 8. Any history of intracerebral arteriovenous malformation, cerebral aneurysm, CNS mass lesion, or history of severe head trauma, intracranial surgery or stroke <3mo before study. 9. Gastrointestinal bleeding, <6wks before study, which required medical intervention, unless definitive surgery has been performed. 10. Trauma patients at increased risk of bleeding (eg flail chest, significant contusion to lung). 11. Women with known oesophageal varices, chronic jaundice, cirrhosis, chronic ascites, or chronic renal failure on either haemodialysis or peritoneal dialysis. 12. Women with acute clinical pancreatitis without a proven source of infection.

v. Current heparin therapy in greater than prophylactic doses. Therapeutic heparin defined as: unfractionated heparin dosed to treat an active embolic or thrombotic event within 8h; LMWH used at any dose greater than the recommended dose for prophylaxis in the 12h prior to study drug infusion. Prophylactic heparin up to 15,000IU/d was permitted in PROWESS (Recombinant human protein C Worldwide Evaluation in Severe Sepsis) (167).

vi. Women with a known hypercoagulable state such as activated protein C resistance, a hereditary deficiency of protein C, protein S, or antithrombin; presence of anticardiolipin antibody, antiphospholipid syndrome, lupus anticoagulant, or homocysteinaemia, or patients recently documented (within 3mo of study entry) or highly suspected of having deep venous thrombosis (DVT) or pulmonary embolism.

vii. Inability to give informed consent because of language or similar issues. Women who are moribund and where death is perceived to be imminent (within 24h). Presence of an advanced directive to withhold life-sustaining treatment.

viii. Other significant immune disorders. HIV positive women. Women who have
undergone bone marrow, liver, pancreas, or small bowel transplantation.

ix. Other: Age <18y of age. Weight >135kg.

Note: Exclusion from the antenatal trial has not excluded woman from postnatal enrolment.

2.3.2 Cohort 2 - Postpartum pre-eclampsia

The target population was women >30min postpartum, who either had severe pre-eclampsia antenatally, first develop pre-eclampsia postpartum, or whose condition deteriorates postpartum. Following placental delivery, there is often a transient postnatal deterioration after which there is usually (in 95% of cases) maternal recovery by 72h. However, some women will succumb (194-197), and the postpartum period corresponds more closely to treated sepsis, in which rhAPC has been shown to be effective (167).

2.3.2.1 Inclusion criteria

i. Severe antenatal pre-eclampsia with perturbation of central nervous, cardiorespiratory, coagulation, hepatic and/or renal systems, at least 30min postpartum (3rd stage) with normal lochia.

ii. Postpartum deterioration of clinical state, in terms of central nervous, cardiorespiratory, coagulation, hepatic and/or renal systems with normal lochia.

iii. De novo postpartum pre-eclampsia, with perturbation of central nervous, cardiorespiratory, coagulation, hepatic and/or renal systems, at least 30min postpartum (3rd stage), and with normal lochia. Organ dysfunction was defined as above for the
cardiovascular, renal, coagulation, central nervous, respiratory, and hepatic systems.

2.3.2.2 Exclusion criteria (in addition to those listed for the antenatal trial).

**Ongoing postpartum haemorrhage** (PPH, estimated blood loss >500ml at vaginal delivery, or >1L at Caesarean section). Once a PPH has been controlled for at least 4h, women could then be enrolled in the study.

2.4 Infusion protocol

The infusion protocol was based on the PROWESS trial (167). Once women were eligible and had consented and the on-call perinatologist, obstetric anaesthetist, and obstetric internist had agreed, the Pharmacist on-call was contacted and asked to prepare the rhAPC for infusion, and the rhAPC infusion was started. All health care professionals received in-service training before the trial began. The obstetric resident staff were kept informed at all times and were included in the decision making surrounding the enrolment of any woman into the trial. The Charge Nurse was informed of the eligibility of a woman for the trial and arranged for one-on-one nursing for that woman.

2.4.1 Starting rules

**Antenatally:** Women without bleeding started the infusion immediately. Women with suspected/confirmed antepartum haemorrhage (APH) will commence the infusion >48h after last concern about active bleeding. There was no concern if a low dose of heparin is
properly used. A wait time of >8h after most recent dose of unfractionated heparin or >12h since last dose of LMWH occurred (167). For regional anaesthesia, the infusion commenced >4h after an epidural catheter is sited.

Postnatally: Following spontaneous or assisted vaginal delivery, the infusion started >30min after the 3rd stage with normal lochia. For Caesarean section, >12h postoperatively, with normal lochia. For regional anaesthesia, the infusion commenced >4h after the withdrawal of an epidural catheter.

2.4.2 Infusion rules

Early-onset pre-eclampsia with dismal fetal prognosis: The rhAPC infusion ran at 24 \( \mu g/kg/h \) for up to 96h antenatally. If there was a wash out period >36h, then a repeat infusion was started for up to 96h postpartum. Delivery decisions were made by the attending obstetrician, and were based on criteria of worsening maternal or fetal status.

Postpartum pre-eclampsia: The rhAPC infusion ran at 24 \( \mu g/kg/h \) for up to 96h.

2.4.3 Stopping rules

Under the following circumstances the infusion was be stopped:

i. Patient request;

ii. One-on-one nursing cannot be provided;

iii. Suspected/confirmed APH or PPH;
iv. Evidence of bleeding from any other site;

v. Onset of labour;

vi. Commencement of an induction of labour;

vii. 120min prior to a scheduled Caesarean section;

viii. 120min prior to a regional anaesthetic (where possible, women will be managed with intravenous opiates for intrapartum pain relief);

ix. 120min prior to the removal of an epidural catheter;

x. Immediately, should an emergency Caesarean section be indicated. Average time to commence an emergency Caesarean section (>20min) is greater than the half-life of rhAPC (15min);

xi. The occurrence of eclampsia;

xii. INR >3.0;

xiii. Platelet count <30 x 10⁹/L;

xiv. Ultrasound evidence of a hepatic haematoma.

2.4.4 Restarting rules

i. Women with suspected or confirmed APH: >48h since last concern about active bleeding.

ii. PPH: >4h since last abnormal level of bleeding.

2.5 Potential risks and management of adverse events

2.5.1 Obstetric haemorrhage
Our primary concern has been that of obstetric haemorrhage, both antenatal and postpartum. From PROWESS (167), we know that rhAPC was associated with a trend towards increased haemorrhage (p=0.06). These bleeding events occurred in patients with a previous risk factor for bleeding (e.g. gastric stress ulcers) or with thrombocytopenia <30 x 10^9/L. For 3991 ICU patients with sepsis treated with open label rhAPC, there were 34 cases of significant bleeding (0.85%) (195). For the 2786 women enrolled in clinical trials and who received rhAPC, 71 (2.6%) had serious bleeding episodes (195). The total risk of bleeding for all treated groups is 1.5% (105 of 6777) (195). The excess bleeding was limited to patients suffering from trauma, significant anticoagulation, platelet counts <30 x 10^9/L, and undergoing procedures. 42% of the significant bleeds were procedure-related (195). In total, in the scenario of systemic sepsis in an older population than being studied in this trial, 11 of 6777 (0.16%) suffered from fatal episodes of bleeding (seven intracranial and four non-intracranial) (195). The guidelines for rhAPC use now limit it to patients with a platelet count >30 x 10^9/L.

Outside pregnancy, the bleeding risks associated with rhAPC use are similar to those associated with medium dose heparin (15U/kg/h (20-30,000IU/d)) (195), a dose used in late pregnancy for venous thromboembolism (VTE) prophylaxis, as recommended by the Society of Obstetricians and Gynaecologists of Canada (196). The use of both prophylactic and therapeutic heparin with both unfractionated and LMWH is well-established in obstetrics. Ginsberg et al (197) reviewed the risks of unfractionated heparin therapy. Two women (2% [95%CI 0%, 7.1%]) suffered significant bleeding complications; one APH (medium dose
heparin), the other a PPH (full anticoagulation dose). These risks are consistent with those observed with LMWH, where 13 of 486 pregnancies (2.7% [1.4%, 4.5%]) were complicated by only minor bleeding (198, 199).

On the other hand, in an exploratory phase II clinical study (historical controls), lyophilised pasteurised APC was found effective in treating postpartum DIC associated with moderate to severe placental abruption in 16 women (184). Laboratory evidence of DIC was reversed within 24h of initiating APC in all, without any adverse events including significant PPH. As the half-life of APC in the circulation is 15min (158), it is administered by continuous intravenous infusion. These bleeding risks can be reduced by avoiding intrapartum and intraoperative (Caesarean) anticoagulation (197). We have avoided intrapartum, intraoperative, and immediately postoperative rhAPC, administering a compound with a plasma half-life of 15min, as against the 6h of unfractionated heparin and 12h for LMWH.

In response to concerns about obstetric and/or other haemorrhage, this trial has been designed as an open-label, single arm trial. Should bleeding have occurred, interventions could be applied immediately without spending time breaking randomization codes. We recognised that the sample size for each subtrial is quite small (20 per subtrial, with some of the women in the antenatal subtrial receiving a second postnatal course). This trial was not designed to preclude any increase in risk, but to identify any increase that would make it unsafe to continue. It is our belief that the planned, and yet to be achieved, sample size was
adequate to detect any increase in bleeding risks that would preclude extending this line of enquiry to a multicentre RCT. The subsequent RCT would be powered to clarify the risk-benefit equation for women who might eventually be offered this intervention outside the confines of a clinical trial.

2.5.2 Teratogenicity and fetotoxicity

There are no teratogenic concerns with rhAPC use for pre-eclampsia, as pre-eclampsia occurs after 20wks' gestation (1, 175, 189). Fetotoxicity would require transplacental transport of protein C. Activated protein C has a MW of 56kD, making it too large for passive transplacental diffusion. For comparison, the MW of LMWH is 3kD (198), and heparins are not transferred across the placenta. Also, APC will not be actively transported across the placenta, as there is fetal synthesis of coagulation factors from early in gestation. No components of the haemostatic system are believed to cross from mother to fetus (189, 200). Although theoretical concerns could be raised about the possibility of 'breaks' in the materno-fetal placental barrier occurring in pre-eclampsia, there is no evidence for this in the literature with IUGR (179), pre-eclampsia, or heparin (182, 201). As stated, heparin is a smaller molecule, is used routinely in pregnancies where pre-eclampsia is more likely to occur, and has a reassuring safety profile. In in vitro isolated placental cotyledon perfusion experiments, protein C neither crosses nor is degraded by the placenta (183). These data are in contrast with the concerns raised about repeated maternal steroid administration, which is known to be effective transplacental therapy, in single courses, to reduce the burden of neonatal morbidity and mortality associated with birth before 34wks' gestation.
2.5.3 Other adverse events

Adverse events that were collected across all phase 1/1B studies are described in Table 2.1 (202). None of these adverse events should be of such significance to deter women from consenting to the trial.
Table 2.1 Number and percentage of subjects reporting adverse events for events occurring in >1% of subjects in phase 1/1B studies (n=243).

<table>
<thead>
<tr>
<th>*COSTART terms</th>
<th>Number of subjects reporting event</th>
<th>% of total subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecchymosis</td>
<td>70</td>
<td>28.8</td>
</tr>
<tr>
<td>Headache</td>
<td>67</td>
<td>27.5</td>
</tr>
<tr>
<td>Pain</td>
<td>26</td>
<td>10.6</td>
</tr>
<tr>
<td>Rhinitis</td>
<td>17</td>
<td>6.9</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>11</td>
<td>4.5</td>
</tr>
<tr>
<td>Accidental injury</td>
<td>11</td>
<td>4.5</td>
</tr>
<tr>
<td>Dizziness</td>
<td>9</td>
<td>3.7</td>
</tr>
<tr>
<td>Asthenia</td>
<td>8</td>
<td>3.2</td>
</tr>
<tr>
<td>Back pain</td>
<td>8</td>
<td>3.2</td>
</tr>
<tr>
<td>Rash</td>
<td>8</td>
<td>3.2</td>
</tr>
<tr>
<td>Pruritis</td>
<td>8</td>
<td>3.2</td>
</tr>
<tr>
<td>Nausea</td>
<td>7</td>
<td>2.8</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>6</td>
<td>2.4</td>
</tr>
<tr>
<td>Vomiting</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>Rectal disorder</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>Injection site oedema</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>Myalgia</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>Pharyngitis</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Sinusitis</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>Chills</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Cough increased</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Eye disorder</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Injection site pain</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Malaise</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Sweating</td>
<td>3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*COSTART: coding symbols for thesaurus of adverse reaction terms*
2.5.4 Management of adverse events

APH, intrapartum haemorrhage, and PPH: The infusion was to be stopped immediately. Management was to be controlled by the attending obstetrician. Procoagulant therapy was to be administered for the usual indications. Should a woman have suffered from two or more episodes of PPH related to a consumptive coagulopathy then the infusion was not be recommenced; to date this has not been an issue.

Other bleeding complications: The infusion was to be stopped immediately. Management will be controlled by the attending obstetrician. Internal medicine consultation was to occur.

Were the infusion stopped in response to any bleeding complications, given the short half life of the medication, one would expect almost complete washout of the drug within 1-1.5h. APC promotes bleeding by inactivating Va and VIIIa and reducing fibrinolysis by inhibiting PAI-1. Factors V, VIII and PAI-1 are present in fresh frozen plasma (FFP). In the event of major haemorrhage, IL of FFP would have been administered urgently. Further procoagulant management would have been directed by haemostatic tests (PTT, INR, fibrinogen, platelet counts). Prolonged PTT (≥1.5 x upper limit of normal) or prolonged INR (≥1.3) would have prompted additional FFP infusion. Fibrinogen less than 1.5 would have prompted infusion of at least 10U of cryoprecipitate. Platelet counts less than 50 would result in infusion of 5U of random donor platelets.
2.6 Sample size and statistical analysis

The unit of analysis will be women (not babies) and twenty women will be recruited to each cohort. Descriptive statistics (means, standard deviation, medians, 95% CI, and %) will be calculated and compared with historical and contemporaneous controls within the Pre-eclampsia Integrated Estimate of Risk Score (PIERS) database. For the antenatal pre-eclampsia subtrial, we will examine data for all women in the PIERS database who meet the antenatal entry criteria but were not enrolled in the trial, and compare pregnancy prolongation from admission. The same approach will be taken for the women who receive postnatal rhAPC, where the outcome will be 'days alive and free of illness' (free of illness defined by the absence of any Canadian Hypertension Society (CHS) adverse features) (183). The PIERS database has existed since December 2002, during which time there have not been any substantive changes in either the management of women with pre-eclampsia in the Children's and Women's Health Centre of British Columbia (CWHCBC), or in the management of their infants in the neonatal intensive care unit (NICU). These women can be considered contemporaneous for the purposes of the trial.

The influence of rhAPC on the basic science markers was examined non-parametrically. The statistical tests used were Mann Whitney U, Wilcoxon, and Kruskal-Wallis ANOVA (with Dunn's post hoc test (including Bonferroni correction)) analyses. These results will be further used to determine the sample size of a planned RCT of rhAPC in pre-eclampsia.
2.7 Experimental plan of basic science

Samples were drawn for thromboelastogram (TEG), ELISA, Western immunoblotting, Luminex, real-time RT-PCR and flow cytometry, etc., with each clinical sample outlined above. These experiments have provided mechanistic insights into the modification (if any) of the processes of pre-eclampsia in response to rhAPC.
Chapter 3

Recombinant human activated protein C (rhAPC) may act as a potent modifier of haemostasis in women with pre-eclampsia
3.1 Hypothesis

The effects of rhAPC on coagulation parameters may be monitored by thromboelastography (TEG). Moreover, rhAPC may influence TEG indices and plasma haemostatic parameters, reflecting its action as both an anticoagulant and a fibrinolytic agent in women with pre-eclampsia.

3.2 Materials and methods

3.2.1 Study cohorts

With approval obtained from the Research Ethics Committee and informed consent, we have studied 22 patients admitted to the delivery suite with the diagnosis of severe pre-eclampsia, seven of them (Cohort 1) are antenatal, seventeen of them (Cohort 2) are postnatal (two patients were enrolled into both of these cohorts).

3.2.2 Thrombelastography (TEG)

TEG assessments were performed on a 5000 series Thrombelastograph Haemostasis Analyzer (Hemoscope Corporation, Niles, IL). The sample was handled and processed according to the guidelines laid out in the Thrombelastograph® user manual (203). Level I and Level II controls were run and confirmed to be within specified limits before the real tests.

As routine antenatal/postnatal blood tests, an additional 3 ml of sodium-citrated whole
blood was taken and transported to the laboratory from the Delivery Suite or maternity ward within 30 min of venipuncture. Citrated whole blood (340 μl) and calcium chloride (20 μl of 0.2 M) were placed in a rotating cup warmed to 37°C. The cup oscillates 4°45' in either direction every 4.5 s (Fig 3.1). A pin was suspended in the cup of blood from a torsion wire that is electrically transduced to a computer monitor, and the rotational motion was transferred to the pin as fibrin strands formed between the wall of the cup and the pin. The electronic device enabled the characteristic tracing to be recorded in the computer.

Figure 3.1 A schematic diagram of the TEG® analyzer technology *.

(*The source of the original figure:
http://www.haemoscope.com/technology/teg_analyzer.html)

The resulting haemostasis profile is a measure of the time it takes for the first fibrin strand to be formed, the kinetics of clot formation, the strength of the clot (in shear elasticity units of dyn/cm²) and dissolution of clot
Since its first description in 1948, the thrombelastograph (TEG) has been successfully used in the point-of-care assessment of haemostasis. TEG monitors haemostasis as a complete dynamic process, compared with isolated conventional coagulation screens (204, 205). TEG measures the viscoelastic properties of blood as it is induced to clot under a low shear environment resembling sluggish venous flow. Initially, a straight-line trace is produced. However, as the blood in the cup clots through fibrin-platelet bonds, the motion of the rotating cup is transmitted to the pin. As the clot retracts or lyses, transfer of cup motion is diminished. Thus, TEG can measure the in vitro life of a clot; the time to initial clot formation, then evaluate a developing clot (acceleration phase, strengthening and retraction), and, finally, clot lysis (Fig 3.2) (206).
Figure 3.2 A schematic diagram of the thromboelastography trace* (206)

(*The source of the original figure: Fig 1; Salooja N, Perry DJ. Thrombelastography. Blood Coagul Fibrinolysis. 2001; 12: 327-37.)

Reaction (R) time is the period of time of latency from the time that the blood was placed in the TEG cup until the initial fibrin formation. K time is a measure of the speed to reach a certain level of clot strength (amplitude=20 mm) from the beginning of clot formation. Alpha (a) angle measures the rapidity of fibrin build-up and cross-linking (clot strengthening). MA, or Maximum Amplitude, is a direct function of the maximum dynamic properties of fibrin and platelet bonding. MA represents the ultimate strength of the fibrin clot. The whole blood clot lysis index is the amplitude 60 min after the MA is achieved (A60) as a percentage of MA. In comparison with a normal trace (A), fibrinolysis (B) is associated with a prolonged r time, reduced MA and a reduced A60, while hypercoagulability (C) is associated with a short r time and an increased MA.
3.2.3 Enzyme-linked immunosorbent assays (ELISA)

4.5 ml of whole blood were collected by venipuncture tube with sodium citrate at 0.5 hr before rhAPC infusion, 4 hr, 28 hr, 52 hr and 76 hr of infusion, and 4 hr after infusion was stopped. The blood samples were immediately centrifuged at 4000rpm (Eppendorf Centrifuge 5810R, Swing-Bucket Rotor, Hamburg, Germany) for 20 minutes at 4°C; and the plasma was stored at -80°C until tested. Plasma levels of TAT, F1+2, PAI-1 were measured in duplicate with the use of commercial kits (TAT and F1+2: Enzygnost, Behringwerke, Germany; PAI-1: ADI, Greenwich, CT) according to manufacturers’ protocol.

In brief, the ELISA procedure (mainly performed at room temperature) was as follows: the wells of a 96-well plate were precoated overnight with 100 μl of coating antibody, diluted 1:100 with coating buffer. Thereafter the wells were washed 4 times with 400 μl of phosphate buffered saline (PBS) containing 0.05% Tween 20 (washing buffer) and then blocked with 200 μl of blocking buffer (1% Bovine serum albumin (BSA) in PBS) for 1 hour on a shaker. After washing the plate four times with washing buffer, 100 μl of plasma samples (diluted in different ratios with assay dilution buffer) or standards were pipetted into the wells. The plate was incubated overnight on a shaker in the cold room (4°C). After washing the plate four times with washing buffer, 100 μl of biotinylated antibody (diluted in different ratios with assay dilution buffer) was pipetted into the wells and incubated 2 hours on the shaker. After washing the plate, the wells were incubated 30 minutes on a shaker with 100μl of streptavidin-HRP conjugate. Then the plate was washed for the last four times with washing buffer and incubated with 100μl of tetramethylbenzidine substrate.
solution. The reaction was stopped around 30 minutes later with 100μl of stop solution (0.5M H₂SO₄). The absorbance per well was measured at 450 or 490 nm by a Wallac 1420 VICTOR3 Multilabel Counter (Perkin-Elmer Wallac). Sample concentrations were calculated using the respective standard calibration lines.

3.2.4 Statistical analysis

The statistical analysis was calculated using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). The significance of differences between the baseline and other infusion time points was assessed by means of a one-way analysis of variance for nonparametric values (Kruskal-Wallis test) and a following multiple-comparison post test (Dunnett's test). As biological data were not likely to follow Gaussian distribution, therefore, median were compared rather than means. A value of p < 0.05 was considered significant.

Kruskal-Wallis test

This is a non-parametric method for testing equality of population medians among groups. Intuitively, it is identical to a one-way analysis of variance (ANOVA) with the data replaced by their ranks and it is an extension of the Mann-Whitney U test to three or more groups. Since it is a non-parametric method, the Kruskal-Wallis test does not assume a normal population and does not require homoscedasticity (population variabilities among groups do not have to be equal), unlike the regular ANOVA. If P value is small, the possibility that the data sets do not differ ('difference coincides') will be rejected. Although it does not mean that every group differs from other groups, but at least there would be one
3.3 Results

3.3.1 Clinical characteristics of the study patients

To date, 22 women have been enrolled in the trial. The characteristics of the patients before treatment are summarized in Table 3.1; the demographic data collected included the patient's date of birth, race, sex, and ethnic origin (if known).
Table 3.1 Demographic and Baseline Clinical Characteristics of the Study Patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Women with Antenatal PET</th>
<th>Women with Postnatal PET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 7)</td>
<td>(n = 17)</td>
</tr>
<tr>
<td>Age — yr</td>
<td>30 (24-38) *</td>
<td>31 (21-43)</td>
</tr>
<tr>
<td>Height — cm</td>
<td>162 (158-173)</td>
<td>165 (155-178)</td>
</tr>
<tr>
<td>Weight — kg</td>
<td>73.5 (49-113)</td>
<td>78.5 (52-102)</td>
</tr>
<tr>
<td>Systolic blood pressure — mm Hg</td>
<td>130 (100-165)</td>
<td>120 (100-162)</td>
</tr>
<tr>
<td>Diastolic blood pressure — mm Hg</td>
<td>85 (70-110)</td>
<td>80 (60-114)</td>
</tr>
<tr>
<td>Primigravida — no. (%)</td>
<td>42.9</td>
<td>35.3</td>
</tr>
<tr>
<td>Current smoker— no. (%)</td>
<td>28.6</td>
<td>23.5</td>
</tr>
<tr>
<td>Ever married — no. (%)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Race or ethnic group — no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>42.9</td>
<td>41.2</td>
</tr>
<tr>
<td>Other or unknown</td>
<td>57.1</td>
<td>58.8</td>
</tr>
<tr>
<td>Other medications — no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>100</td>
<td>47.1</td>
</tr>
<tr>
<td>Oral antihypertensives</td>
<td>100</td>
<td>64.7</td>
</tr>
<tr>
<td>Short-acting antihypertensives for</td>
<td></td>
<td></td>
</tr>
<tr>
<td>actually elevated blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO4</td>
<td>57.1</td>
<td>76.5</td>
</tr>
<tr>
<td>Aspirin (ASA)</td>
<td>14.3</td>
<td>5.9</td>
</tr>
<tr>
<td>Narcotics/Opiates</td>
<td>28.6</td>
<td>35.3</td>
</tr>
<tr>
<td>Others</td>
<td>14.3</td>
<td>35.3</td>
</tr>
<tr>
<td>Adverse maternal outcomes — no. (%)</td>
<td>0</td>
<td>5.9 **</td>
</tr>
<tr>
<td>BW — g</td>
<td>545 (425-820)</td>
<td>1785 (545-3865)</td>
</tr>
<tr>
<td>GA — wk ***</td>
<td>25.3 (25.0-26.1)</td>
<td>33.1 (25.1-41.6)</td>
</tr>
<tr>
<td>WBC — x 10^9/L</td>
<td>12.8 (6.5-17.9)</td>
<td>11.7 (6.9-16.5)</td>
</tr>
<tr>
<td>WBC &gt; 10 x 10^9/L — no. (%)</td>
<td>71.4</td>
<td>64.7</td>
</tr>
<tr>
<td>Platelets — x 10^9/L</td>
<td>232.1 (101.0-285.0)</td>
<td>201.5 (103.0-262.0)</td>
</tr>
<tr>
<td>Platelets &lt; 100 x 10^9/L — no. (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ALT — U/L</td>
<td>19.5 (5.0-321.0)</td>
<td>25.5 (3.0-363.0)</td>
</tr>
<tr>
<td>ALT &gt; 40 U/L — no. (%)</td>
<td>28.6</td>
<td>41.2</td>
</tr>
<tr>
<td>AST — U/L</td>
<td>54 (12-278)</td>
<td>38 (17-304)</td>
</tr>
<tr>
<td>AST &gt; 40 U/L — no. (%)</td>
<td>28.6</td>
<td>35.3</td>
</tr>
<tr>
<td>Uric Acid — μM</td>
<td>423 (295-546)</td>
<td>375 (271-592)</td>
</tr>
<tr>
<td>Uric Acid &gt; 345 μM — no. (%)</td>
<td>57.1</td>
<td>64.7</td>
</tr>
<tr>
<td>LDH — U/L</td>
<td>655 (342-1642)</td>
<td>609 (450-1511)</td>
</tr>
</tbody>
</table>

* Values are expressed as median and range
** One case had postpartum haemorrhage
*** Antepartum cohort: gestational age at rAPC infusion started; Postpartum cohort: gestational age at delivery

PET, pre-eclampsia; BW, birth weight; GA, gestational age; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase
3.3.2 TEG indices

The effects of rhAPC on TEG indices are summarized in Table 3.2 and Figure 3.3. The TEG yields data that characterize clot formation and retraction, analyzed parameters included the R-time (R), K-time (K), Angle (α) and MA. R signifies the time to initial fibrin formation and can be reduced by hypercoagulable conditions. The K time and Angle represent the dynamics of clot formation and are both affected by the availability of fibrinogen, which determines the rate of clot build-up, and to a lesser extent, by platelets. The MA is a measure of maximum strength of the clot, it is primarily determined by platelet and fibrinogen (205).
<table>
<thead>
<tr>
<th></th>
<th>Baseline (-0.5 hr)</th>
<th>4 hr</th>
<th>28 hr</th>
<th>52 hr</th>
<th>76 hr</th>
<th>4 hr post-infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AP (n = 7)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-time (min)</td>
<td>5.4 (3.5-7.5)</td>
<td>6.5</td>
<td>6.4</td>
<td>5.0</td>
<td>6.1</td>
<td>5.4 (3.5-9.1)</td>
</tr>
<tr>
<td>K-time (min)</td>
<td>1.3 (1.1-1.4)</td>
<td>1.7</td>
<td>1.4</td>
<td>1.3</td>
<td>1.3</td>
<td>1.4 (1.1-1.9)</td>
</tr>
<tr>
<td>Angle (deg)</td>
<td>71.8 (69.0-74.6)</td>
<td>66.2</td>
<td>67.6</td>
<td>68.9</td>
<td>71.1</td>
<td>68.1 (51.2-73.6)</td>
</tr>
<tr>
<td>MA (mm)</td>
<td>68.1 (62.9-72.4)</td>
<td>69.4</td>
<td>66.2</td>
<td>67.8</td>
<td>66.0</td>
<td>68.2 (65.7-71.6)</td>
</tr>
<tr>
<td><strong>PP (n = 17)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-time (min) *</td>
<td>6.4 (2.3-9.6)</td>
<td>6.4</td>
<td>8.3</td>
<td>10.3</td>
<td>10.3</td>
<td>7.8 (5.8-10.3)</td>
</tr>
<tr>
<td>K-time (min)</td>
<td>1.7 (1.2-4.3)</td>
<td>1.7</td>
<td>1.6</td>
<td>3.0</td>
<td>1.9</td>
<td>1.5 (1.0-2.3)</td>
</tr>
<tr>
<td>Angle (deg)</td>
<td>66.5 (44.9-73.0)</td>
<td>66.7</td>
<td>65.2</td>
<td>52.7</td>
<td>64.1</td>
<td>68.4 (49.4-74.7)</td>
</tr>
<tr>
<td>MA (mm)</td>
<td>66.8 (44.6-74.4)</td>
<td>69.4</td>
<td>70.8</td>
<td>67.4</td>
<td>72.1</td>
<td>71.4 (57.6-77.4)</td>
</tr>
</tbody>
</table>

AP: Antepartum    PP: Postpartum
Values are expressed as median and range
* p< 0.05, Kruskal-Wallis one-way ANOVA
In this study, R-time (clotting time) in postnatal patients was significantly delayed by rhAPC ($p = 0.0454$, Kruskal-Wallis analysis of variance; 28hr: 29.7%; 52hr and 72hr: 60.9%). K-time levels (52hr and 76hr) tended to be increased in this cohort, meanwhile, Angle (28hr, 52hr and 76hr) was inclined to be decreased, but both of them did not reach statistical significance. No significant trend for these indices was detected in antepartum patients nevertheless. At the same time, there was no significant change on MA during therapy with rhAPC in both antenatal and postnatal cohort.

### 3.3.3 Haemostatic parameters measured by ELISA

The effects of rhAPC on plasma TAT, F1+2 and PAI-1 in women with severe pre-eclampsia are shown in Figure 3.4. In the present study, we have shown that the infusion of rhAPC resulted in significant decreases in postnatal F1+2 (4hr: 2.9%; 28hr: 22.4%; $p = 0.0218$, Kruskal-Wallis analysis of variance) and PAI-1 (4hr: 23.6%; 28hr: 69.0%; $p = 0.0078$, Kruskal-Wallis analysis of variance) concentration respectively. TAT levels also tended to be down-regulated in this cohort (4hr: 10.1%; 28hr: 31.2%), but they did not reach
statistical significance \( (p=0.0628, \text{Kruskal-Wallis analysis of variance}) \). For these data, it is unclear what the normal resolution of pre-eclampsia-related changes is in the immediate puerperium. We have no control data to examine, at this time. However, no similarly significant changes were observed in antepartum patients.
Figure 3.4 (A, B, C) Recombinant human activated protein C (rhAPC) decreases plasma TAT, F1+2 and PAI-1 in women with severe pre-eclampsia. (A) TAT, (B) F1+2, (C) PAI-1. Left green boxes indicate results for antenatal patients (n = 7) and right red boxes for postnatal ones (n = 17). The boundary of the box indicates the 25th and 75th percentiles and the line within the box marks the median. The whiskers (error bars) indicate the minimum and maximum values.

Note: AP: Antepartum; PP: Postpartum.

-0.5h: baseline, 0.5hr before the infusion began; +4h: 4h after the rhAPC ended

KW: Kruskal-Wallis one-way ANOVA test

D: Dunnett’s post test, compared with baseline value (-0.5 hr)
3.4 Discussion

3.4.1 RhAPC influences TEG indices as an anticoagulant agent

TEG has been used successfully to guide the transfusion of blood components in hepatic and, more widely, in cardiac surgery. Recently, certain applications also indicate that TEG is likely to become a very valuable tool in pharmaceutical monitoring and clinical investigations. The graph tracing and corresponding values allow for targeting the specific haemostatic blood component(s) required to treat coagulopathy. For example, when the reactive time (R) parameter is prolonged, such as with anticoagulants, administration of FFP may be warranted. Since the clot formation time (K) measures the time it takes for the strength of the clot to reach a certain level, a prolongation would indicate a lack of fibrinogen. The Angle (α), reflecting the rapidity of the formation of the clot, also reflects decreased fibrinogen levels when it is narrow. Maximum amplitude (MA), or width of the thromboelastography tracing, is affected by the platelet number and function and to a lesser extent by fibrinogen level. K time, Angle and MA, are correlated because of the interaction between fibrinogen and platelets, which together form the fibrin-platelet bonding to produce the final clot. However, no studies concerning TEG have been performed during rhAPC infusions.

The maintenance of haemostasis involves a balance between thrombosis and fibrinolysis, which is provided by the mutual activity of pro-coagulant proteins, natural anticoagulants and the regulator proteins of the fibrinolytic system. In pre-eclampsia, these two opposing processes of anticoagulation and haemostasis must be managed carefully and modified with respect to the patient's haematological status and desired haemostatic outcome. The results of
Xie's toxaemia study show that in early-onset pre-eclampsia (EoPET), two major TEG parameters, R-time and K-time are significantly shortened compared with matched normal pregnancy controls (NPC) (p<0.05) (207).

Therefore, during the infusion of rhAPC, careful attention must be paid to monitoring haemostasis. Our observations are the first to provide evidence supporting the use of TEG as a practical sensitive method in haemostasis management in patients receiving rhAPC. It demonstrates that rhAPC has a significant anticoagulant effect in women with postnatal pre-eclampsia as shown by a change in several TEG indices, R-time, K-time, Angle (α), without an alteration in the MA. It may reflect that rhAPC could effectively decrease the enzymes mediating clotting and the fibrinogen levels in women with pre-eclampsia. But the number and function of platelets in pre-eclamptic patients, both antepartum and postpartum, may not be influenced by rhAPC.

3.4.2 RhAPC influences plasma haemostatic parameters as an anticoagulant and fibrinolytic

In this study, the second major finding of interest was that our ELISA results on plasma haemostatic factors were compatible with anticoagulant effect of rhAPC in TEG indices. F1+2 and TAT levels were both reduced around 30% in postnatal patients at 28 hour after rhAPC infusion, though the decreased trend of TAT did not reach statistical significance. During the same period, the levels of PAI-1 in the same cohort were significantly decreased 70% (Fig. 3.4). PAI-1 didn’t reach statistical significance in antepartum patients, however,
they clearly showed a decreased trend. We consider sample size could be a major bias factor.

As we mentioned above, pregnancy itself is a hypercoagulable state, at least in part, due to the physiological changes in the coagulation and fibrinolytic systems. There is growing evidence implicating alterations in blood coagulation and fibrinolysis having an important role in the pathogenesis of pre-eclampsia. Hypercoagulability may be related with various characteristics seen in pre-eclampsia, such as consumptive thrombocytopenia, fibrin deposition in a diversity of organs, and placental hypoperfusion, ischaemia, or infarction. As a result of increased thrombin generation, TAT complex and F1+2 levels increase in normal pregnancy, and even more in pre-eclampsia (121, 208). PAIs, members of the serpin (serine protease inhibitor) family of protease inhibitors (208), suppress t-PA function and limit plasmin generation. Through this mechanism PAIs block fibrinolysis. PAI-1 is synthesized at high levels by the endothelium, but is also secreted by other tissue types, such as adipose tissue (209-212), whereas PAI-2 is known as the “placental PAI” due to its high levels of expression at this site (213). The binding affinity of PAI-1 to t-PA is approximately 1000-fold that of PAI-2 (209, 214), indicating that PAI-1 plays a more critical role in the regulation of fibrinolysis. Dhainaut JF et al tested these three biomarkers above in the PROWESS trial, and they also found that the anti-thrombotic and profibrinolytic activities of rhAPC were demonstrated through significantly decreased TAT, F1.2 and PAI-1 levels as compared to placebo treatment in patients with severe sepsis (215).
In conclusion, rhAPC may influence postnatal TEG indices and plasma haemostatic parameters as an anticoagulant; it may also stimulate fibrinolysis activity, at least in part, by its ability to form a complex with or to degrade PAI. In the future, control postpartum group with resolving pre-eclampsia need to be investigated, to determine what changes may be independent of rhAPC. At the same time, we should recognize that the results in antepartum patients are less compelling, but are still supportive enough to encourage us to complete the trial on the 20 women.
Chapter 4

The effects of rhAPC on circulating cytokines in the women with severe pre-eclampsia
4.1 Hypothesis

The therapeutic mechanisms of rhAPC to severe pre-eclampsia might partly depend on its regulatory effects on the activities of maternal circulating cytokines.

4.2 Materials and methods

4.2.1 Study cohorts

See Chapter 3 (3.2.1 Study cohorts)

4.2.2 Multiplexed fluorescent microsphere immunoassay (Luminex assay)

4.5 ml of whole blood were collected by venipuncture tube without sodium citrate or ethylenediamine tetraacetic acid (EDTA) at 0.5 hr before rhAPC infusion, 4 hr, 28 hr, 52 hr and 76 hr following the start of infusion, and 4 hr after the infusion stopped. The blood samples were centrifuged after clotting at 4000rpm in a centrifuge (Eppendorf Centrifuge 5810R, swing-out rotor, Hamburg, Germany) for 20 minutes at 4°C; then serum was removed from the red cells and stored at -80°C until tested. Serum levels of TNF-α, interferon (IFN)-γ, IL-1β, IL-2, IL-6 and IL-10 were evaluated by means of a multiplexed fluorescent microsphere immunoassay, using the Luminex 100 System (Luminex Corporation, Austin, TX). Multiplex bead kits were purchased from the LINCO Research, Inc. (St. Charles, MO, USA), the measurements were performed in duplicate according to manufacturer’s protocol.

The “Luminex assay” is based on conventional sandwich assay technology. The
antibody specific to each cytokine was covalently coupled to Luminex microspheres, and which were uniquely labelled with a fluorescent dye. The microspheres were incubated with standards, controls, and samples (25 μl) in a 96-well microtiter filter plate overnight at 4°C. After incubation, the plate was washed to remove excess reagents, and detection antibodies, in the form of a mixture containing each of the six antibodies, were added. After 30 min incubation at room temperature, streptavidin-phycoerythrin was added for an additional 30 min. After the final wash step, the beads were resuspended in buffer and read on the Luminex 100 instrument to determine the concentrations of the cytokines of interest. Results were reported as the average of the replicates.

4.2.3 Statistical analysis

The statistical analysis was calculated using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). The significance of differences between the baseline and other infusion time points was assessed by means of a one-way ANOVA for nonparametric values (Kruskal-Wallis test) and a multiple-comparison test (Dunn's test). A value of p < 0.05 was considered significant.

4.3 Results

4.3.1 Clinical characteristics of the study patients

The characteristics of the patients before treatment are summarized in Table 3.1.
4.3.2 Maternal circulatory cytokines measured by Luminex assay

The effects of rhAPC on serum TNF-α, IL-1β, IL-2, IL-6, IL-10 and IFN-γ in women with severe pre-eclampsia are shown in Figure 4.1. In the present study, we have shown that the infusion of rhAPC resulted in significant decreases in postnatal IL-6 (4hr: 18.8%; 28hr: 49.8%; p = 0.0319, Kruskal-Wallis analysis of variance) and IL-10 (4hr: 30.7%; 28hr: 36.6%; p = 0.0317, Kruskal-Wallis analysis of variance) concentration respectively. TNF-alpha, IL-1β and IL-2 levels also tended to be reduced in this cohort, but they did not reach statistical significance. No similarly significant phenomena, however, have been observed in antepartum patients. In addition, there were even higher expression levels of TNF-α and IL-10 observed in antenatal cohort (Figure 4.2), though the differences were not significant. Moreover, no significant trend was detected for IFN-γ in either the antepartum or postpartum cohort.
Luminex

(A) IFN-gamma

(B) IL-2

(C) TNF-alpha

(D) IL-1beta

(E) IL-6

(F) IL-10

Infusion Time (hr)

Infusion Time (hr)

Infusion Time (hr)

Infusion Time (hr)

Infusion Time (hr)

Median IFN-γ (pg/ml)

Median IL-2 (pg/ml)

Median TNF-α (pg/ml)

Median IL-1β (pg/ml)

Median IL-6 (pg/ml)

Median IL-10 (pg/ml)

KWP = 0.0319

KWP = 0.0317
Figure 4.1 (A, B, C, D, E, F). RhAPC decreased serum TNF-α, IL-1, IL-2, IL-6 and IL-10 in postnatal pre-eclampsia. (A) IFN-γ, (B) IL-2, (C) TNF-α, (D) IL-1β, (E) IL-6 and (F) IL-10. Left green boxes indicate results for antenatal patients (n = 7) and right red boxes for postnatal ones (n = 17). The boundary of the box indicates the 25th and 75th percentiles and the line within the box marks the median. The whiskers (error bars) indicate the minimum and maximum values.

Note: AP: Antepartum; PP: Postpartum
-0.5h: baseline, 0.5hr before the infusion began; +4h: 4h after the rhAPC ended
KW: Kruskal-Wallis one-way ANOVA test

Luminex

Figure 4.2 (A, B). The effects of rhAPC on serum levels of TNF-α and IL-10 in women with antepartum pre-eclampsia. (A) TNF-α, (B) IL-10. The boundary of the box indicates the 25th and 75th percentiles and the line within the box marks the median. The whiskers (error bars) indicate the minimum and maximum values.

Note: AP: Antepartum; PP: Postpartum
-0.5h: baseline, 0.5hr before the infusion began; +4h: 4h after the rhAPC ended
4.4 Discussion

Clinical and basic researches have demonstrated that late pregnancy is characterized by mild maternal systemic inflammatory changes (151, 155). There is evidence of widespread activation of innate (not ‘specific’) elements of the maternal immune system, including the activation of endothelial system (216), stimulation of circulating leukocytes (151), and the associated acute phase response (139). Pre-eclampsia is accompanied by a more intense systemic inflammatory response (151) and it is thought that exaggeration of this response causes the distinguishing characteristics of this disease (155).

Mammalian immune systems play important roles in resistance to infections and cancer. Immunity has traditionally been classified into two major branches: a more primitive innate immune system, and an acquired or adaptive immune system of vertebrates. The innate immune system comprises the cells (granulocytes, monocytes, dendritic cells, NK cells, mast cells, eosinophils and basophils, etc.) and the complement system (a biochemical cascade composed of many plasma proteins that helps the antibodies or other cells to mark or clear pathogens). The innate immune system defends the host from infection by other organisms in a rapid and non-specific manner (217). The adaptive immune system is composed of highly specialized, systemic cells (T and B lymphocytes) and processes that eliminate pathogenic challenges. It provides the vertebrate immune system with the ability of antigen recognition and clonal expansion, leads stronger attacks each time the pathogen is encountered, and generates an antigen-specific response.
Among the components of the adaptive immune system, CD4-positive T helper (Th) lymphocytes are very special. They can be divided into two different functional subgroups based on their profile of cytokine production. Type 1 Th (Th1) cells produce IFN-γ, IL-2, and TNF-α, and promote the production of opsonizing and complement-fixing antibodies, NK cell and macrophage activation, antibody-dependent cell cytotoxicity and delayed hypersensitivity. On the other hand, type-2 Th (Th2) cells produce IL-4, IL-5, IL-10 and IL-13, which provide assistance for humoral immune responses, induce B-cell activation and antibody production (216). Wegmann’s group suggested that during successful pregnancy there is a clear bias towards Th2-type cytokines as Th1-type immunity is incompatible with placental formation (217). It has been proposed that women with pre-eclampsia show so many excessive inflammatory changes, and that these are associated with abnormal Th1-type cytokine production toward adverse Th1-dominated immune response (217-221), although the sources and mechanisms of these harmful pro-inflammatory cytokines in the circulation have not been completely elucidated.

4.4.1 Th1-type cytokines

As discussed above, IFN-γ and IL-2 are two principal cytotoxic Th1 type cytokines (222). Th1 cells and the pathway they dominate are heavily reliant on IFN-γ, and to a lesser extent IL-2 and IL-12.

Interferons are major contributors to the first line of antiviral defense, which by itself is sufficient to make them of great interest, but they exert many other important effects on cells
in addition to inhibiting virus replication. They belong to the network of cytokines that are involved in the control of cellular function and replication and that become actively engaged in host defense during an infection. The interaction of IFNs and the immune system has probably received more attention than any other aspect of IFN activity: by virtue of its mode of production, antigen-specific induction of sensitized T cells, IFN-γ acts from within the immune system. IFN-α and IFN-β are made by macrophages and lymphocytes as well as by other nucleated cells in the organism and act from within or without. IFN-γ released by Th1 cells, recruits leukocytes to a site of infection, resulting in increased inflammation. It also stimulates macrophages to kill bacteria that have been engulfed and is also important in regulating the Th2 response. As IFN-γ is implicated to be central in the regulation of the innate immune response, its exaggerated production can lead to autoimmune disorders.

IL-2 was first identified in 1975 as a growth-promoting activity for bone-marrow-derived T lymphocytes. Since then, the spectrum of IL-2 biological activity has expanded significantly to include direct effects on the growth and differentiation not only of T cells but also of B lymphocytes, NK cells, lymphokine-activated killer (LAK) cells, monocytes, macrophages and oligodendrocytes.

Many studies have shown consistently elevated levels of IFN-γ or IL-2 in women with pre-eclampsia (223, 224), though in our laboratory, Xie’s research has found IFN-γ increased in EoPET compared with NPC groups (207). In the present study, we have demonstrated that the infusion of rhAPC resulted in reduced IL-2 levels during the infusion of rhAPC in the
postnatal cohort, but these levels did not reach statistical significance. No similarly significant phenomena, however, have been observed in antepartum patients. Moreover, no significant trend detected for IFN-γ in either the antepartum or postpartum cohort; however, we remain underpowered to be able to exclude any significant effects at this time.

4.4.2 Pro-inflammatory cytokines

TNF-α, IL-1β and IL-6 are three crucial pro-inflammatory cytokines with multiple functions in normal pregnancy and pre-eclampsia. TNF-α could also be categorized as a Th1-type cytokine, because one of its sources is Type 1 Th (Th1) cell.

TNF-α is an extremely potent peptide cytokine which serves as an endogenous mediator of inflammatory, immune and host defense functions. It participates as an autocrine regulator of its cells of origin and their progenitors, and is implicated as a central paracrine mediator in a diversity of localized responses, not only as a direct effector but also as part of the network of cytokines and other mediators which interact to coordinate and control the responses of both resident and recruited cells at the tissue level. In other words, TNF-α is capable of acting independently and in conjunction with a wide variety of other factors to affect the phenotype and metabolism of cells in every tissue of the body. Macrophages/monocytes are thought to be the cells which contribute most to the local and systemic TNF-α response to bacterial, viral and parasitic organisms and products, although other cell types participate in certain scenarios.
IL-1 is the term for two polypeptides (IL-1α and IL-1β) that possess a wide spectrum of inflammatory, metabolic, physiological, haematopoietic and immunological activities. Although the two forms of IL-1 are distinct gene products, they recognize the same receptor and share biological properties. They belong to a group of cytokines with overlapping biological properties, including TNF and IL-6. These cytokines increase the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes to sites of infection and reset the hypothalamic thermoregulatory center, leading to an increased body temperature which expresses itself as fever. The up-regulated body temperature could assist the body's immune system in fighting infection. IL-1 is therefore called an endogenous pyrogen. IL-1 is produced by a variety of cells (macrophages, monocytes and dendritic cells) in response to infection, microbial toxins, inflammatory agents, products of activated lymphocytes, complement and clotting components.

Similar to IL-1, IL-6 is also one of the most important mediators of fever, stimulating energy mobilization in the muscle and fatty tissue which leads to elevated body temperature. IL-6 is a multifunctional cytokine which is produced by both lymphoid and nonlymphoid cells and regulates immune response, acute-phase reactions and haematopoiesis. IL-6 is secreted by macrophages, endothelial cells, fibroblasts and activated T-helper (Th) cells. It induces the acute-phase response in liver and activates the differentiation of B-cells and their consequent production of immunoglobulin. Unlike TNF-α, IL-1 and IL-2, however, IL-6 does not augment cytokine expression directly or indirectly; its primary effects are to enhance the responses of immune system to other cytokines.
Some previous literature reported increased maternal circulating TNF-α, IL-1β and IL-6 concentrations in women with pre-eclampsia, indicating that three of them are involved in the pathogenesis of this disorder (223-226). Based on the currently tested samples in Xie’s research, serum IL-6 concentration was indeed significantly increased in EoPET group compared with matched NPC (p<0.05) (207). In this study, we found that the infusion of rhAPC resulted in significant decreases in postnatal IL-6 (4hr: 18.8%; 28hr: 49.8%; p =0.0319, Kruskal-Wallis analysis of variance). At the same time, TNF-α and IL-1β levels also tended to be down-regulated in this cohort, but they did not reach statistical significance. No similarly significant phenomena, however, were observed in antepartum patients.

4.4.3 Regulatory (anti-inflammatory) cytokines

The human immune system is regulated by a highly complex and intricate network of control elements and there is a dynamic and ever-shifting balance between proinflammatory cytokines and anti-inflammatory components. IL-10 is one of the most important regulatory cytokines, and is also a major Th2-type cytokine. It is primarily synthesized by CD4-positive Th2 cells, monocytes and B cells. It is a potent inhibitory factor of Th1 cytokines and pro-inflammatory cytokines.

Theoretically, the enhanced levels of Th1-type and pro-inflammatory cytokines observed in pre-eclampsia could be relative with reduced concentration of IL-10. But the reality of cytokine signaling and inflammatory response is not as simple as we supposed. Some
authors reported a reduction in maternal IL-10 concentrations during pre-eclampsia (223-226), however, most studies to date were unable to observe any significant changes (227, 228). Moreover several studies detected elevated IL-10 secretion in this disorder (229, 230), including the preliminary results of Xie’s toxaemia study in our laboratory (207). Of interest, all data from cultured peripheral blood mononuclear cells (PBMCs) showed the reduction in IL-10 concentrations, while studies that tested actual IL-10 levels in maternal serum or plasma generally showed the elevation. It may well reflect a truly reciprocal relationship between the pro-inflammatory and anti-inflammatory cytokines in women with pre-eclampsia, while the nature of the immune response in individual patients depends on the net effect of interactions between these two kinds of molecules over time. In this study, we found that the infusion of rhAPC resulted in significant reduction in postpartum IL-10 (4hr: 30.7%; 28hr: 36.6%; p = 0.0317, Kruskal-Wallis analysis of variance) levels. However, no similarly significant phenomena were detected in antenatal cohort. There were even higher serum levels of IL-10 detected in this cohort, though the elevations were not significant. Thereby we could not conclude that rhAPC has negative impact on the production of cytokines in antenatal pre-eclampsia.

Dhainaut JF et al once investigated the effects of rhAPC on TNF-α, IL-1β, IL-6 and IL-10 in the patients with severe sepsis (215). Their findings demonstrated that levels of IL-6 were reduced more rapidly in rhAPC-treated patients than placebo-control in change from baseline and percent change from base line analyses, although there were no significant differences between rhAPC and placebo group in levels of TNF-α, IL-1, or IL-10. Here, we
consider the possibility that both sepsis and pre-eclampsia are severe conditions with very complicatedly biological dynamics, there are multiple stages or assorted components of response involved in these two disease, so the ignition or alleviation of may be associated with two different pathways and under two different schedule conditions. We have to further identify those candidates of inflammatory activity that can be used to monitor the rhAPC effect on the severe pre-eclampsia in any stages during the whole process. Interestingly, the range of our IL-6 data is nearly ten times less than Dhainaut's results, it may be caused by the difference of laboratory methods (LUMINEX vs. ELISA) or the effects of steroid administered for the promotion of fetal lung maturity in mothers with pre-eclampsia.

In summary, the therapeutic mechanisms of rhAPC to postnatal pre-eclampsia might partly depend on its inhibitory effect on the deleterious Th1 type cytokines and pro-inflammatory cytokines (IL-2, TNF-α, IL-1β and IL-6), while it may also down-regulates the expression of IL-10, a powerful regulatory (anti-inflammatory) cytokine. Therefore, rhAPC may exert the negative impact on most of components within the cytokine network in women with postpartum pre-eclampsia (we still require control postpartum women with resolving pre-eclampsia to determine what changes may be independent of rhAPC). The effect of rhAPC on IL-10 in ante-partum patients needs further evaluation when a larger sample size has been obtained.
Chapter 5

The mechanisms of cell activation in peripheral blood neutrophils during pre-eclampsia and the effects of rhAPC on the neutrophil activation
5.1 Hypothesis

Increased neutrophil activation has been demonstrated in women with pre-eclampsia, and rhAPC inhibits the activation of neutrophils by regulating their anti-inflammatory and anti-apoptotic signaling pathways.

5.2 Materials and methods

5.2.1 Study cohorts

See Chapter 3 (3.2.1 Study cohorts)

5.2.2 Separation of peripheral blood neutrophils

25 ml (5 x 5ml) of whole blood were collected by venipuncture tube with sodium citrate at 0.5 hr before rhAPC infusion, 4 hr and 28 hr after the start of the infusion, and 4 hr after the infusion was stopped. The five blood samples of each time-point were transferred to 50-ml polypropylene tubes (BD Falcon), then Ca\(^{2+}\) - and Mg\(^{2+}\)-free Dulbecco PBS (Gibco, Grand Island, NY) was added to a total volume of ~40ml. After 7ml of 6% Dextran was added to the tube with blood (1:6 ratio), the tube was inverted a few times gently. The mixture was allowed to stand at room temperature for 20–30 min to let red blood cells (RBCs) settle down until red cell margin fell to below 50% of the total volume. Leukocyte-rich plasma (LRP) was transferred into a new Falcon 50ml tube. Then cells were spun down for 7 min at 2500 rpm (Eppendorf Centrifuge 5810R, Swing-Bucket Rotor, Hamburg, Germany) at room temperature. After supernatant was aspirated, the pellet cells were washed with
40ml of PBS. The tube was centrifuged for 5 minutes at room temperature at 3000 rpm, and then after the supernatant was decanted, the cell pellet was dispersed by gentle tapping and flicking of the tube to prevent cell clumping. The cells were resuspended in 20ml of PBS and layered onto 10 ml of Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) in a new Falcon 50ml tube. After centrifugation (Eppendorf Centrifuge 5810R, Swing-Bucket Rotor, 700 x g, room temperature, 20 min, with brake), the neutrophil-enriched cell pellet was kept and washed with PBS for 2 times. After 5ml of 1x RBC Lysis Buffer (0.155M Ammonium Chloride, 0.01M Potassium Bicarbonate and 0.1 M EDTA) was added, the tube was vortexed briefly and incubated for 5-7 minutes, then was quickly filled up with PBS to dilute lysis buffer. The neutrophils was spun down at 3000 rpm and washed 2 times with PBS. Cells were counted using a haemocytometer.

For mRNA analysis, neutrophils were pooled at a density of 10M per vial in 500 ul RNAlater (Qiagen, Valencia, CA), stored at -80°C until used.

For Western blot detection, 450ul cell lysis buffer (PBS, 1%NP40, 0.5%Sodium Deoxycholate, add fresh phenylmethylsulphonylfluoride (PMSF) 1:100, Proteinase Inhibitors 1:100) was added to each 30M cells. The samples were then incubated on ice for 30 min, centrifuged at 12,000 rpm (Eppendorf Centrifuge 5810 R, Fixed-Angle Rotor) for 10 min at 4°C; the supernatant was collected and stored at -80°C.
5.2.3 RNA isolation and reverse transcription

Total RNA was isolated from neutrophils in RNAlater by using the RNeasy kit (Qiagen, Valencia, CA), treated with RNase-free DNase (Qiagen) for an additional 30 min at room temperature to remove DNA contamination, and quantitated by absorbance at 260 nm. The first-strand cDNA reaction was synthesized by ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA) and primed by random hexamers. 9 μl of intact total RNA (containing 0.5-5μg) was used in each 20 μl reverse transcription reaction. The RNA templates were added to a 0.2 ml PCR tube containing 1 μl of random hexamers, 2 μl of 10mM Deoxyribonucleotide triphosphate (dNTP) mix. The RNA was denatured at 65°C for 5 min, and placed on ice for 10 min. Then 4 μl of 5x cDNA synthesis buffer, 1 μl of 0.1M dithiothreitol (DTT), 1 μl of RNaseOUT (40U/μl), 1 μl of DEPC-treated water, and 1 μl of ThermoScript RT (15 units/μl) were added to the RNA/primer mixture and the reaction was carried out at 25°C for 10 min, followed by 50 min at 50 °C in PCR system (Eppendorf, Hamburg, Germany). The RT reaction was terminated at 85°C for 5 min and chilled on ice for 10 min. After 1 μl of RNase H was added, cDNA synthesis reaction was incubated at 37°C for 20 min, then stored at -20°C.

5.2.4 Quantitative Real-Time Reverse-Transcription PCR

Quantitative reverse-transcription PCR (RT-PCR) was performed in an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR green PCR Master Mix kit (Applied Biosystems). Primers targeting exons of the caspase-3, toll-like receptor (TLR)-2, TLR-4, cryopyrin and glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) genes were designed by Primer Express software (Applied Biosystems) and synthesized Invitrogen Life Technologies (Carlsbad, CA) (for sequences, see Table 5.1). GAPDH was used as the reference housekeeping gene, in order to account for the variability in the initial concentration of the total RNA and conversion efficiency of the reverse-transcription. Thermocycling for each reaction was carried out in a final volume of 25 µl containing 200 ng of cDNA, forward and reverse primers at 300 nmol/L final concentration, and 2 x SYBR GREEN PCR Master Mix. After 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. All reactions were done in triplicate. Dissociation curve analysis was done after every run to confirm the primer specificity.

Table 5.1 The sequences of the primer pairs used in RT-PCR:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>human GAPDH-sense</td>
<td>5'-ATGGAAATCCCATCACCATCTTT-3'</td>
</tr>
<tr>
<td>human GAPDH-antisense</td>
<td>5'-CGCCCCACTTGATTGG-3'</td>
</tr>
<tr>
<td>human TLR2-sense</td>
<td>5'-GAATCCTCCAATCAGGCTTCTCTT-3'</td>
</tr>
<tr>
<td>human TLR2-antisense</td>
<td>5'-CCTGAGCTGCCCTTGCA-3'</td>
</tr>
<tr>
<td>human TLR4-sense</td>
<td>5'-GGCATGCTGCTGCTGAGTT-3'</td>
</tr>
<tr>
<td>human TLR4-antisense</td>
<td>5'-GGACGGACACACCAAATGATG-3'</td>
</tr>
<tr>
<td>human cryopyrin-sense</td>
<td>5'-GAAGCGTGCTGAGGAAAG-3'</td>
</tr>
<tr>
<td>human cryopyrin-antisense</td>
<td>5'-TGCCCCGACCCAAACC-3'</td>
</tr>
<tr>
<td>human caspase3-sense</td>
<td>5'-GCCTACAGCCATTCCAT-3'</td>
</tr>
<tr>
<td>human caspase3-antisense</td>
<td>5'-GCGCCCTGACCAT-3'</td>
</tr>
</tbody>
</table>

5.2.5 Preparation of protein samples

Total protein concentrations of clarified homogenates were determined using the Dc assay kit (Bio-Rad, Hercules, CA) employing BSA (Calbiochem, San Diego, CA) as a standard, and samples were further diluted in lysis buffer to a concentration of 0.5 µg/µl and heated in boiling water for 5 min. Control and samples were then loaded onto hand cast
5.2.6 SDS gel electrophoresis and immunoblotting techniques

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (231) using 10% gels in a Bio-Rad Mini Protean 3 system (Hercules, CA). Protein extracts (20 ug) were separated on gels with 140V for 90min in running buffer (0.1% SDS, 1 mM EDTA, 50 mM 3-(N-morpholino) propanesulfonicacid, 50 mM Tris, pH 7.2). Afterwards, they were electroblotted to nitrocellulose membranes (Bio-Rad) with 100V for 100min with blotting buffer (20% methanol, 192 mM glycine, 25 mM Tris, pH 8.3).

Subsequently, membranes were blocked for 1 hour at room temperature in blocking buffer (1x Tris-buffered saline (25 mM Tris, 150mM Sodium Chloride, pH 7.5) containing 0.05% Tween 20 and 5% skim milk). The respective primary antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 and 1.5% skim milk and membranes were incubated with primary antibody overnight at 4°C with gentle agitation.

The primary antibodies used included: a polyclonal mouse anti-human caspase-3 antibody (1:3000) (BD, San Diego, CA); a monoclonal goat TLR-2 antibody (1:500) (R&D Systems, Minneapolis, MN); a polyclonal rabbit TLR-4 antibody (1:500) (Santa Cruz); a monoclonal mouse GAPDH antibody (1:2000) (Santa Cruz).

After washing four times with TBST solution (Tris-buffered saline containing 0.05%
Tween 20) and incubation with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, or goat anti-rabbit, Bio-Rad, Hercules, CA; and donkey anti-goat, Santa Cruz, Santa Cruz, CA) in a concentration of 1:3000 in blocking buffer for 1 hr at room temperature, four further washing steps of 15 min each at room temperature in TBST solution followed. After washing, the blots were probed with the enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer’s instructions and exposed to film. To minimize the problem with non-linearity, series images with different exposure times were taken for each individual protein to ensure that all densitometry was performed on images taken in the linear exposure range, and the maximal signal intensity was close to the maximal dynamic range but not saturated.

5.2.7 Digital imaging

For quantification of band intensities, Western blots were scanned with a high-resolution flatbed CanoScan 600 scanner (Canon Computer Systems, Costa Mesa, CA), and the density of bands was determined using the software Scion Image (Scion Corp., Fredrick, MD).

5.2.8 Statistical analysis

The statistical analysis was calculated using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). The significance of differences between the baseline and other infusion time points was assessed by means of a one-way ANOVA for nonparametric values (Kruskal-Wallis test) and a multiple-comparison test (Dunnett’s test). A value of $p < 0.05$ was considered significant.
5.3 Results

5.3.1 Clinical characteristics of the study patients.

The characteristics of the patients before treatment are summarized in Table 3.1.

5.3.2 Measurement of mRNA levels with real-time RT-PCR

The effects of rhAPC on TLR-2, TLR-4, cryopyrin and caspase-3 mRNA expression of neutrophils in women with severe pre-eclampsia are shown in Figure 5.1. In this study, we have shown that the infusion of rhAPC resulted in significant reduction in postnatal neutrophil TLR-2 (p =0.0002, Kruskal-Wallis analysis of variance; 4hr: 30%; 28hr: 23%; 4hr post-infusion: 56%, p <0.001, Dunnett’s post test), TLR-4 (p<0.0001, Kruskal-Wallis analysis of variance; 4hr: 18%; 28hr: 42%, p<0.001, Dunnett’s post test; 4hr post-infusion: 53%, p<0.001, Dunnett’s post test) and cryopyrin (p<0.0001, Kruskal-Wallis analysis of variance; 4hr: 8%; 28hr: 38%, p<0.001, Dunnett’s post test; 4hr post-infusion: 62%, p<0.001, Dunnett’s post test) mRNA expression respectively. Trends of TLR2, TLR4 and cryopyrin were in the same direction in the underpowered antepartum cohort, although there was no significant difference detected.

At the same time, caspase-3 levels tended to be elevated in the postpartum cohort, but they did not reach statistical significance (p=0.0558, Kruskal-Wallis analysis of variance). Significant up-regulation of caspase-3, however, was observed in antepartum patients (p=0.0323, Kruskal-Wallis analysis of variance; 4hr: 6%; 28hr: 30%; 4hr post-infusion: 45%).
Real-time RT-PCR

Figure 5.1 (A, B, C, D). RhAPC decreased neutrophils TLR-2, TLR-4 and cryopyrin mRNA in women with severe pre-eclampsia, while increased the mRNA expression of caspase-3. (A) TLR-2, (B) TLR-4, (C) Cryopyrin, (D) Caspase-3. Data are represented as scatter dot plot with the line at the median. Left green symbols indicate results for antenatal patients (n = 7) and right red ones for postnatal (n = 17).

Note: AP: Antepartum; PP: Postpartum.
-0.5h: baseline, 0.5hr before the infusion began; +4h: 4h after the rhAPC ended
KW: Kruskal-Wallis one-way ANOVA test
D: Dunnett's post test, compared with baseline value (-0.5 hr)
5.3.3 Measurement of protein levels with Western blotting

The effects of rhAPC on TLR-2, TLR-4 and caspase-3 protein expression of neutrophils in women with severe pre-eclampsia are shown in Figure 5.2. There were no similarly significant effects of rhAPC, however, observed on TLR-2, TLR-4 and caspase-3 protein expression of neutrophils in both antepartum and postpartum patients.
Figure 5.2 (A, B, C). The effects of rhAPC on TLR-2, TLR-4 and caspase-3 protein expression of neutrophils in women with severe pre-eclampsia. (A) TLR-2, (B) TLR-4, (C) Caspase-3. No similarly significant effects of rhAPC have been observed on TLR-2, TLR-4 and Caspase-3 protein expression of neutrophils in both antepartum and postpartum patients. Data are represented as scatter dot plot with the line at the median. Left green symbols indicate results for antenatal patients (n = 7) and right red ones for postnatal (n = 17).

Note: AP: Antepartum; PP: Postpartum.

-0.5h: baseline, 0.5hr before the infusion began; +4h: 4h after the rhAPC ended
5.4 Discussion

Many researchers have postulated that an immune maladaptation may link placental abnormalities with the maternal syndrome of pre-eclampsia (232). This maladaptation is speculated to lead to cellular activation and release of substances that alter maternal endothelial function in a direction that causes vascular leakage, coagulopathy, and hypertension. The innate immune system mainly comprises neutrophils and monocytes. Evidence suggests that neutrophils are activated in both the placental bed (233) and maternal circulation of women with pre-eclampsia (151, 234-237) and that this activation resolves after delivery (238). Neutrophils may contribute to vascular dysfunction as part of a maternal inflammatory response by releasing damaging enzymes from intracellular stores. Toxic oxygen radicals are also released, and these can encourage lipid peroxidation, lysis of endothelial cells and increased vascular permeability and reactivity. In addition to promoting endothelial damage, neutrophils also interact with platelets and coagulation and complement systems, all elements integral to the syndrome of pre-eclampsia. As we mentioned before, the generalized priming and activation of neutrophils have been widely accepted as features of pre-eclampsia, possibly in response to proinflammatory cytokines, such as IL-6 (239, 240). Before these primed and activated cells can mediate vascular damage, they must first undergo adhesion to the endothelial cells. The major adhesion molecules necessary for this recruitment are selectins and integrins. As expression of adhesion molecules is associated with diseases of leukocyte activation, the possibility arises that receptors may also be involved in the pathogenesis of pre-eclampsia. In support of this proposal, circulating concentrations of both selectins and integrins are higher in women with
pre-eclampsia than in those with normal pregnancy (241). Thus, activated neutrophils play a significant role in the vascular endothelial pathophysiology in this disorder of pregnancy.

It became clear recently that cells of the innate immune system are far less nonspecific than previously assumed, as they were shown to express a series of receptors known as pattern-recognition receptors (PRRs) that collectively recognize carbohydrate, lipid, peptide and nucleic-acid structures (pathogen-associated molecular patterns, PAMPs) that are broadly expressed by different groups of microorganisms (216). The Mammalian Toll-like receptor (TLR) family includes at least 12 membrane proteins, they all trigger innate immune responses through nuclear factor-κB (NF-κB)-dependent and interferon-regulatory factor (IRF)-dependent signaling pathways. They are exact PRRs and central components of the innate immune system (242, 243).

Although TLRs are an important system for microbial sensing, they are not the only PRRs with this function. Components of viruses or bacteria that penetrate into the cytoplasm are recognized by cytosolic receptors, through which they cause cytokine production and cell motivation (244, 245). These cytosolic receptors appear to be divided into two subgroups: the NLR family (nucleotide-binding oligomerization domain (NOD)-like receptor family), which includes at least 23 members that are either NALPs (NACHT-, LRR- and pyrin-domain-containing proteins) or NOD receptors; and a family of receptors that have an RNA-helicase domain connected to two caspase-recruitment domains (CARDs). NALP3 (cryopyrin) has been shown to form the assembly of the inflammasome, a crucial part of the
innate immune response and a cytosolic complex of proteins that activates caspase-1 activation to promote the processing and secretion of pro-inflammatory cytokines IL-1β and IL-18, with NALP1 and NALP2.

In addition to be the proximal sensor of cellular stress and danger signals, researchers have provided insight that the pathophysiological mechanisms underlying pre-eclampsia may also involve TLR-2 and TLR-4 (246). Using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot techniques, therefore, we investigated the effects of rhAPC on transcription of mRNA and protein expressions in peripheral blood neutrophils during pre-eclampsia, for TLR-2, TLR-4 and cryopyrin (NALP3) (innate immune response to danger signals) and caspase-3 (pro-apoptotic). Caspase-3 is known as a protease, which plays essential roles in apoptosis and inflammation. Spontaneous neutrophil apoptosis is retarded both in normal and, more so, in pre-eclamptic pregnancies (247).

The results from our study show that the infusion of rhAPC resulted in significant reduction in postnatal neutrophil TLR-2, TLR-4 and cryopyrin mRNA expression respectively. It compares with the data of Fang Xie, in our laboratory: TLR-2 and -4 gene expressions were significantly increased in EoPET (p<0.05) compared to matched NPC; EoPET was also associated with elevated cryopyrin mRNA expression (p<0.05) (207). At the same time, significant up-regulation of caspase-3, however, was observed in antepartum patients. Therefore, rhAPC may repress the activation of neutrophils in postnatal pre-eclampsia through TLR-2, -4 and cryopyrin signaling pathways, it may also promote of
human neutrophils apoptosis via caspase-3, especially in women with antenatal pre-eclampsia. However, we should admit that further investigation is needed to examine what effects may be independent of rhAPC.
Chapter 6

Summary
6.1 Aims

We have postulated that rhAPC will safely modify the pathophysiology of pre-eclampsia, permitting the safe prolongation of pregnancies complicated by severe early-onset disease and the rapid resolution of severe postnatal disease, without a significant increase in bleeding complications.

The overall hypothesis will be proved by clinical observations, laboratory testing and basic science investigations. This thesis has focussed on the basic science part and we have tested our hypothesis by: 1) determining whether rhAPC may act as is a potent modifier of haemostasis in women with pre-eclampsia; 2) identifying the effects of rhAPC on temporal changes of proinflammatory and regulatory cytokine activities in the serum of women with pre-eclampsia; 3) investigating the basic mechanisms of cell activation in peripheral blood neutrophils during pre-eclampsia.

6.2 Conclusions

6.2.1 The effects of rhAPC in women with postnatal pre-eclampsia

During the infusion of rhAPC, careful attention must be paid to monitoring haemostasis. Our observations are the first to provide the evidence supporting that TEG may be a practical sensitive method of managing haemostasis in patients receiving rhAPC, and rhAPC has a significant anticoagulant effect in women with postnatal pre-eclampsia. This anticoagulant effect was demonstrated by the change in several major TEG parameters, R-time, K-time,
Angle (α), but not in MA. Therefore, it may reflect that rhAPC could effectively decrease the enzymes mediating clotting and the fibrinogen levels in women with pre-eclampsia. But the number and function of platelets in pre-eclamptic patients, both antepartum and postpartum, may not be influenced by rhAPC.

In this study, the second major finding of interest was that our ELISA results on plasma haemostatic factors were compatible with anticoagulant effect of rhAPC in TEG indices. F1+2 and TAT levels were both reduced around 30% in post-natal patients at 28 hour after rhAPC infusion, though the decreased trend of TAT did not reach statistical significance. During the same period, rhAPC may also stimulate fibrinolysis activity, at least in part, by its ability to form a complex with or to degrade PAI.

Thirdly, we found the therapeutic mechanisms of rhAPC to postnatal pre-eclampsia might partly depend on its inhibitory effect on the deleterious Th1 type cytokines and pro-inflammatory cytokines (IL-2, TNF-α, IL-1β and IL-6), while it also down-regulated the expression of IL-10, a powerful regulatory (anti-inflammatory) cytokine. Therefore, rhAPC may exert the negative impact on most of components within the cytokine network in women with postpartum pre-eclampsia.

The results from our study also showed that the infusion of rhAPC resulted in significant reduction in post-natal neutrophil TLR-2, TLR-4 and cryopyrin mRNA expression respectively. rhAPC may repress the activation of neutrophils in postnatal pre-eclampsia,
therefore, through TLR-2, -4 and cryopyrin signaling pathways.

6.2.2 The effects of rhAPC in women with antenatal pre-eclampsia

The similar trends of several principal TEG indices (R-time, K-time and Angle) and PAI-1, TLR2, TLR4, cryopyrin have been observed in antepartum patients, though the differences were not statistically significant. Meanwhile, there was significant up-regulation of caspase-3 in antepartum cohort. RhAPC may promote apoptosis of human neutrophils through this signaling pathway, especially in women with antenatal pre-eclampsia. At this preliminary stage, we should recognize that the results in antepartum patients are less compelling, but are still supportive enough to encourage us to complete the trial on the 20 women.

6.3 Significance

The clinical part of our study has provided several pieces of information to support the hypotheses that rhAPC safely prolongs pre-eclamptic pregnancies remote from term, safely accelerates the postnatal resolution of the maternal syndrome, and is not associated with a significant increase in bleeding complications. At the same time, the preliminary results of basic science in this thesis have partly improved our understanding of the molecular mechanisms underlying the formation, developing and amelioration of pre-eclampsia.

Assuming that there are no significant safety concerns following this open-label safety
and efficacy trial, the results from this study will be used to determine a sample size for a placebo-controlled RCT of rhAPC for both early-onset pre-eclampsia with dismal fetal prognosis, and severe postpartum pre-eclampsia. In addition, the patient and partner satisfaction questionnaires and the economic analyses piloted in the clinical part of this trial will be used in the definitive phase III, international, multi-centre randomized controlled trial.

6.4 Future directions

From the results and discussions above, we could see the expected changes on haemostatic indices, circulating cytokines or neutrophil components are mainly detected in postnatal cohort. It could be resulted from two aspects: the pharmacological effects of rhAPC or natural recovery from pre-eclampsia after delivery; in other words, we could not completely exclude the influence of parturition on these parameters. Therefore, appropriate post-Caesarean section control samples need to be obtained and analyzed. Same analysis on placebo-controlled RCT in postnatal patients will also provide information to differentiate the effect.

In antenatal patients, results in PAI-1, TLR2, TLR4, cryopyrin and several TEG indices showed the same directions as postpartum cohort, but did not reach statistical significance. Increasing sample size is critical from this point of view. Meanwhile similar analysis on placebo-controlled RCT in antepartum patients will facilitate us to accurately evaluate the effects of this drug.


9. Lo J. Wang Dui Me: Schou Schen Hsiau Bu (Translation), Vol 2, Abhandl Med Faculty Sun Yat Sen University, 1930


61. Atallah AN, Hofmeyr GJ, Duley L. Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems. (Cochrane Review). In: The Cochrane Library, Issue 1


65. CLASP (Collaborative Low-Dose Aspirin Study in Pregnancy) Collaborative Group. CLASP: a randomized trial of low-dose aspirin for the prevention and treatment of
pre-eclampsia among 9364 pregnant women. Lancet 1994; 343:619-29


78. Bateman BT, Schumacher HC, Bushnell CD, Pile-Spellman J, Simpson LL, Sacco RL,


127. Bel L, Santos-Silva A, Rumley A, et al. Elevated tissue plasminogen activator as a potential marker of endothelial dysfunction in pre-eclampsia: Correlation with


146. G.P. Sacks, C.W. Redman and I.L. Sargent, Monocytes are primed to produce the Th1 type cytokine IL-12 in normal human pregnancy: an intracellular flow cytometric analysis of peripheral blood mononuclear cells, Clin Exp Immunol 2003, 131: 490-497


151. Sacks GP, Studena K, Sargent IL, Redman CWG. Normal pregnancy and pre-eclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of


171. Taylor FB Jr, Wada H, Kinaseitiz G. Description of compensated and uncompensated disseminated intravascular coagulation (DIC) responses (non-overt and overt DIC) in baboon models of intravenous and intraperitoneal Escherichia coli sepsis and in the human model of endotoxemia: Toward a better definition of DIC. Crit Care Med 2000; 28 (Suppl): 12-9


201. Dimitrakakis C, Papageorgiou P, Papageorgiou I, Antzakis A, Sakarelou N, Michalas S.


207. F Xie, PhD candidate (von Dadelszen lab); unpublished data.


214. Loskutoff DJ, Sawdey M, Keeton M, Schneiderman J. Regulation of PAI-1 gene...


