Vascular dysfunction in preeclampsia
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Chapter 12

Epilogue and conclusions
Introduction

In this chapter we will first comment on the sample size of the studies described in this thesis. Secondly, the role of microparticles in the pathogenesis of in preeclampsia will be discussed. The studies described in part II of this thesis direct us towards the hypothesis that microparticles are causally involved in the generation of vascular dysfunction in preeclampsia. In this section we will combine the knowledge gathered in the studies in this thesis with information from the literature in order to further develop this hypothesis. Possibilities for future research will be described. Thirdly, the contribution of vascular smooth muscle to vascular dysfunction in preeclampsia will be discussed. The evidence from studies described in part III of this thesis will be combined with information from the literature on this subject to evaluate the implications of these findings for future research and treatment options.

Sample size of the studies in this thesis

At the start of the project described in this thesis, only very limited information was available on vascular smooth muscle function in preeclampsia and practically no information was available on the role of microparticles in vascular dysfunction in preeclampsia. Therefore, many of the studies in this thesis were aimed at unraveling fundamental differences in these aspects between normal pregnancy and preeclampsia, rather than quantitating possible more subtle differences. We therefore chose to use rather low sample sizes (typically n=10) in several of the studies presented in this study. It is well appreciated that these sample sizes limit the statistical power of the experiments. It should however be realized that the applied techniques, notably the cannulation of arterioles, is quite time-consuming. Also, we applied strict inclusion criteria for the patient groups in order to reduce intra-group variability as much as possible. Therefore, collecting tissue and performing these studies on larger groups would have extended the studies considerably, and would have prevented us from following the large set of approaches described in the previous chapters. In several of the studies trends were detected (e.g. endothelial cell-derived microparticles in chapter 5) that we believe warrant future studies with a larger sample sizes. With due observance of the above discussed, we were cautious with the interpretation of the results of the studies described in this thesis.

Microparticles

As described in the introduction, a central mechanism in the pathogenesis of preeclampsia is the release of an unknown factor from the placenta into the maternal blood that causes a generalized vascular dysfunction. In the studies described in this thesis we investigated whether microparticles could be this unknown factor. The results of these studies indeed direct us towards a causal role for microparticles in the generation of vascular dysfunction in preeclampsia.
Numbers of circulating microparticles derived from granulocytes and lymphocytes were increased in women with preeclampsia (Chapter 5) and the total group of circulating microparticles isolated from the blood of women with preeclampsia induced endothelial dysfunction in resistance arteries isolated from healthy pregnant women in contrast to microparticles isolated from the blood of normal pregnant (Chapters 6 and 7) or nonpregnant women (Chapter 7). This damage did not seem to be directly related to the phospholipid composition of the microparticles (Chapter 7). Furthermore, microparticles were not directly involved in the increased coagulation activation in preeclampsia. Thus, many questions remain, including: which subpopulation(s) of microparticles are the damaging ones? How and when are these damaging microparticles formed? How do they exert their effects? Which components of the microparticles cause the damage? How could microparticle-induced damage be prevented \textit{in vivo}? The possible answers to these questions and possibilities for research that will help to answer them will be discussed in the next section.

\textbf{Which subpopulations of microparticles are the damaging ones?}

Several possible subpopulations of microparticles could be involved in the generation of vascular dysfunction in preeclampsia. The largest portion of circulating microparticles in nonpregnant, normal pregnant and preeclamptic women was derived from platelets (Chapter 5). Furthermore, microparticles from erythrocytes, leukocytes and endothelial cells were present (Chapter 5). The subgroup, however, that is most likely to be involved in the generation of vascular dysfunction in preeclampsia is formed by leukocyte-derived microparticles. We found that their numbers are increased in the blood of women with preeclampsia (Chapter 5). We also investigated the role of STBM in the pathogenesis of vascular dysfunction in preeclampsia. Previously, STBM numbers have been reported to be increased in women with preeclampsia [64] and high concentrations of artificially prepared STBM were found to damage endothelial function in isolated subcutaneous arteries [66]. However, in our experiments the antibody, previously used to detect microparticles in venous blood from women with preeclampsia, proved to be non-specific (Chapter 5). Since no syncytiotrophoblast-specific antibodies are currently available we were unable to establish the possible presence of STBM in our samples. However, since we were capable of determining the cellular origin of virtually all circulating microparticles, STBM - if present in the maternal blood - can only comprise a modest percentage of the total (Chapter 5). The assumption that STBM are not the microparticle subgroup involved in generation of vascular dysfunction was strengthened by the results of the study described in chapter 4, in which we found that artificially prepared STBM did not cause vascular dysfunction in isolated myometrial arteries.

\textbf{How and when are these damaging microparticles formed?}

There are several possibilities to answer the question how and when these damaging microparticles are formed. One possibility is that cells are activated while they pass through the placenta. As described in chapter 2 there is a substantial amount of evidence that placentation is
suboptimal in preeclampsia. This results in a placenta with specific characteristics, such as increased cytokine production, adhesion molecule- and antigen expression [49,333]. During passage of this “activated” preeclamptic placenta, blood cells may also become activated. Activation of leukocytes during passage through preeclamptic placentas has recently been reported [334]. Furthermore, culture medium incubated with placentas from women with preeclampsia stimulated the interaction between neutrophils and endothelial cells [258], indicating that something is derived from these placenta’s that stimulates leukocytes and/or endothelial cells and their interaction. Lymphocyte microparticles could also be derived from activated lymphocytes that are present in increased amounts in the placental tissue during preeclampsia [257].

**How do the damaging microparticles exert their effects?**

After their formation, leukocyte microparticles could cause endothelial activation and/or dysfunction directly or indirectly by interacting with other cells that could then in turn start forming microparticles, thus creating a vicious cycle. So far, the only experiments that have determined microparticle numbers in relation with preeclampsia, measured when preeclampsia had already developed. As a consequence, we do not have any information about microparticles in relation to the time-course of the disease.

It is also possible that the population of circulating microparticles substantially differs from the population of microparticles bound to endothelial cells. It seems reasonable that the latter group forms the culprit. The fact that microparticles contain cell-specific adhesion molecules or adhesion molecule ligands enables them to bind to cells that expose either ligands or adhesion molecules (Chapter 3). Binding of microparticles to cells has indeed been shown [197] as well as direct transfer of molecules from microparticles to cells [196], which implies that there must have been direct contact between microparticles and cells. [200]. In the experiments described in chapter 6 we observed that microparticles affected vascular function only after prolonged incubation. Since this incubation was performed at 4°C, a temperature at which enzymatic and biochemical processes are largely inactive in contrast to physical processes such as diffusion and attachment, we hypothesize that attachment of microparticles to the endothelium is an important step in the generation of vascular dysfunction in preeclampsia. We also observed that preeclamptic microparticles affected endothelial function when minimal amounts of plasma were present, but endothelial function was unaffected when higher concentrations of plasma were present (Chapter 6). We assume this protective effect of plasma is due to inhibition of microparticle attachment. The most likely substances involved in such protection are plasma proteins. Albumin protects endothelial function in isolated arteries [265] and albumin and other plasma proteins bind to the glyocalyx – a layer on the endothelial cell membrane that consists of specific proteoglycans and glycoproteins - to form a protective layer that is essential for maintenance of the vascular endothelium [335]. Albumin also binds arachidonic acid and platelet-activating factor [336,337]. Both arachidonic acid and platelet-activating factor can be
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present in or on microparticles [157,338]. Therefore, it is possible that plasma proteins support a glycocalyx layer around microparticles or on the endothelium that prevents the interaction between them. The reduced albumin concentrations and altered albumin characteristics in preeclampsia [266] fit in this theory, as well as the fact that the effect of plasma was concentration-dependent (Chapter 6).

The presence of oxidative stress in preeclampsia [274] could be involved. Oxidative stress could further enhance the damaging effect of microparticles. Oxidative stress also diminish the endothelial glycocalyx, thereby facilitating adhesive interactions of blood cells with the endothelium [267].

**Which components of the microparticles cause the damage?**

Which components of the microparticles are involved in generation of vascular dysfunction in preeclampsia and other diseases remains unclear. As discussed in chapter 3, the composition of microparticles not only depends on the “parent cell”, but also on the underlying cellular process, the stimulus that initiated microparticle generation and on the presence of oxidative stress. Not only the adhesive capacity of microparticles could be important, but also their protein and phospholipid composition. We found that the phospholipid composition of the circulating microparticle population in women with preeclampsia did not significantly differ from that in normal pregnant women (Chapter 7). Thus, the phospholipid composition of microparticles seems not to be of importance for the induction of vascular dysfunction in preeclampsia. Whether the oxidation status of the phospholipids in microparticles is of importance remains to be investigated.

**The role of microparticles in coagulation and inflammation**

Microparticles have not only been implicated in the generation of vascular dysfunction but also in coagulation and inflammation (Chapter 3). Both these processes are activated in preeclampsia. We investigated the possible involvement of microparticles in the hypercoagulation in preeclampsia. Although microparticles were involved in thrombin generation, and different pathways of thrombin generation initiation were involved in preeclampsia when compared to microparticles from normal pregnant and nonpregnant women, the hypercoagulation did not directly result from an altered thrombin generating capacity of the circulating microparticles (Chapter 8). Several characteristics indicate an exaggerated inflammatory state in preeclampsia; leukocytes are activated, there is increased leukocyte adhesion and circulating cytokine levels are increased [256]. The increased numbers of circulating leukocyte microparticles and the increased elastase concentrations we observed, also point towards this inflammatory state (Chapter 5). No experiments have been performed to study the contribution of microparticles to inflammation in preeclampsia.
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Hypothesis
Considering the above-mentioned evidence, we hypothesize that microparticles are involved in the pathogenesis of vascular dysfunction in preeclampsia. We propose that leukocytes become activated during passage through the preeclamptic placenta or are already present in the placenta and start generating microparticles with specific characteristics. These microparticles then either bind other cells, stimulating them to start microparticle generation or directly bind to the endothelium, thereby causing endothelial dysfunction. Obviously, further investigations are needed to confirm this hypothesis.

Possibilities for future research
Firstly, the microparticle subgroup that causes endothelial dysfunction has to be identified. Therefore, microparticles from women with preeclampsia have to be separated based on cellular origin. This separation can be achieved using affinity beads. After separation, the effect of the microparticle subgroups can be tested according to the protocol described in chapter 6, by overnight incubation of isolated arteries with the different microparticle groups and analysis of endothelial function after incubation.

Secondly, after identification of the microparticle group(s) that causes endothelial dysfunction, the characteristics of these microparticles will have to be determined. Furthermore, it will be of interest to investigate which mechanism resulted in production of microparticles with these specific characteristics. Analysis of the antigen composition of microparticles can be done by flowcytometry using various antigen-specific antibodies directed against e.g. adhesion molecules and receptors. The oxidation status of phospholipids in microparticles can be determined with electrospray ionization mass spectrometry. To identify the stimulus that generates microparticles with specific damaging characteristics, stimulation of cells, either cultured cells or cells isolated from blood of healthy subjects, with various stimuli, such as apoptosis inducers, oxidative stress or cytokines can be performed. Analysis of the resulting microparticles by flowcytometry, high performance thin layer chromatography and electrospray ionization mass spectrometry could clarify which stimulus is responsible for the generation of microparticles with characteristics that resemble those isolated from women with preeclampsia. Clarification of this mechanism could help to unravel the hypothesis on the pathogenesis of preeclampsia further.

Longitudinal studies are also essential to reveal when leukocyte microparticle numbers and characteristics start to change. The longitudinal course of microparticle numbers in pregnancy and preeclampsia has not been established yet. It will be interesting to determine when in the process of development of preeclampsia leukocyte microparticle numbers start to increase and/or when characteristics of microparticle subgroups start to alter, e.g. phospholipid content of microparticles. Furthermore, the correlations of numbers and characteristics of (subgroups of) microparticles with disease severity, including their behavior during episodes of deterioration of the clinical symptoms, e.g. during a HELLP episode, should be determined.
Thirdly, it should be established whether adhesion of microparticles to endothelial cells is indeed an important step in mediating their damaging effect on endothelial function. In order to investigate whether there is a difference in adhesion of microparticles from women with preeclampsia and from normal pregnant women we performed pilot experiments. In these experiments we labeled preeclamptic microparticles and normal pregnant microparticles irreversibly with two different fluorescent dyes, with different emission spectra (Dil and DiO (Molecular Probes, Leiden, The Netherlands)). After the labeling procedure we mixed the two types of microparticles and then perfused them simultaneously through an isolated subcutaneous artery from a normal pregnant woman. We monitored binding of fluorescent microparticles to the vascular wall under a confocal microscope (Leica). We envision that the total number of microparticles attached to the vessel wall can be counted from 3D reconstructed images. Figure 1 depicts an example of a single optical section showing emission of both Dil- and DiO-labeled particles at the vascular wall. Using Dil and DiO as fluorescent tracers, we had several problems. Firstly, it is very difficult to dissolve these lipophilic tracers adequately in the water-rich solution in which microparticles are suspended and, therefore, to label the microparticles adequately. Furthermore, Dil and DiO have co-emission at the same absorption wavelength, as visible on the overlay projection in figure 1.

Thus, before definite experiments can be started, other tracers will have to be tested that do not have problems with dissolving in a water-rich environment and with emission spectra that are further apart. In these experiments, the origin of the used bioassay artery may also be of importance. Endothelial activation or dysfunction may result in an altered capacity of the endothelium to bind microparticles. Therefore, it will be of importance not only to test adhesion of the different microparticle types to vessels from normal pregnant women, but also to vessels from women with preeclampsia or to vessels that have been exposed to e.g. oxidative stress and thus lack their glycocalyx. [267]. Also, as previously discussed, plasma proteins may be of importance to prevent microparticle adhesion to the endothelium. Thus, microparticle adhesion also needs to be tested in the presence of plasma or with plasma proteins, such as albumin, in different concentrations. An alternative approach could be to investigate microparticle adhesion using intravital microscopy in an animal model. In this approach fluorescent-labeled microparticle behavior could be investigated in e.g. the cremaster microcirculation in the hamster.

**Figure 1.** Overlay projection of an isolated subcutaneous artery after perfusion with normal pregnant microparticles labeled with DiO, and microparticles from women with preeclampsia labeled with Dil. The extra bright spots are emitting in the Dil and DiO spectrum.
This technique as previously been used in our laboratory to evaluate the effect of intravascular oxidative stress on the glycocalyx and the resulting adhesive interactions of blood cells with the microvascular endothelium [267] and is probably also applicable for microparticles [339].

A second alternative study strategy that could provide information whether adhesion of microparticles is involved in the pathogenesis of preeclampsia is to try to envision them on the vascular wall of isolated resistance arteries from women with preeclampsia. This could be done by scanning electron microscopy. It may also be possible to determine the cellular origin of adherent microparticles in these isolated arteries by fixing them for electron microscopy and to label them with cell specific antibodies, e.g. CD 66e, which is specific for granulocytes.

**Intervention strategies**

After the mechanisms through which microparticles induce vascular dysfunction in preeclampsia have been clarified, development of prevention or intervention strategies has to be initiated. A role for oxidative stress in the pathogenesis of preeclampsia and in the generation of damaging microparticles seems likely. Therefore, antioxidant therapy may prove useful. Previously, it has been shown that treatment of women at increased risk for preeclampsia with antioxidant vitamin C and E reduces the prevalence of preeclampsia [77]. Furthermore, treatment of patients with congestive heart failure with vitamin C decreased the number of circulating microparticles [242]. Thus, the effect of treatment of women at increased risk of preeclampsia with vitamins C and E may be (partially) due to a reduction in microparticle formation. Another possible effect of antioxidants may be the prevention of phospholipid oxidation. The fact that treatment of isolated arteries from women with preeclampsia with the antioxidant estrogen improves endothelial function partially (Chapter 11) may imply that it interferes with ongoing oxidative damage. In the near future a collaborative study will hopefully start, in which the numbers and characteristics of microparticle will be determined longitudinally in women at high risk for preeclampsia, that were either treated with vitamin C and E or placebo. In this way, we may get a clear picture of the effects of antioxidant therapy on microparticle numbers and composition and the effect on pregnancy outcome.

Another possible future therapeutic target is prevention of microparticle generation. In this respect, abciximab (Reopro®), a GP IIb-IIIa receptor antagonist that is currently used as an antiplatelet drug in prevention of ischemic complications after percutaneous coronary intervention, is interesting since this drug also blocks platelet vesiculation [240]. Alternatively, calcium channel blockers are of interest, since they can also decrease microparticle generation, as was shown in patients suffering from a transient ischemic attack [241].

Another possible therapeutic target could be formed by albumin. As discussed in chapter 6 and in this chapter the presence of plasma prevented the damaging effect of preeclamptic microparticles on vascular function and albumin, as a protector of endothelial function and the integrity of the glycocalyx, could be responsible for this effect. The reduced albumin concentrations in preeclampsia and the altered albumin characteristics [266] could amplify the
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damaging effect of microparticles. Albumin replacement therapy has been evaluated in preeclampsia, although not in a randomized controlled setting. No clear beneficial effects were observed from albumin supplementation on maternal blood pressures and need for antihypertensives, nor on the fetal condition [340]. Presently, albumin replacement therapy in disease states with hypoalbuminemia has become less used with the upcoming of alternative colloid solutions [341].

Obviously, when interventions are done to prevent the development of vascular dysfunction in preeclampsia, they need to be done as early in the course of the disease as possible, preferably even before pregnancy. This necessitates early identification of patients at increased risk for preeclampsia. Thus far, two-stage uterine artery doppler analysis has been used to identify women at increased risk, which works reasonably [77]. Ongoing research in the Academic Medical Center into the pre-pregnancy and early pregnancy hemodynamic function of women that later develop preeclampsia may provide us with such early markers.

Vascular dysfunction

The role of vascular smooth muscle

From the studies described in part 3 of this thesis, regarding the nature of the vascular dysfunction in the resistance vasculature in preeclampsia, we can conclude that vascular smooth muscle function is altered in preeclampsia. We found that vascular smooth muscle calcium sensitivity of the resistance vasculature in preeclampsia is increased (Chapter 9). Four mechanisms are involved in regulating vascular smooth muscle function; intrinsic vascular smooth muscle characteristics, endothelial function, sympathetic nervous activity and circulating factors in the blood. In the currently studied isolated vessels effects of the sympathetic nervous system and substances circulating in the maternal blood could be excluded. Therefore, the increased sensitivity had to stem from either intrinsic changes in vascular smooth muscle cells or endothelial factors. Based on single dose dilatations to ACh and BK, endothelial function was not affected in these vessels. It thus seems that the intrinsic calcium sensitivity of the vascular smooth muscle cells was increased in preeclampsia. The pathways involved in generation of this effect have not been clarified. In view of the previously discussed microparticle theory, we hypothesize that circulating microparticles in the maternal blood not only affect the endothelium, but also intrinsic vascular smooth muscle cell characteristics. Since there is most likely no direct contact between microparticles in the blood and vascular smooth muscle cells, endothelial processes are probably involved in mediation of this effect. A second vascular smooth muscle function that we investigated is vascular remodeling, the process in which the structure of the vascular wall, in specific the vascular smooth muscle and the extracellular matrix, is altered in order to capacitiate the altered hemodynamic conditions in the vessels. We found no direct evidence for remodeling of the subcutaneous resistance vasculature in preeclampsia. However, we did find evidence for ongoing extracellular matrix degeneration (Chapter 10). This indirectly supports the presence of vascular remodeling somewhere in the vasculature in preeclampsia. Other vascular beds in preeclampsia may therefore show signs of vascular remodeling. Aalkjaer
and coworkers indeed reported remodeling in the omental resistance circulation in preeclampsia [150].

**Improving vascular dysfunction**

The fact that vascular dysfunction in preeclampsia not only comprises endothelial dysfunction, but also vascular smooth muscle dysfunction could have therapeutical implications. Current therapy consists of symptomatic treatment of hypertension with antihypertensive drugs and ultimately delivery. Modulation of calcium sensitivity in experimental systems is possible by e.g. NO donors, such as nitroprusside, or rho-associated protein kinase inhibitors. NO donors have been used for treatment of hypertension in preeclampsia. Clinical studies with NO donors in women with preeclampsia have shown to cause an adequate blood pressure reduction and an improved umbilical cord flow to the fetus [124,125]. However, no large controlled trials have evaluated the long-term effects. This may be due to the possible side effects; maternal and fetal cyanide poisoning has been reported to result from the use of NO donors in animal studies, although strategies for prevention of this complication have also been developed [342]. An inhibitor of rho-associated protein kinase, Y-27632, has been developed by Uehata and coworkers [343] and was tested *in vitro* on rat mesenteric arteries [344]. Y-27632 inhibited basal tone and vascular smooth muscle calcium sensitivity in those isolated bioassay arteries [344]. In general, the vascular component of antihypertensive treatment acts on a spectrum from pure reduction of vascular smooth muscle intracellular calcium (e.g. blockers of voltage-operated calcium channels) to reduction of calcium sensitivity. Towards which end of this spectrum antihypertensive treatment in preeclampsia should be directed is not clear and this deserves further research. In particular, future *in vivo* testing of the rho-associated protein kinase inhibitor Y-27632 will have to reveal whether treatment of calcium sensitivity is effective for *in vivo* treatment of preeclampsia.

In the last chapter of part III we describe that flow-mediated dilatation and myogenic tone of myometrial arteries from women with preeclampsia can be improved by incubation of these arteries with estrogens, while BK-mediated dilatation was unaffected (Chapter 11). Estrogens have antioxidant effects [313], and also affect vascular function. They can cause short-term endothelium-independent [314] and endothelium-dependent dilatation [315], and longer-term endothelium-dependent effects, which may be genomic and primarily involve NOS [316]. The beneficial effect of estrogens on vascular function is probably due to their effect on vascular smooth muscle cell calcium metabolism [325]. This effect is mediated through endothelial NO and cyclic GMP [326] (Chapter 11), substances that can affect vascular smooth muscle calcium sensitivity. Whether estrogens indeed affect vascular smooth muscle calcium sensitivity has not been investigated. The fact that estrogens do improve flow- but not BK-mediated dilatation indicates that vascular dysfunction could not completely be converted and that additional damage remains. Prolonged incubation with estrogens may improve this remaining dysfunction or supplementation of estrogens during pregnancy may prevent this damage due to their
antioxidant effect. Possibly, the antioxidant effect of estrogens could also prevent generation of oxidized phospholipids in microparticles.

Conclusions

In conclusion, there are two major conclusions that can be drawn from the studies described in this thesis:

1. Microparticles form a good candidate for the unknown circulating factor, involved in the pathogenesis of vascular dysfunction in preeclampsia

2. The dysfunction of the resistance vasculature in preeclampsia does not solely consist of an endothelial dysfunction, but also has a vascular smooth muscle contribution. Vascular function can, at least partially, be improved by treatment with estrogens.