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**EFFECTS OF IN VITRO  
FERTILIZATION ON  
THROMBOSIS AND  
HAEMOSTASIS AND THE  
RELATIONSHIP BETWEEN  
INFERTILITY AND  
CARDIOVASCULAR DISEASE**

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*“There is a crack in everything.  
That’s how the light gets in.”*

Leonard Cohen

## ABSTRACT

**Background:** Infertility afflicts more than 10% of all couples worldwide. In vitro fertilization (IVF) is now performed at an ever increasing rate. There is scarce information concerning the associations between cardiovascular disease (CVD), venous thromboembolism (VTE) and infertility.

**Aims:** To assess the incidence of pulmonary embolism (PE) and VTE in pregnancy after IVF. To investigate whether there is an association of CVD and infertility. To study effects of IVF on global and individual markers of haemostasis.

**Methods and Results:** We studied all women who had given birth to a child after IVF (n=23,498) between 1990 and 2008 and individually matched women by age and calendar year (n=116,960). Information from the Swedish Medical Birth Register was linked to the Swedish National Patient Register in a cross-sectional study. The incidence of VTE was found to be increased during all pregnancy after IVF compared to pregnancy after natural conception. During the first trimester the risk was increased fourfold for VTE and sevenfold for PE.

We also studied the two groups after delivery until occurrence of hypertension, stroke, coronary heart disease, diabetes mellitus or until end of follow-up (average follow-up time eight years). Both univariable and multivariable analyses showed a higher incidence of hypertension after IVF pregnancy compared to control. There was a tendency towards a higher incidence of stroke, whereas the incidence of coronary heart disease and diabetes did not differ.

Furthermore we studied 31 women undergoing IVF at maximal downregulation (DR) and during high-level stimulation (HLS) of oestradiol synthesis. Antigen levels and activities of both von Willebrand factor (VWF) and ADAMTS13 in plasma were determined at DR and at HLS. Haemostasis was also assessed with 1) the calibrated automated thrombogram (CAT; measures thrombin generation), 2) overall haemostasis potential (OHP; measures fibrin formation and degradation) and 3) fibrin gel permeability measurements (assesses fibrin network characteristics). The increments in oestradiol during IVF were paralleled by an increase in VWF antigen and activity respectively a decrease in circulating ADAMTS13 antigen and activity. We found both an increased thrombin generation and fibrin formation from DR to HLS, whereas fibrin gel permeability did not change.

**Conclusion:** IVF pregnancy is associated with an increased risk of PE and VTE, in particular during the first trimester. The risk of PE is low in absolute terms but because the condition is a leading cause of maternal mortality and clinical suspicion is critical for diagnosis, an awareness of this risk is important. The mechanistic studies identified procoagulable changes in haemostasis during the IVF procedure.

Hypertension was more prevalent after IVF pregnancy. This association of CVD and infertility suggests that infertility and CVD could share common pathophysiological mechanisms.

## LIST OF PUBLICATIONS

- I. **Westerlund E**, Antovic A, Hovatta O, Eberg K, Blombäck M, Wallén NH, Henriksson P.  
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- II. **Westerlund E**, Henriksson P, Wallén NH, Hovatta O, Rodriguez-Wallberg K, Antovic A.  
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- III. Henriksson P, **Westerlund E**, Wallén NH, Brandt L, Hovatta O, Ekbohm A.  
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- IV. **Westerlund E**, Brandt L, Hovatta O, Wallén NH, Ekbohm A, Henriksson P.  
Increased incidence of hypertension in women after IVF pregnancy: A population-based cohort study from Sweden.  
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## LIST OF ABBREVIATIONS

Abs-sum	A summation of the absorbance levels
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
APC	Activated protein C
APTT	Activated partial thromboplastin time
AT	Antithrombin
BMI	Body mass index
CAT	Calibrated automated thrombogram
CLT	Clot lysis time
CRP	C-reactive protein
CVD	Cardiovascular disease
DR	Down regulation
DVT	Deep venous thrombosis
ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous thrombin potential
F1+2	Prothrombin fragment 1+2
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HR	Hazard ratio
HRT	Hormone replacement therapy
ICD	International classification of diseases
IVF	In vitro fertilization
LIA	Latex immunoagglutination assay
HLS	High level stimulation
Ks	Fibrin gel permeability coefficient
MBR	Medical Birth Register
MP	Microparticles
nAPCsr	Normalized activated protein C sensitivity ratio
OC	Oral contraceptive
OCp	Overall coagulation potential
OFp	Overall fibrinolysis potential
OHp	Overall haemostasis potential
OHSS	Ovarial hyperstimulation syndrome
PAI	Plasminogen activator inhibitor
PAP	Plasmin-antiplasmin complex
PCOS	Polycystic ovarian syndrome
PE	Pulmonary embolism
Plt	Platelets
PT	Prothrombin time
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complex
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
tPA	Tissue plasminogen activator
ttPeak	Time to peak
VTE	Venous thromboembolism
VWF	Von Willebrand factor
VWF:RCof	Von Willebrand factor ristocetin cofactor

# 1 BACKGROUND

## 1.1 VENOUS THROMBOEMBOLISM (VTE)

In the blood stream there should under normal conditions be a balance between blood clotting and lysis of blood clots. Imbalance between these entities can result in thrombosis or bleeding. Rudolf Virchow's triad from 1856 still remains true <sup>1</sup>. It states that 1) alterations in the vessel wall, 2) disturbances in blood flow or 3) disturbances in the constitution of blood are present in thrombosis.

Two major clinical manifestations of thrombosis on the venous side are deep venous thrombosis (DVT) and pulmonary embolism (PE). These conditions are collectively called venous thromboembolism (VTE).

There are several risk factors for VTE and many of them mechanistically belong to one of the "factors" identified by Virchow. Examples of risk factors are surgery, malignancy, immobilization, pregnancy, oestrogen treatment and thrombophilia (any coagulation disorder associated with increased tendency to VTE). Inherited thrombophilia includes activated protein C (APC) resistance (Factor V Leiden mutation), prothrombin mutation or deficiencies of either antithrombin (AT), protein S or protein C. The most common forms of acquired thrombophilia are phospholipid antibodies (lupus anticoagulant or cardiolipin antibodies) and acquired APC resistance. In addition, smoking, high levels of FVIII and previous venous thrombosis increase the VTE risk <sup>2-4</sup>.

It still remains controversial whether atherosclerosis and cardiovascular disease (CVD) risk factors are associated with risk of VTE <sup>5</sup>. Patients with unprovoked VTE were, however, recently reported to have an increased incidence of CVD <sup>6</sup> and an association between atherosclerotic disease and spontaneous VTE has also been observed <sup>7</sup>. Furthermore, the fact that the statin rosuvastatin significantly reduced the occurrence of symptomatic VTE in apparently healthy persons could favour such a contention <sup>8</sup>.

### 1.1.1 Risk of VTE in fertile women

The risk for a healthy fertile woman to suffer from VTE is fortunately low. The observed incidence of VTE in a population-based study in Sweden was 0.11, 0.26 and 0.97 per 1000 women at the ages 20-29, 30-39 and 40-49 years, respectively <sup>9</sup>.

Since the oral contraceptives (OC) were introduced in the 1960s and the awareness that exogenous oestrogens are associated with VTE, the risk of VTE among OC users has

been extensively studied. Thus, OC with low oestrogen content seems to cause less VTE compared with OC with high oestrogen contents<sup>10, 11</sup>. However, the absolute risk of VTE associated with OC use is lower (one in 3000 to 5000) than the risk of pregnancy-associated VTE<sup>12</sup>.

### **1.1.2 Risk of VTE during pregnancy**

VTE is one of the leading causes of maternal death in the industrialized countries with an incidence of 1.1 per 100.000 deliveries<sup>13</sup>. The risk of VTE is approximately 5-fold increased during normal pregnancy, especially during the third trimester and in the postpartum period (six weeks after delivery)<sup>14</sup>. One pregnant woman out of 1000 suffers from VTE according to data from Sweden and Norway<sup>15, 16</sup>.

The increased risk of VTE during pregnancy is most likely due to several factors, many of them directly related to haemostasis. These include gradually increasing plasma levels of coagulation factors and von Willebrand factor (VWF)<sup>17, 18</sup> as well as decreasing levels of natural anticoagulants<sup>19</sup>, altogether causing a procoagulable state. Furthermore, fibrinolysis is depressed as circulating levels of plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) are elevated and plasma levels of tissue type plasminogen activator (tPA) are reduced<sup>20, 21</sup>. This shift in haemostasis towards a procoagulant state is of course important for minimizing blood loss during delivery, but unfortunately it also contributes to an increased VTE risk during pregnancy<sup>22</sup>. Of note, inherited and acquired thrombophilia increase the risk of VTE during pregnancy even further<sup>23</sup>. Alterations in blood flow during pregnancy may also contribute to an increased risk of VTE. Stasis of blood flow in the left iliac vein due to pressure from the right iliac artery causes an overrepresentation of DVT in the iliofemoral veins of the left leg<sup>17</sup>. Another contributing cause of VTE during pregnancy is the 50% reduced venous flow velocity in the legs during the third trimester<sup>24</sup>.

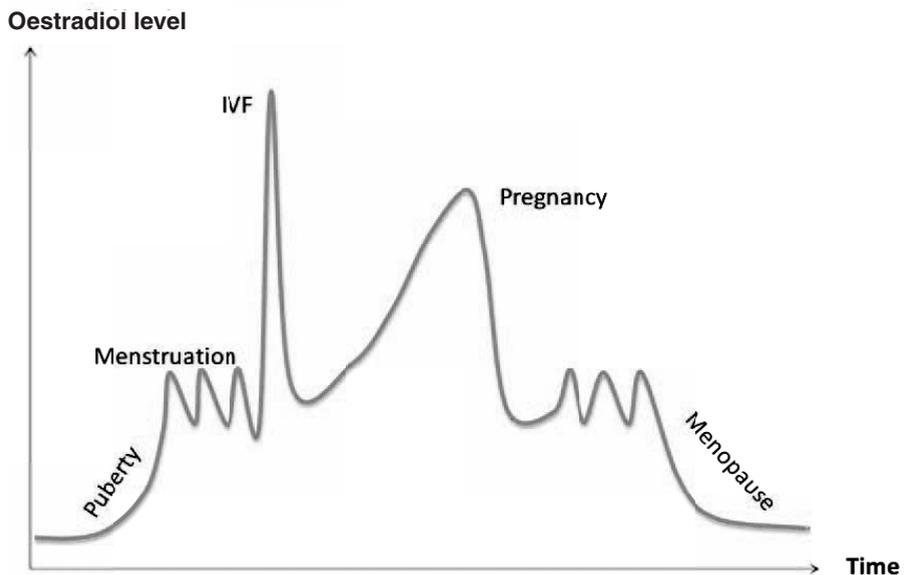
The more common proximal localization of the thrombosis during pregnancy and its association with more unspecific symptoms clearly constitute diagnostic and therapeutic challenges. See table 1 below for further data on effects of pregnancy and OC on various aspects of haemostasis.

### **1.1.3 Risk of VTE during in vitro fertilization**

Occurrence of VTE during in vitro fertilization (IVF) pregnancies has been reported in several case reports and two small consecutive series [two out of 2500 (0.8/1000 respectively three out of 2748 (1.1/1000)]<sup>25, 26</sup>. However, the incidence estimates of

VTE after IVF have been regarded to be comparable to the incidence rates of VTE during normal pregnancy. Interestingly, a recent Norwegian study showed that IVF increased the risk of VTE during pregnancy. The risk was more increased antepartum than postpartum<sup>15</sup>.

**Figure 1.** Schematic presentation of changes in oestradiol levels during IVF and pregnancy.



**Table 1.** Different effects of in vitro fertilization (IVF), pregnancy, oral contraceptives (OC) and hormone replacement therapy (HRT) on haemostasis.

		IVF	Pregnancy	OC	HRT	
<b>Coagulation factors</b>	Fibrinogen	↑ 27	↑ 28	↑ 29	↔ ↓ 30, 31	
	FII	↑ 32	↔ 33	↑ 29		
	FV	↑ 32	↔ 33	↓ 29	↔ 34	
	FVII	↓ 35	↑ 28	↑ 36	↔ 31	
	FVIII	↑ 35	↑ 28	↔ 29, 37	↔ 38	
	FIX	↑ 32	↔ 33	↑ 39	↑ 31	
	FX		↑ 28	↑ 36	↔ 30	
	FXI		↓ 40	↑ 41	↓ 42	
	FXII	↓ 43	↑ 40	↑ 44	↑ 30	
	FXIII		↓ 28	↓ 45		
	<b>Coagulation inhibitors</b>	AT	↓ 27	↓ 28	↓ 46	↓ 47
		Heparin cofactor 2	↓ 48	↑ ↔ 49, 50	↑ 49	
		Protein S	↔ ↓ 35, 48	↓ 51	↓ 46	↓ 31
Protein C		↓ 35	↓ 51	↑ 46	↔ 47	
APC resistance		↑ 52	↑ 53	↑ 54	↑ 55	
TFPI		↓ 66	↑ 50	↓ 67	↓ 65	
<b>Fibrinolysis</b>	t PA	↔ ↓ 48, 56	↑ 51	↑ 57	↓ 31	
	PAI-1	↔ ↓ 48, 56	↑ 51	↓ 58	↓ 59	
	PAI-2		↑ 60			
	Plasminogen	↑ 48	↓ 61	↑ 57	↑ 47	
	α-antiplasmin	↔ 48	↑ 40		↔ 47	
	TAFI	↑ 62	↔ 63	↑ 64	↔ 65	
	PAP complex	↑ 68	↑ 69	↑ 70	↔ 71	
	<b>Global methods</b>	TEG	↑ 72	↑ 73	↑ ↔ 74, 75	
OHP		↑ 76	↑ 77			
TGA		↑ 76	↑ 78	↑ 79	↑ 80	
Fibrin gel		↔ 76				
<b>Platelets</b>	Aggregation	↔ 81	↑ 82	↔ 83	↔ 84	
<b>Micro-particles</b>	Platelet MP		↑ 85		↑ 86	
	Endothelial MP		↑ 85		↔ 86	
<b>Miscellaneous</b>	PT (INR)	↓ 32	↓ 51	↓ 37	↔ 47	
	PTT	↓ 32	↓ 51	↓ 37	↔ 31	
	D-dimer	↔ 35	↑ 60	↑ 70	↑ ↔ 31, 87	
	Soluble fibrin	↔ 35	↑ 60	↔ 29	↑ 87	
	F 1+2	↑ 48	↑ 51	↑ 29	↑ 88	
	TAT complex	↔ ↓ 48, 56	↑ 89	↔ 29	↔ 90	
	VWF	↑ 35	↑ 28	↔ 37	↔ 38	
	ADAMTS13	↓ 91	↓ 92	↔ 93		

Interestingly, VTE during IVF has been reported to be predominantly located in the upper extremities and jugular veins <sup>25</sup>. There are different hypotheses behind this finding. One is that the high oestradiol concentration in the lymphatic fluid of the thoracic duct causes a local inflammation in the veins which in turn precipitates thrombus formation <sup>94</sup>.

There is little information available about the risk profile of PE following IVF. Indeed, this is an important issue since PE is a potentially lethal condition <sup>95, 96</sup>.

## **1.2 IN VITRO FERTILIZATION**

IVF is the most common procedure performed to assist reproduction. Since the first attempt in 1978 IVF treatment has been used at an increased frequency <sup>97</sup>, resulting in the birth of approximately five million “test tube babies” worldwide. The IVF procedure is regarded both effective and safe with about 30% of the attempts resulting in a pregnancy and 25% in a live birth <sup>98, 99</sup>.

Gonadotropin-releasing hormone (GnRH) agonists and follicle-stimulating hormone (FSH) are administered during the IVF pretreatment, causing a rapid increase in circulating oestradiol levels from very low concentrations during the down-regulation phase (GnRH) to supraphysiological concentrations during the stimulation phase (FSH). This results in the production of multiple ovarian follicles. Notably, the maximal oestradiol levels during IVF are usually equal to or sometimes even exceeding late pregnancy oestradiol concentrations <sup>100</sup> (figure 1).

Several studies demonstrate that the rapid increase in oestradiol levels in plasma attained during IVF may induce a procoagulable state through direct effects on several haemostatic variables as shown in table 1. Indeed, IVF pretreatment is a unique model to study short-term effects of endogenous oestrogens on haemostasis.

A major complication of IVF is the ovarian hyperstimulation syndrome (OHSS), characterized by ovarian enlargement and fluid shift to the third space due to capillary leakage <sup>101</sup>. Depending on the severity of OHSS, its incidence rate has been described to vary between 0.5% and 23% <sup>102, 103</sup>. Severe cases are associated with arterial and venous thrombotic complications <sup>104</sup>, but because of lack of a systematic registration there are no existing precise figures of incidence rates of thrombotic complications. High serum oestradiol concentrations, increased number of small ovarian follicles at the time of ovulation induction, polycystic ovarian syndrome (PCOS), young age and a low BMI are factors known to relate to the development of OHSS <sup>105</sup>. The predictive value

of any of these factors for the development of OHSS is not high, and there are conflicting reports in the literature on the relation of these variables to OHSS <sup>106,107</sup>.

### **1.3 OESTROGEN**

Oestrogen is the main female sex hormone and essential for menstruation and reproduction. The three major naturally occurring oestrogens in women are oestrone (E<sub>1</sub>), oestradiol (E<sub>2</sub>), and oestriol (E<sub>3</sub>). Oestradiol, which is the predominant oestrogen during reproductive years, reaches the highest levels in plasma and has the greatest oestrogenic activity. Luteinizing hormone (LH) from the pituitary gland stimulates the oestrogen production in the ovaries, while increase of follicle-stimulating hormone (FSH) from the pituitary gland causes ovulation. For reproduction these two hormones have to act synergistically. Progesterone, the major progestogen, is involved in the menstrual cycle and supports gestation and embryogenesis.

During the fertile period of a woman's life, almost all biologically active oestradiol is secreted from the developing follicles in the ovaries and during pregnancy even from the placenta. The plasma level of oestradiol varies during the menstrual cycle, with the lowest levels in the early follicular phase and the highest levels at the time of ovulation. The majority of oestrogen (40-90%) is bound to a carrier protein in the blood, sex hormone-binding globulin, which is produced in the liver. About 10-30% of oestrogen in plasma is bound to circulating albumin, and only about 1% remains as a free fraction exerting biological effects. There is also some production of oestrogen in the liver, adrenal glands, breasts and fat cells, the latter potentially being the reason why underweight or overweight are risk factors for infertility <sup>108</sup>.

### **1.4 CARDIOVASCULAR DISEASE**

#### **1.4.1 Infertility and cardiovascular disease**

In women, heart disease is the leading cause of death while stroke is the third most common cause of death <sup>109</sup>. Infertility afflicts more than 10% of all couples worldwide <sup>110</sup> and the cause of infertility is usually divided into female factors, male factors and unknown factors. Both obesity as well as underweight seems to be associated with reduced fertility in women <sup>111</sup>. In addition, smoking, increasing age and environmental factors seem to impair development and function of the reproductive organs, which could lead to hormonal imbalance and fertility problems in both men and women <sup>112</sup>. The most common cause of female infertility is the polycystic ovarian syndrome

(PCOS)<sup>113, 114</sup>. Interestingly, women with PCOS have a sevenfold increased risk of myocardial infarction<sup>115</sup>. Endothelial dysfunction is an early marker of CVD<sup>116</sup> and has been found in women with PCOS as well as in animal models of this condition<sup>117, 118</sup>. Possible associations between endothelial dysfunction in the ovarian vasculature and infertility have, however, to our knowledge not yet been studied. In addition, women with PCOS have menstrual irregularities, which have also been associated with subfertility<sup>119</sup> and an increased risk of CVD<sup>120</sup>.

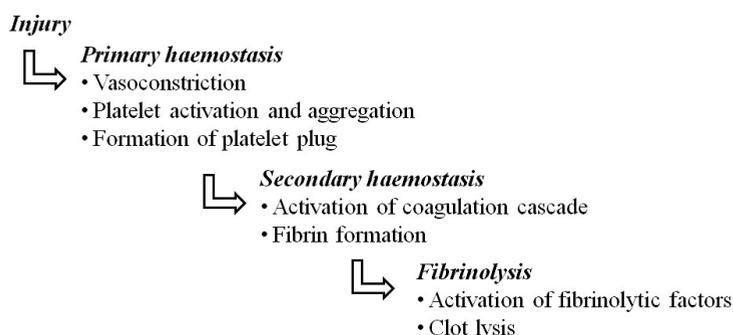
#### 1.4.2 Hormone replacement therapy and cardiovascular disease

There are a lot of conflicting reports regarding hormone replacement therapy (HRT) and CVD risk. Some register studies suggested that HRT reduced the risk of coronary heart disease<sup>121</sup>, whereas randomized double blind trials [Heart and Estrogen/progestin Replacement Study (HERS) and Women’s Health Initiative (WHI)] suggested that HRT on the contrary increase the risk of VTE and coronary heart disease<sup>122, 123</sup>.

### 1.5 HAEMOSTASIS

A well balanced haemostatic system is necessary to prevent thrombosis or bleeding. Haemostasis includes the “clotting process” from the formation of platelet plugs and clots to lysis of the clot, and is traditionally divided into primary and secondary haemostasis (figure 2).

**Figure 2.** Schematic summary of haemostasis.

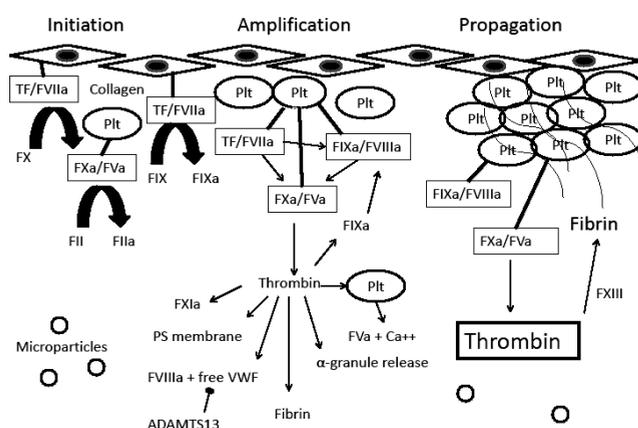


There are many important components involved in the haemostatic process, including vessel wall, platelets (Plt), white blood cells, coagulation factors and inhibitors, fibrinolytic factors and inhibitors, calcium ions, phospholipids as well as the blood flow<sup>124, 125</sup>. Most interactions between the factors of the haemostatic system occur on

surfaces, i.e. on surfaces of activated cells (e.g. platelets or endothelial cells), on microparticles released from cells, or on collagen exposed in the injured vessel wall <sup>126</sup>. In fact, a “cell-based model of coagulation” is used nowadays. This view is probably the most accurate as it involves not only the plasma factors but also the complex interactions between cellular components and plasma factors (see figure 3 and text below). However, blood coagulation has traditionally been described as a cascade of distinct enzymatic reactions where proteolytic activation of a proenzyme leads to activation of the next proenzyme <sup>126</sup>, and this view has still some value in terms of understanding of this very complex system. The coagulation proenzymes or cofactors are either vitamin K-dependent (FII, FVII, FIX and FX) or thrombin-dependent (fibrinogen, FV, FVIII, FXI and FXIII).

Coagulation is tightly controlled and sequences of interactions result in the formation of thrombin and subsequently fibrin. A series of events that comprise a sequence of proteolytic cleavages at very specific sites on the proenzymes or cofactors occur. These activation steps cause conformational changes of the proteins, which in turn expose their enzymatically active sites and/or binding sites for cofactors, phospholipids and other specific receptors located on various cell surfaces <sup>125</sup>. In some situations, the precursor protein can be activated not only by coagulation factors but also by other proteases, for example enzymes from tumour cells, microorganisms and white blood cells or proteins released from surfaces that have become exposed due to vessel damage.

**Figure 3.** Cell-based coagulation.



### **1.5.1 The coagulation process**

The cell-based model has three distinct but overlapping steps; the initiation, the amplification and the propagation phases <sup>127</sup>. A schematic presentation of the three steps in coagulation is shown in figure 3.

#### *1.5.1.1 The initiation of blood coagulation*

During the initiation phase of haemostasis, the damage to the vessel wall brings plasma into contact with cells bearing tissue factor (TF). A small amount of circulating coagulation factor VII (FVII) binds to TF and is rapidly activated. The FVIIa/TF complex, together with the cofactors  $Ca^{2+}$  and phospholipids exposed on activated cells or microparticles, activates more FVII but also FX and FIX. FIXa, in turn, forms a complex with FV on TF-bearing cells, and together the two activated proteins produce small amounts of thrombin. Depending on the strength of the stimuli, the initiating process will either proceed or will be stopped through the action of inhibiting factors such as tissue factor pathway inhibitor (TFPI).

#### *1.5.1.2 The amplification of blood coagulation*

In the amplification phase, small amounts of initially generated thrombin activate circulating platelets, enabling platelets to adhere to the site of injury. Platelet adhesion to subendothelial surfaces is mediated by VWF, which acts like a glue between platelets and collagen. Circulating VWF/FVIII complexes bind to activated platelets and more FVIII is released and activated by thrombin. Thrombin-induced platelet activation also results in release and activation of FV from the platelets.

#### *1.5.1.3 The propagation of blood coagulation*

The last phase includes formation of procoagulant complexes on platelets and microparticle phospholipid surfaces. The tenase complex (FIXa/FVIIIa) activates FX, which in turn forms the prothrombinase (FXa/FVa) complex and induces a thrombin burst. The cleavage of prothrombin into thrombin occurs through several steps, producing the active enzyme alpha-thrombin and prothrombin fragment 1+2 (F1+2). Thrombin converts fibrinogen to fibrin in the last step of the coagulation cascade, and the fibrin fibrils are cross-linked by Factor XIII into an insoluble fibrin network which stabilizes the platelet plug.

Strict regulation of the coagulation process and activation only at a local site of injury is essential to avoid massive systemic fibrin deposition. The regulation of this process is

very complex, with strong negative and positive feedback systems. The most important positive feedback is considered to be activation of FV by thrombin, while various anticoagulant proteins and cofactors also contribute and act as important inhibitors of this system.

### **1.5.2 Contact pathway**

The contact pathway starts with the activation of FXII from either collagen or polyphosphates secreted from platelets. FXIIa starts the cascade by activating FXI, which subsequently activates FIX. The contact pathway then merges into the initiation phase of the cell-based model as FIXa activates FX. The contact pathway is an interesting target for future antithrombotic treatment as animal models have shown that FXII deficient mice have a normal haemostatic capacity but a defect thrombus formation protecting them against both venous and arterial thromboses<sup>128</sup>.

### **1.5.3 Coagulation inhibitors**

The coagulation inhibitors balance the reactions in the coagulation process. Tissue factor pathway inhibitor (TFPI) inhibits both the produced FXa and the FVIIa/TF complex during the initiation phase. Antithrombin (AT) is the main inhibitor of thrombin and binds irreversibly to its target forming the thrombin-antithrombin complex (TAT). AT also inactivates FIXa, FXa, FXIa and FXIIa. Heparin used in anticoagulant therapy binds to AT causing a conformational change that leads to activation of the protein<sup>129</sup>.

After binding to thrombomodulin on the surface of endothelial cells, thrombin initiates the anticoagulant pathway by activating protein C. Activated protein C (APC) together with Protein S as cofactor inhibits thrombin formation through proteolytical inactivation of FVa and FVIIIa<sup>130</sup>.

### **1.5.4 APC resistance**

APC resistance is a decreased response to the anticoagulant function of APC and an important risk factor for the development of VTE<sup>131</sup>. This resistance can also be acquired in some conditions such as IVF<sup>52</sup>, pregnancy<sup>53</sup> and use of oral contraceptives<sup>54</sup> and is associated with increased thrombin generation<sup>132</sup>.

### 1.5.5 Fibrinogen

Fibrinogen is a soluble glycoprotein, which is synthesized in the liver. Approximately 75% of total fibrinogen is present in plasma<sup>133</sup> and under normal conditions at a concentration of 2-4 g/L. The rest is distributed in interstitial fluid and in the lymph. Fibrinogen is an important regulator of thrombin activity in clotting blood and the fibrinogen plasma level is an independent risk factor for both arterial and venous thrombosis<sup>134</sup>. Plasma fibrinogen is the “substrate” of the fibrin network and influences the structure of the fibrin network, as tighter networks are formed in the presence of higher fibrinogen levels<sup>135, 136</sup>.

### 1.5.6 Fibrinolysis

The fibrinolytic system dissolves and removes blood clots from the circulation. tPA is the main regulator of fibrinolysis, cleaving plasminogen into plasmin. Plasmin in turn cleaves fibrin into degradation products, i.e. d-dimers. The main inhibitors of fibrinolysis are PAI-1 and PAI-2 (which inhibit tPA) and  $\alpha$ 2-antiplasmin (which inhibits plasmin). PAI-2 is produced only by the placenta of pregnant women and its plasma level increases with gestational age<sup>137</sup>.

### 1.5.7 Endothelial related factors

#### 1.5.7.1 Von Willebrand factor

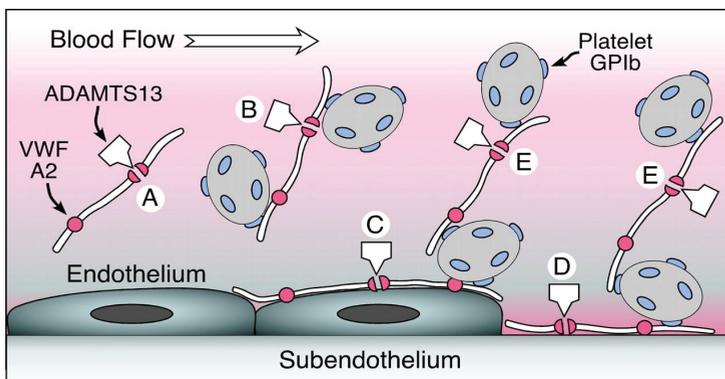
Von Willebrand factor (VWF), formerly called factor VIII-related antigen, is an adhesive plasma glycoprotein synthesized in endothelial cells and megakaryocytes. It plays a major role in primary haemostasis by serving as a link between platelets and the damaged vessel wall, and indirectly contributes to coagulation by binding, stabilizing and protecting circulating FVIII from degradation<sup>138</sup>. VWF is stored in Weibel-Palade bodies in endothelial cells and in  $\alpha$ -granules of platelets. In response to vascular damage, the rapid release of ultra-large VWF multimers mediates platelet adhesion. VWF multimers circulate in a “folded” inactive form<sup>139</sup>, but under shear stress the molecule exposes its binding sites in the stretched and unfolded form<sup>140</sup>.

Endothelial dysfunction is associated with increased circulating levels of VWF in both arterial and venous vessels<sup>3, 141, 142</sup>. During pregnancy, both VWF and FVIII levels raise two- to threefold during the second and third trimesters<sup>28, 40</sup>, and increased VWF and FVIII levels are also seen during IVF treatment<sup>35, 143</sup>. Interestingly, high plasma levels of VWF are associated with severe OHSS. It has been argued that assessment of VWF may be of value in predicting OHSS<sup>144</sup>.

### 1.5.7.2 ADAMTS13

An important regulatory mechanism of VWF levels in plasma is the activity of a zinc-containing metalloprotease enzyme, ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type I motifs), also known as VWF-cleaving protease. As shown in figure 4, this enzyme cleaves the unfolded VWF at a single site in the A2 domain, thereby converting ultra-large VWF multimers into lower molecular weight forms with reduced adhesive potential<sup>145, 146</sup>.

**Figure 4.** Possible sites of ADAMTS13 action on VWF.



From Sadler et al. with permissions from publisher<sup>147</sup>. Copyright 2013 National Academy of Sciences, U.S.A.

There is a negative association between plasma levels of VWF and the activity of ADAMTS13. The latter tends to be low in plasma when VWF is high<sup>148-150</sup>.

ADAMTS13 appears to be produced in the liver, but reports also indicate platelets and endothelium as production sites<sup>151, 152</sup>. Thrombin and plasmin can inactivate ADAMTS13 by cleavage and thereby regulate its activity at the site of thrombus formation<sup>153</sup>. Interestingly, patients with thrombotic microangiopathies like thrombotic thrombocytopenic purpura have a reduced function of ADAMTS13<sup>154-156</sup>, and ADAMTS13 is therefore a target for diagnosis and treatment of this serious disease<sup>157</sup>.

There are some studies indicating a moderate decrease of this protease during pregnancy and puerperium<sup>158</sup>. However, ADAMTS13 has not been investigated previously in patients undergoing IVF.

Immunologic assays can measure ADAMTS13 antigen concentrations, but measuring the activity of the enzyme is associated with methodological difficulties. Kokame et al.

have developed a new method which involves the use of a small fluorogenic labelled substrate for ADAMTS13 called FRETS-VWF73<sup>155</sup>. This substrate is a VWF fragment consisting of 73 amino acids. The method is commercially available, easy to perform, has a high specificity for ADAMTS13 and is able to detect decreased activity due to different causes.

### **1.5.8 Global markers of coagulation**

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) are the two routine assays for coagulation screening with a detectable clot as end point. The use of aPTT and PT in detecting prothrombotic states and in the monitoring of new antithrombotic agents is not optimal<sup>159</sup>. These methods are based on the formation of a detectable fibrin clot, which occurs already when 3-5% of the total amount of thrombin is produced. Haemostatic reactions occurring later on in the process may thus not be detected.

From a clinical point of view it is better to have functional methods as general indicators of coagulability. Ideally, such methods could be used to assess the balance between coagulation and fibrinolysis in the patient's plasma.

Very little is known about the haemostatic balance during IVF treatment. Although there is a wide spectrum of recently developed global haemostatic assays, only thromboelastography (TEG) has, to our knowledge, so far been used to assess changes during IVF<sup>72</sup>. TEG is a global method performed in whole blood and carried out bedside. It monitors clot formation as well as clot stability and dissolution<sup>160</sup>. An advantage with this method is the possibility to study interactions between blood cells, platelets and plasma constituents, but the test has to be performed within a few hours after blood sampling which limits its use in larger clinical studies.

#### *1.5.8.1 Calibrated Automated Thrombogram*

Calibrated Automated Thrombogram (CAT) is another commercially available global haemostatic method. By using tissue factor (TF) as a trigger, the thrombin generation potential of the patient's plasma is assessed, forming a temporal curve of the total thrombin generation. The area under the curve is called the endogenous thrombin potential (ETP)<sup>161</sup>. The CAT assay is based on the assumption that thrombin generation in examined plasma reflects the sum of the activities and concentrations of pro- and anticoagulant substances. The investigated variables include ETP and related parameters, namely lag time (i.e. time to start of detectable thrombin formation), peak

height (i.e. maximal thrombin concentration attained) and time to peak (i.e. the time taken to obtain peak height).

Increasing evidence shows that CAT may be a useful assay both in the diagnosis of procoagulable states and in the detection of certain bleeding disorders<sup>162, 163</sup>. Patients with thrombotic disorders, e.g. DVT and stroke, have higher ETP<sup>164, 165</sup>. CAT may provide additional information beyond what is obtained through measurements of traditional markers of thrombin generation, such as F1+2 or TAT. As stated by Al Dieri, Laat and Hemker: thrombin generation as assessed by ETP “indicates a fire hazard, while F1+2 and TAT are smoke detectors”<sup>166</sup>.

However, even though this method gives information about the coagulation capacity in plasma through assessment of thrombin generation, it does not measure the final step of coagulation, i.e. fibrin formation. Furthermore, fibrin degradation, i.e. fibrinolysis, is not assessed.

#### *1.5.8.2 Overall Haemostasis Potential*

He & Blombäck developed the Overall Haemostasis Potential (OHP) assay as a quantitative method for determination of the fibrin formation and degradation in plasma<sup>167</sup>. Spectrophotometric measurements reflect the fibrin fibril formation through changes in turbidity during the test period of 40 minutes. Previous investigations have shown that this approach can be used to detect a variety of procoagulable<sup>167-169</sup> as well as hypocoagulable disorders<sup>169, 170</sup>.

Fibrinogen and FVIII are increased during IVF<sup>35, 48</sup> and seem to correlate to the variables of the above mentioned global haemostasis assays<sup>161, 167</sup> which supported their assessment in study II.

The clot lysis time (CLT) may be viewed upon as a global assay of fibrinolysis in which triggers of both coagulation (TF, phospholipids and calcium) and fibrinolysis (recombinant tPA) are added to plasma. The changes in absorbance attained are measured during clot formation and lysis. We performed the CLT assay as another part of the OHP assay.

#### *1.5.8.3 Fibrin network*

To study the characteristics of the fibrin network, we used the liquid-permeation technique which investigates the permeability of a fibrin gel formed after addition of thrombin (or TF) to a plasma sample<sup>171, 172</sup>. The test thus provides information about

fibrin network characteristics including the fibre mass/length ratio and the permeability coefficient of the fibrin gel *in vitro*<sup>171, 173</sup>. The permeability coefficient (Ks) is an established variable that provides information on the network structure and reflects the size and shape of the pores in the fibrin gel formed. Collet et al. studied the fibrin network by using real-time confocal microscopy techniques *in vitro*, demonstrating that a tight fibrin network formation with smaller pores was more difficult to lyse<sup>174</sup>. Low values of Ks indicate a tighter, less porous fibrin network, which is more resistant to fibrinolysis<sup>173</sup> and may be associated with thrombotic complications<sup>175</sup>.

Patients with diabetes, stroke and VTE have tighter fibrin networks<sup>176-178</sup>. However, as of today there are no large prospective clinical trials studying fibrin network structure and its relationship to future thrombotic complications.

## 2 AIMS

- To evaluate the effects of IVF on haemostasis.
- To assess the incidence of pulmonary embolism and venous thromboembolism in pregnant women after IVF.
- To estimate whether cardiovascular disease and some related risk factors have a higher incidence in women after IVF pregnancy as compared to women who delivered after natural conception.

## **3 MATERIAL AND METHODS**

### **3.1 PATIENTS**

#### **3.1.1 Studies I-II**

We investigated plasma samples of 31 consecutive women undergoing IVF treatment at the Fertility Unit of Karolinska University Hospital, Huddinge, during 2006-2008. Upon inclusion, patients were interviewed regarding smoking habits, ongoing medication and own or family history of VTE, diabetes mellitus and CVD. Patients with polycystic ovarian syndrome (PCOS) or ongoing anticoagulation treatment were excluded.

#### **3.1.2 Studies III-IV**

IVF group (exposed women) consisted of 23,498 Swedish mothers who had their first child born as a result of IVF during 1990-2008 and were retrieved from the Swedish IVF Register at the National Board of Health and Welfare.

A control group (unexposed women) was retrieved from the Medical Birth Register (MBR). Each IVF woman was matched with five unexposed women from this register by calendar year of delivery  $\pm 2$  years and maternal age  $\pm 1$  year. This resulted in 116,960 unexposed women.

For all women we also retrieved information on their country of birth, pre-pregnancy body mass index, family situation, cigarette smoking habits, singleton/multiple births, and estimated length of gestation from MBR. Trimester dates were calculated from length of gestation.

Follow-up in study IV started at the time of delivery. End of follow-up was 31<sup>st</sup> of December 2009 or the date of first registered event of one of the following: a CVD diagnosis (coronary heart disease or stroke), a diagnosis of a related risk factor (hypertension or diabetes), emigration from Sweden or death.

### **3.2 BLOOD SAMPLING**

In study I and II venous blood sampling was always performed under fasting conditions, through direct venipuncture and after a 20 minutes supine rest.

Blood was drawn into vacuum tubes containing trisodium citrate (0.13 mol/L, pH 7.4). The tubes were immediately centrifuged for 20 minutes (2000g, room temperature), and platelet-poor plasma aliquots were stored at  $-80^{\circ}\text{C}$ .

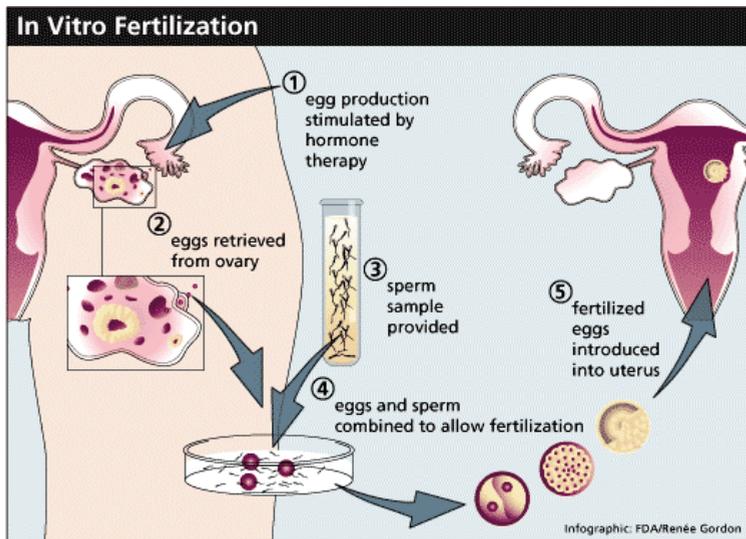
We performed two blood samplings during the IVF procedure: the first at time of maximal down-regulation (DR) of oestradiol synthesis (<150 pg/mL) and the second at time of high level stimulation (HLS) of oestradiol synthesis preceding human chorionic gonadotropin (hCG) administration (7-10 days after the first blood test).

During the first blood sampling, the following routine blood tests were performed: haemoglobin, platelet count, white blood cell count, haematocrit, creatinine, sodium, potassium, plasma glucose and C-reactive protein (CRP).

### 3.3 IN VITRO FERTILIZATION

The IVF procedure is illustrated in figure 5. To induce controlled ovarian hyperstimulation, the oestrogen production was first down-regulated by using a GnRH agonist administered as a nasal spray and starting on the 21<sup>st</sup> day of menstrual cycle. After the menstrual bleeding, two weeks later, an oestradiol measurement was carried out to verify down-regulation. Acceptable baseline oestradiol concentration for initiating the IVF cycle was <150 pg/ml. After that, the GnRH dose was decreased.

**Figure 5.** The IVF procedure.



The ovarian stimulation was then initiated by using recombinant human FSH administered subcutaneously. The response was followed up by an oestradiol measurement six days later, and by ultrasound scanning of the ovarian follicles at days

9-10 after the first FSH injection. The oestradiol measurement and ultrasound scanning were repeated when necessary.

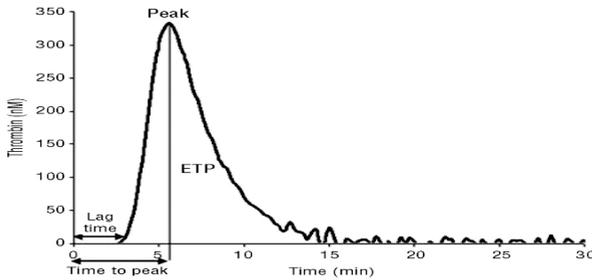
Transvaginal ultrasound-guided oocyte retrieval was planned when the largest follicles had reached a diameter of 18 mm. At that stage, recombinant or urine-purified hCG were given to induce the final maturation of the oocytes, which were retrieved 36-38 hours later. Thus, oocytes were retrieved 10 to 14 days after starting FSH stimulation. The embryo transfer procedure was carried out three to five days after oocyte retrieval. In order to improve fertility outcome the women were given progesterone vaginally during the first weeks after embryo transfer.

### **3.4 GLOBAL HAEMOSTASIS ASSAYS**

#### **3.4.1 Calibrated Automated Thrombogram**

The analysis was performed at the Clinical Research Center at Danderyd Hospital. We used the method described by Hemker et al.<sup>161</sup> and according to the manual provided by Thrombinoscope BV (Maastricht, the Netherlands). Briefly, we triggered coagulation in platelet-poor plasma by adding a reagent giving final concentrations of 5 pM TF and 4  $\mu$ M phospholipids. Fluorescence was measured at 390/460 nm every 30 seconds over a period of 40 minutes. Both calibrator and thrombin generation were analyzed in triplicate using commercially available Thrombinoscope<sup>TM</sup> software, and we calculated both mean levels and standard deviation. We derived the following variables from the thrombin generation curves: lag time (initiation phase, time to reach one-sixth of the peak height), ETP (total amount of thrombin formation over time which is measured as area under the curve), peak height (i.e. peak thrombin concentration attained) and time to peak (i.e. time to peak thrombin concentration), as shown in figure 6. The CAT assay is convenient to perform and it takes only one hour to run 16 samples in a microplate. The method has acceptable reproducibility, with an inter-assay coefficient of variation (CV) less than 5%.

**Figure 6.** A typical thrombin generation curve showing the main variables of the thrombogram.



Obtained with permissions from Haematologica/the Hematology Journal website <http://www.haematologica.org><sup>179</sup>.

Thrombin generation was measured in each plasma sample in both the presence and absence of 4.8 nM/mL APC. This is the concentration of APC sufficient to obtain an 80-90% decrease in ETP. The ETP-based APC sensitivity ratio (APCsr) is defined as the ETP in the presence of APC divided by the ETP in the absence of APC. To reduce variation between assays, results are expressed as normalized APCsr by dividing the APCsr of the sample by the APCsr of pooled normal plasma<sup>180</sup> as described below:

$$\frac{\left( \frac{\text{Sample}_{\text{ETP} + \text{APC}}}{\text{Sample}_{\text{ETP} - \text{APC}}} \right)}{\left( \frac{\text{NPP}_{\text{ETP} + \text{APC}}}{\text{NPP}_{\text{ETP} - \text{APC}}} \right)} = \text{nAPCsr}_{\text{ETP}}$$

### 3.4.2 Overall Haemostasis Potential

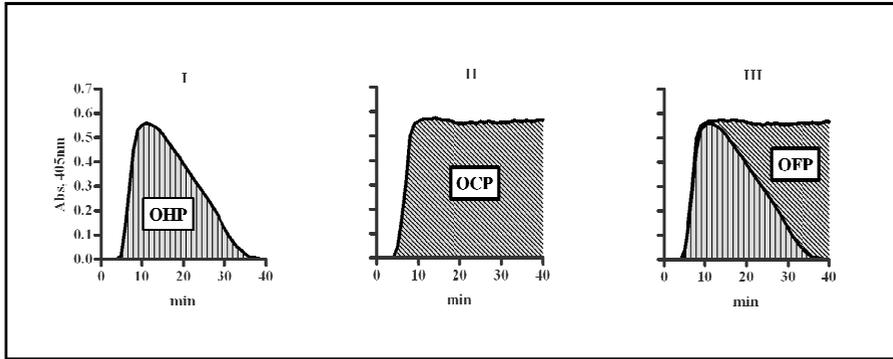
In Study II we used the Overall Haemostasis Potential (OHP) assay described by He et al. This is a functional method which assesses both fibrin formation and lysis<sup>77</sup>. The OHP curve is an absorbance curve formed by repeated spectrophotometric registration of fibrin fibril formation after adding triggers of coagulation (calcium and thrombin) and fibrinolysis (recombinant tPA) to citrated plasma in the well.

The OHP curve forms when both triggers of coagulation and fibrinolysis are present in the sample. The Overall Coagulation Potential (OCP) forms when only triggers of coagulation are present.

Absorbance (Abs) at 405 nm was measured every minute for 40 minutes to construct the two fibrin aggregation curves (OCP and OHP). The area under the curve was expressed by a summation of the Abs values (Abs-sum). The difference between the

two areas reflects the overall fibrinolysis potential (OFP), calculated by  $OFP (\%) = ((OCP - OHP) / OCP) \times 100$  (figure 7). OFP values were expressed as percentages.

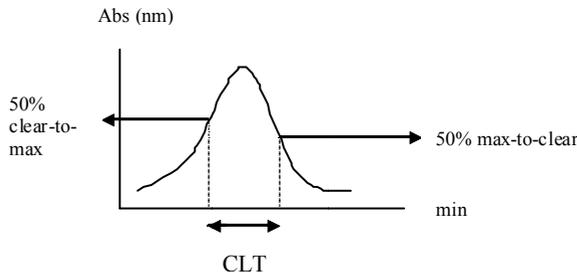
**Figure 7.** Absorbance curves of OHP (overall haemostasis potential), OCP (overall coagulation potential) and OFP (overall fibrinolytic potential).



From Antovic et al. with permissions from publisher<sup>170</sup>.

The OHP assay is easy to perform. By using a spectrophotometric reader about 30 patient plasma samples can be analyzed within one hour. The CVs are low (inter-assay CV 4%, intra-assay CV 3%)<sup>181</sup>. The method is described more in detail in Paper II. Clot lysis time as part of the OHP assay is the estimated time from 50% clear-to-max to 50% max-to-clear turbidity (figure 8).

**Figure 8.** Presentation of the clot lysis time calculation.



### 3.4.3 Fibrin network

Blombäck et al. developed the fibrin network assay<sup>182</sup> and He et al. modified the method further so that it required a smaller plasma volume (250  $\mu$ l instead of 3000  $\mu$ l)<sup>173</sup> and the reproducibility was improved<sup>183</sup>.

The method is qualitative and based on the principle that a fibrin gel is triggered to be formed in a citrated plasma sample by addition of thrombin and calcium.

In brief, a 10 µl TRIS buffer containing 0.42 mol/l CaCl<sub>2</sub> and 8.3 IU/ml thrombin, giving final concentrations of 20 mmol/l and 0.4 IU/ml, respectively, was added to 200 µL dialyzed plasma in a small plastic cylinder (2.5 cm long, opening area 0.1 cm<sup>2</sup>). The fibrin gel cylinder was kept in a standing position in a wet box at room temperature overnight.

Permeability (porosity) measurements of the gel were carried out through measurements of the volume of a buffer (pH 7.4, 0.02 mol/L TRIS, 0.02 mol/L imidazol, 0.1 mol/L NaCl) percolated through the gel under different hydrostatic pressures. The permeability coefficient (Ks) was calculated using the equation by Carr et al. from 1977<sup>184</sup>. The method is both labour and time consuming and it takes one working day to analyze 8-10 samples. The method is described in detail in Paper II.

### **3.5 ENDOTHELIAL RELATED FACTORS**

#### **3.5.1 Von Willebrand factor**

VWF antigen was measured in plasma using the LIATEST from Diagnostica Stago (Asnieres, France) and assayed using BCS equipment (Dade Behring, Marburg, Germany) at the Clinical chemistry laboratory of Karolinska University Hospital, Solna. The measurement procedure was performed according to the manufacturer's instructions<sup>185, 186</sup>. Thus, latex particles covered with antibodies against VWF are mixed with the sample building a complex consisting of antigen (VWF) and antibody latex particle. A monochromatic light with the wavelength 570 nm is sent through the suspension. If there is much antigen in the sample, there will also be more complexes resulting in more absorption of light. The increased speed of the light absorption is direct proportional to the VWF concentration in plasma.

VWF activity was assayed as ristocetin cofactor activity in plasma using the BC von Willebrand Reagent from Siemens Healthcare Diagnostics (Deerfield, ILL, USA) at the Clinical chemistry laboratory, Karolinska University Hospital, Solna. The basic principle of the test is to measure the ability of VWF to agglutinate platelets by binding to glycoprotein 1b (GP1b) receptor in the presence of ristocetin (an antibiotic that binds to both VWF and GP1b).

#### **3.5.2 ADAMTS13**

In study I we measured ADAMTS13 activity and antigen concentrations in plasma using the TECHNOZYM ADAMTS13 kit (Technoclone GmbH, Vienna, Austria).

Measurements were carried out at the Clinical Research Center at Danderyd Hospital. The method is based on the fluorogenic method described by Kokame et al. (155) and uses a small fluorogenic substrate for ADAMTS13 called FRETs-VWF73, a VWF-fragment consisting of 73 amino acids. Plasma is incubated in micro wells coated with a monoclonal antibody specific for the extra cellular domain of ADAMTS13 which is thus attached to the wall of the well. After a washing procedure the substrate is added. This consists of both a fluorescent part and a part that extinguishes fluorescence. If the substrate is degraded by ADAMTS13 fluorescence is emitted and detected. The rate of fluorescence development is used to kinetically measure the ADAMTS13 activity, incubated at 30 °C and assayed at 360/460 nm. The antigen concentration is measured in the same samples by incubation with peroxidase labelled monoclonal antibodies towards ADAMTS13.

Results are reported as a percentage of those from a pool of plasma from >100 healthy individuals. According to the manufacturer the reference interval for ADAMTS13 antigen concentration is 75-110% and for ADAMTS13 activity concentration 50-110% of the pool.

The CV for duplicates should not exceed 15% according to the manufacturer. The assay was run using a Tecan Infinite M220 multireader (Männedorf, Switzerland).

The uncertainty was estimated from duplicate measurements of 48 samples in a study performed at our lab, and found to be 10.8% (SD), CV 9.6%, for ADAMTS13 activity and 6.4% (SD), CV 7.1%, for ADAMTS13 antigen<sup>187</sup>.

### **3.6 OTHER LABORATORY METHODS**

Commercially available kits and calibrators were used to measure all the quantities below, and the measurement procedures were carried out according to the manufacturer's instructions and performed at the Clinical chemistry laboratory of Karolinska University Hospital, Solna:

#### **3.6.1 FVIII**

FVIII was measured using the COAMATIC FVIII reagent from Haemochrom Diagnostica (Essen, Germany).

#### **3.6.2 Fibrinogen**

Fibrinogen was analysed by Fibri-Prest Automate from Diagnostica Stago (Asnieres, France).

### 3.6.3 Routine analyses

Concentrations of serum creatinine, sodium, potassium, plasma lipids, plasma glucose, CRP and blood cell counts were all measured by routine laboratory techniques.

## 3.7 REGISTERS

For study III we performed a cross-sectional study using linkage of Swedish population and health registers. We used the same registers for the cohort in study IV.

MBR includes more than 99% of all births in Sweden since 1973 and consists of prospectively collected and validated information from the pregnancy, delivery and neonatal periods<sup>188</sup>.

The Swedish IVF Register is now a part of the Swedish Medical Birth Register (MBR) at the National Board of Health and Welfare and includes information on IVF pregnancies since 1982.

The Swedish National Patient Register encompasses both inpatients and outpatients<sup>189</sup>. Inpatient data in Sweden became available nationwide in 1987. Outpatient diagnoses from hospitals started to be collected in 1997. The register comprises date of admission and discharge, as well as main diagnosis with up to seven contributing diagnoses. Data in the Patient Register can be linked to other registers through the unique personal identity number assigned to all Swedish residents<sup>190</sup>.

Women's education was found in the Register of Education, Statistics Sweden<sup>191</sup>, by linkage through the personal identity number.

For study III + IV the diagnoses were found by linkage to the Swedish Patient Register of the National Board of Health and Welfare<sup>189</sup>. Diagnoses were recorded according to the International Classification of Diagnoses (ICD), ninth version in the time period 1990 to 1996 and the tenth version from 1997 and onwards.

For study III we used the following diagnoses for VTE: 415B, 451B, 452, 453C-D, 453W, 453X, 671D-F and 673C in ICD-9 and I26.0, I26.9, I80.1- 3, I80.8-9, I82.2-3, I82.8-9, O22.3, O22.5, O87.1, O87.3, O87.9 and O88.2 in ICD-10.

For study IV we used the diagnoses of hypertension, stroke, coronary heart disease and diabetes by the same linkage: 250, 401-405, 410, 411B, 412, 431, 433-434, 438, 642A-D, 642X and 648A in ICD-9 and E10-11, E14, I10-13, I15, I20.0, I21-23, I25.2, I61, I63, I69.3-4, O10.0-4, O11.9, O13.9, O16.9 and O24 in ICD-10.

## 4 STATISTICAL ANALYSES

An overview of the statistical analyses used in this PhD thesis is described below.

For further details see Paper I-IV, respectively:

- Students t-test – between groups with normally distributed variables, non-normal distributed data were log-transformed and checked to be normally distributed before analysis.
- Pearson’s correlation coefficient – to estimate if associations were present between normally distributed continuous variables, expressed as r values (Paper I-II).
- Multiple regression – was used to assess the relationship between a dependent variable and one or more explanatory (independent) variables.
- Cox proportional hazard regression - conditioned on matching sets was used to calculate Hazard ratios (HR) and 95% confidence intervals (95% CI) for VTE respectively PE in study III and for diagnosis events (end-points) of hypertension, diabetes, stroke and coronary heart disease in study IV. In study IV, the person-time for each woman was calculated from end of pregnancy to month of diagnosis of the end-point, month of death from other causes, emigration or to end of follow-up.

For in-house statistical calculations Statistica software, version 8, by Statsoft Inc., Tulsa, Oklahoma, USA, was used. Data are presented as mean  $\pm$  SD. Values of  $p < 0.05$  were considered statistically significant.

The conditional logistic regressions were performed in the Proportional Hazards Regression (PHREG) procedure of SAS, version 9.2, by SAS Institute Inc., Cary, NC, USA.

## 5 RESULTS

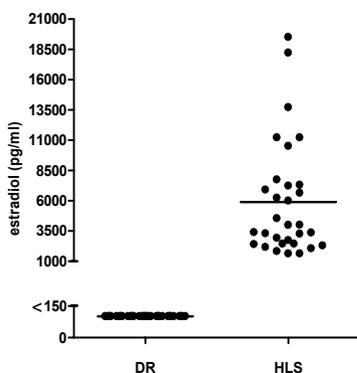
### 5.1 PAPER I + II

All patients were Caucasians in the ages 25-38 years and with normal resting blood pressures ( $\leq 140/90$  mm/Hg). None of them had a history of arterial or venous thrombosis. Basic characteristics of the patients are presented in table 2.

Indications for IVF included female infertility in seven cases (anovulation in four women, ovarian dysfunction in two women and one had endometriosis), male infertility was present in twelve cases and unexplained infertility in twelve.

Oestradiol increased 10-100-fold from  $<150$  pg/mL at DR to a mean of 5889 pg/mL at HLS (range 1620–19500 pg/mL) as illustrated in figure 9.

**Figure 9.** Oestradiol level changes from time of down-regulation (DR) to time of high level stimulation (HLS) in 31 women undergoing IVF treatment.



Twelve patients got pregnant and all except one were primigravidae. Three patients suffered a mild OHSS as defined by the Practice Committee of the American Society for Reproductive Medicine <sup>101</sup>.

#### 5.1.1 Paper I

VWF:Ag and VWF:RCoF activity increased by 41% respectively 49% ( $p < 0.001$ ) from the time of DR to HLS, and FVIII levels increased by 31% during the same time period ( $p < 0.001$ ). These changes were accompanied by a concomitant 6% decrease in ADAMTS13 antigen ( $p < 0.05$ ) and a 9% decrease in ADAMTS13 activity ( $p < 0.01$ , table 3). The relative changes in ADAMTS13 antigen and activity from DR to HLS correlated significantly ( $r = 0.7$ ,  $p < 0.001$ ).

**Table 2.** Baseline characteristics of the 31 women enrolled in the study.

	<b>Patients</b>	<b>Normal range</b>
<b>Age, years</b>	33.0 ( $\pm$ 3.3)	
<b>Body mass index, kg/m<sup>2</sup></b>	24.1 ( $\pm$ 3.6)	<25
<b>Waist-hip ratio</b>	0.8 ( $\pm$ 0.1)	<0.8
<b>Causes and type of infertility</b>		
<b>Female, n (%)</b>	7 (22.6%)	
<b>Male, n (%)</b>	12 (38.7%)	
<b>Unknown, n (%)</b>	12 (38.7%)	
<b>Current smoking, n (%)</b>	2 (11.1%)	
<b>Triglycerides, mmol/l</b>	0.8 ( $\pm$ 0.4)	0.45-2.6
<b>Total cholesterol, mmol/l</b>	4.9 ( $\pm$ 0.7)	3.3-6.9
<b>LDL cholesterol, mmol/l</b>	2.9 ( $\pm$ 0.7)	1.4-4.7
<b>Creatinine, mmol/l</b>	68.9 ( $\pm$ 7.3)	<90
<b>Plasma glucose, mmol/l</b>	4.7 ( $\pm$ 0.5)	4.0-6.0
<b>Haemoglobin, g/L</b>	129.0 ( $\pm$ 8.6)	117-153
<b>Haematocrit, %</b>	0.39 ( $\pm$ 0.02)	0.35-0.46
<b>Platelet count, x10<sup>9</sup>/L</b>	251.2 ( $\pm$ 47.6)	165-387

Data are presented as mean ( $\pm$ SD) or n (%).

We observed an inverse significant correlation between the concentration of VWF:Ag and the concentration of ADAMTS13 antigen and ADAMTS13 activity at the time of HLS ( $r=0.6$ ,  $p=0.015$  respectively  $r=0.5$ ,  $p=0.026$ ).

The three OHSS patients had a 64% increase in VWF:Ag and a 63% increase in VWF:RCoF activity level from the time of DR to the time of HLS. FVIII increased 56% during the same time period. These changes were accompanied by a 9% decrease in ADAMTS13 antigen and a 14% decrease in ADAMTS13 activity.

Nine patients had blood group A (33%), eight had blood group AB (30%), seven had blood group O (26%) and three had blood group B (11%). In four of the women the blood group was unknown. We had few women with blood group O and a high percentage of women with blood group AB, which is probably a chance phenomenon due to the limited number of patients. The women with blood group O seemed to have lower VWF levels as compared to group mean; however, conventional statistical tests were not performed due to the low number of cases.

**Table 3.** Changes in haemostatic variables during IVF.

	<b>Down regulation</b>	<b>High level stimulation</b>	<b>p-value</b>	<b>Reference interval</b>
VWF:Ag, kIU/L	0.75 (±0.22)	1.06 (±0.40)	<0.001	0.6 - 1.6
VWF:RCoF activity, kIU/L	0.83 (±0.26)	1.24 (±0.48)	<0.001	0.5 - 1.5
ADAMTS13 antigen, %	72.2 (±13.5)	67.9 (±9.9)	<0.05	75 - 110
ADAMTS13 activity, %	88.6 (±18.3)	80.8 (±15.7)	<0.01	50 - 110
Factor VIII, kIU/L	0.96 (±0.34)	1.26 (±0.41)	<0.001	0.5 - 1.8
Fibrinogen, g/L	2.8 (±0.7)	3.3 (±0.7)	<0.001	2 - 4
Fibrin gel permeability, Ks	9.5 (±3.5)	8.6 (±3.0)	0.13	9.3 - 11.1
OHP, Abs sum	7.7 (±2.9)	10.2 (±3.4)	<0.001	4.2 - 14.5
OCP, Abs sum	15.3 (±5.4)	19.5 (±5.5)	<0.001	6.8 - 20.0
OFP, Abs sum	48.2 (±11.7)	47.5 (±11.8)	0.75	16.5 - 52.0
Clot lysis time, min	16.4 (±3.1)	17.1 (±3.7)	0.24	-
ETP, nM IIa*min	1542 (±287)	1739 (±288)	<0.001	1430 - 2273
Lag time, min	2.7 (±0.6)	2.5 (±0.5)	0.001	2.1 - 2.9
Peak, nM IIa	290 (±42)	343 (±38)	<0.001	270 - 395
ttPeak, min	5.3 (±0.7)	4.8 (±0.6)	<0.001	4.4 - 5.7
nAPCsr	1.4 (±0.7)	2.1 (±0.6)	<0.001	2.3 - 2.6

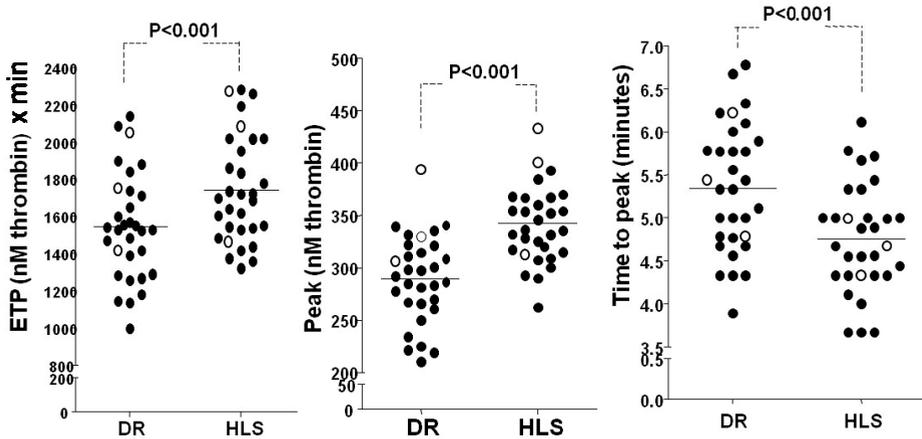
### 5.1.2 Paper II

As shown in figure 10, the thrombin generation variables ETP ( $p<0.001$ ) and peak height ( $p<0.001$ ) were significantly raised at HLS as compared to DR. Concurrently there were decreases in lag time ( $p=0.001$ ) and time to peak ( $p<0.001$ ). We observed a 32% increase in OHP ( $p<0.001$ ) and a 27% increase in OCP ( $p<0.001$ ) whereas OFP remained almost unchanged. Regarding fibrin gel permeability only a small (-9%) statistically non-significant reduction in permeability (Ks) was observed ( $p=0.13$ ). Fibrinogen and FVIII concentrations in plasma increased by 19% ( $p<0.001$ ) respectively 30% ( $p<0.001$ ) from time of DR to HLS. All changes in haemostatic variables during IVF are shown in table 3.

Although significant changes toward a procoagulable state were observed in the variables of thrombin generation as well as of fibrin formation, all remained within the reference interval. The significant results persisted after exclusion of three patients who later developed OHSS.

Multiple regression analyses showed that the change in FVIII from DR to HLS explained a part of the change in OHP ( $r=0.49$ ,  $p=0.024$ ), whereas ETP could explain a part of the variation in OCP ( $r=0.70$ ,  $p<0.001$ ). Further multiple regression analyses showed that fibrinogen and FVIII explained a part of the variation in peak height ( $r=0.47$ ,  $p=0.031$ ). At the time of HLS, plasma fibrinogen significantly explained a part of the variation in OHP ( $r=0.65$ ,  $p=0.002$ ).

**Figure 10.** Changes in ETP, peak and time to peak in 31 IVF women.

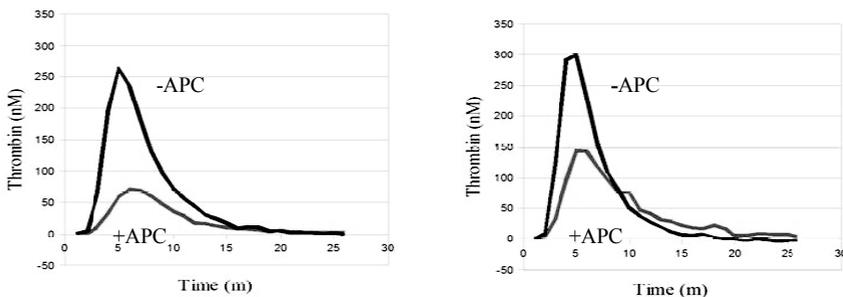


- DR time of down- regulation
- HLS time of high level stimulation
- not ovarian hyperstimulation syndrome
- ovarian hyperstimulation syndrome
- mean

ETP and peak thrombin height were correlated both at time of DR and HLS ( $r=0.85$ ,  $p<0.001$  respectively  $r=0.80$ ,  $p<0.001$ ).

When adding APC to the plasma samples we observed an acquired resistance to APC during IVF treatment (figure 11, unpublished data).

**Figure 11.** One patient's thrombin generation curve at downregulation (left) and high level stimulation (right) in the presence and absence of APC.



## 5.2 PAPER III + IV

We included 23,498 women with pregnancies after IVF (exposed women) and because we matched them by maternal age, the included women with pregnancies after natural

conception (unexposed) had an almost identical mean age ( $33.3 \pm 4.0$  and  $33.4 \pm 3.9$ , respectively).

The proportion of multiple births in the women who had undergone IVF was 16.9%. Women in the IVF group had a higher educational level (47.1% had >12 years of education), were in a less frequency smokers (6.7%) and a higher proportion of them were born in Sweden (86%) as compared to the control group. See table 4 for further details about the two groups of women.

**Table 4.** Characteristics of women from IVF register and Medical Birth Register (MBR) during the time period 1990–2008.

	Women from IVF register	Women from MBR
<b>Total number</b>	n=23,498	n=116,960
Mean age ( $\pm$ SD)	33.3 ( $\pm$ 4.0)	33.4 ( $\pm$ 3.9)
Median age (quartiles)	33 (31;36)	33 (31;36)
<b>Single/multiple birth</b>		
Single birth	83.1%	97.4%
Multiple births	16.9%	2.6%
<b>Mothers born in Sweden</b>	86.2%	81.4%
<b>Prepregnancy BMI</b>		
Missing	16.9%	17.3%
<25	53.2%	53.4%
25-29	21.9%	21.1%
$\geq$ 30	8.1%	8.2%
<b>Smoking habits</b>		
Missing	7.6%	6.6%
No	85.7%	82.9%
<10 cigarettes/day	4.9%	6.7%
$\geq$ 10 cigarettes/day	1.8%	3.8%
<b>Education (years)</b>		
Missing	0.2%	0.9%
$\leq$ 9	7.6%	10.0%
10-12	45.1%	43.2%
>12	47.1%	45.9%
<b>PCOS, n (%)</b>	788 (3.4%)	563 (0.5%)
<b>Preeclampsia, n (%)</b>	1646 (7.0%)	6117 (5.2%)
<b>Hypothyroidism, n (%)</b>	447 (1.9%)	1790 (1.5%)

### 5.2.1 Paper III

The proportion of VTE in the exposed group was 4.2/1000 (n=99) as compared to 2.5/1000 (n=291) in the unexposed women (table 5).

**Table 5.** Venous thromboembolism and pulmonary embolism events in the two groups of pregnant women expressed as numbers and percentages.

<b>Events in relation to pregnancy</b>	<b>IVF pregnancies n (%)</b>	<b>Normal pregnancies n (%)</b>	<b>Proportional hazard regression (95% CI)</b>
<b>Venous thromboembolism:</b>			
Prepregnancy	71 (0.30)	415 (0.35)	0.85 (0.66 - 1.10)
Pregnancy and delivery	99 (0.42)	291 (0.25)	1.77 (1.41 - 2.23)
Days 43-365 postpartum	24 (0.10)	95 (0.08)	1.29 (0.82 - 2.02)
<b>Pulmonary embolism:</b>			
Prepregnancy	21 (0.09)	103 (0.09)	1.04 (0.65 - 1.66)
Pregnancy and delivery	19 (0.08)	70 (0.06)	1.42 (0.86 - 2.36)
Days 43-365 days postpartum	3 (0.01)	26 (0.02)	0.60 (0.18 - 1.98)

As shown in table 5, the incidence of VTE after IVF was increased during all pregnancy ( $P < 0.001$ ; HR 1.77, 95% CI 1.41-2.23) and differed between the trimesters ( $P = 0.002$ , figure 12 and table 6). The risk was in particular increased during the first trimester (1.5/1000 v 0.3/1000, HR 4.05, 95% CI 2.54-6.46). The risk did not differ between the two groups of women before pregnancy (HR 0.85, 95% CI 0.66-1.10) and during the year after delivery (HR 1.29, 95% CI 0.82-2.02).

PE occurred in 19 women in the IVF group (8.1/10,000) as compared to 70 of the matched women (6.0/10,000). The incidence was increased after IVF ( $P < 0.01$ ; HR 1.42, 95% CI 0.86-2.36) and differed between the trimesters ( $P = 0.0092$ ). The incidence was in particular increased during the first trimester (3.0/10 000 v 0.4/10 000, HR 6.97, 95% CI 2.21-21.96), see figure 12 and table 7.

**Table 6.** Time to first venous thromboembolic event by trimester in the two groups of women. The effect of different levels of the effect modifier body mass index (BMI) is also given.

	IVF pregnancies (n=23 498)	Normal pregnancies (n=116 960)	Hazard ratio (95% CI)	Proportional hazard regression (95% CI)*			
				Total	BMI <25	BMI 25-29.9	BMI >30
First trimester	36 (0.15)	38 (0.03)	4.61 (2.95 - 7.21)	4.05 (2.54-6.46)	6.64 (3.6-12.23)	2.61 (0.97-7.07)	1.01 (0.22 - 4.6)
Second trimester	23 (0.10)	63 (0.05)	1.00 (0.51 - 1.97)	1.11 (0.54 - 2.29)	1.04 (0.40- 2.72)	2.13 (0.66 - 6.93)	—†
Third trimester	33 (0.14)	94 (0.08)	1.04 (0.64 - 1.69)	1.30 (0.77-2.19)	1.30 (0.61- 2.80)	1.80 (0.81-4.01)	0.26 (0.04 - 1.91)
Three days before to 42 days after birth	49 (0.21)	192 (0.16)	1.69 (1.15 - 2.48)	1.59 (1.03- 2.45)	1.66 (0.90 - 3.05)	1.55 (0.75-3.24)	1.45 (0.49 - 4.27)
P value for test of difference‡	—	—	—	<0.001	<0.001	0.07	0.53
P value for test of equal effect in all time periods§	—	—	—	0.002	0.0006	0.86	0.33

BMI=body mass index.

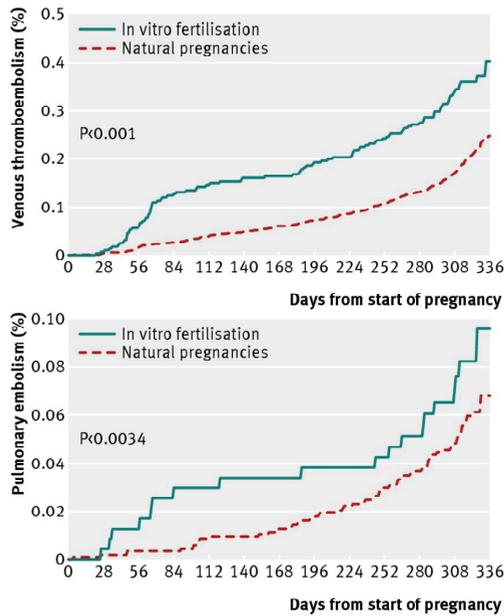
\*Women with complete data on BMI.

†Weeks 13-25 are merged with week 26 until three days before delivery owing to few events.

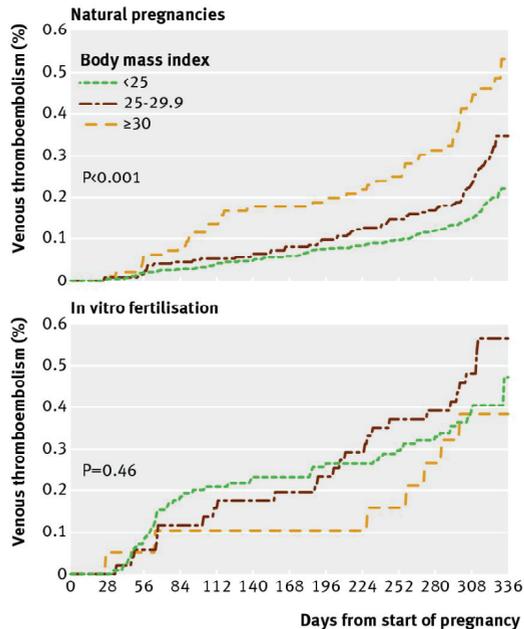
‡Wald  $\chi^2$  test for difference between IVF and no IVF.

§Wald  $\chi^2$ .

**Figure 12.** Proportional hazard regression of venous thromboembolism and pulmonary embolism in pregnant women after IVF (n=23 498) and in natural pregnancies (n=116 960).



**Figure 13.** Proportional hazard regression of venous thromboembolism in three strata for body mass index (<25, 25-29.9, and  $\geq 30$ ) in pregnant women after IVF (n=23 498) and in women with natural pregnancies (n=116 960).



**Table 7.** Time to first event of pulmonary embolism in the two groups of women. Values are numbers (percentages) unless stated otherwise.

<b>Variables</b>	<b>IVF Pregnancies (n=23 498)</b>	<b>Normal pregnancies (n=116 960)</b>	<b>Proportional hazard regression (95% CI)</b>
First trimester (weeks 1-12)	7 (0.03)	5 (0.004)	6.97 (2.21 - 21.96)
Second trimester (weeks 13-25)	5 (0.02)	13 (0.01)	0.42 (0.05 - 3.20)
Third trimester (week 26 to <3 days before birth)	6 (0.03)	18 (0.02)	0.40 (0.10 - 1.68)
≤3 days before to 42 days after birth	11 (0.05)	49 (0.04)	1.79 (0.86 - 3.74)
P value for test of difference*	—	—	0.0034
Test of equal effect in all time periods†	—	—	0.0092

\*Wald  $\chi^2$  for difference between IVF and controls.

†Wald  $\chi^2$  test.

No significant interaction was observed between BMI and IVF concerning incidence of VTE (P=0.21). The incidence in women who did not undergo IVF, however, increased as expected by BMI level (P<0.001, figure 13), but no such effect was observed in women after IVF (P=0.46, figure 13).

Further multivariable analyses taking calendar period, parity, single or multiple births, smoking, education, maternal age, country of birth, and marital status into account were carried out and we stratified on BMI in two categories: <25 and 25-29.9 (table 8). These adjustments did not alter the significance of the main finding.

**Table 8.** Multivariable analysis stratified on body mass index (BMI) in pregnant women with BMI <30.

Variables	Hazard ratio (95% CI)		
	BMI <25	BMI 25-29.9	Adjusted by conditioning on BMI
Normal pregnancy	1=reference	1=reference	1=reference
<b>IVF pregnancy:</b>			
First trimester	5.21 (2.68 - 10.14)	2.40 (0.85 - 6.80)	4.13 (2.37 - 7.17)
Second trimester	0.91 (0.34 - 2.46)	1.78 (0.53 - 5.93)	1.18 (0.55 - 2.51)
Third trimester	1.17 (0.53 - 2.58)	1.38 (0.57 - 3.33)	1.25 (0.69 - 2.25)
Three days before to 42 days after birth	1.10 (0.56 - 2.17)	1.27 (0.59 - 2.75)	1.16 (0.69 - 1.93)
P value for test of difference*	<0.001	0.77	<0.001
Test of equal effect in all time periods†	0.001	0.42	0.0017
<b>No of older siblings:</b>			
None	1=reference	1=reference	—
≥1	0.92 (0.65 - 1.30)	1.36 (0.58 - 3.19)	0.83 (0.63 - 1.1)
Single birth	1=reference	1=reference	
<b>Multiple births</b>	2.52 (1.49 - 4.25)	1.33 (0.57 - 3.13)	2.06 (1.32 - 3.21)
<b>Smoking at start of pregnancy:</b>			
No	1=reference	1=reference	—
Yes	0.91 (0.49 - 1.68)	0.89 (0.45 - 1.77)	0.89 (0.56 - 1.41)
<b>Education (years):</b>			
≤9	1=reference	1=reference	—
10-12	1.16 (0.57 - 2.39)	0.55 (0.27 - 1.12)	0.88 (0.53 - 1.44)
>12	1.26 (0.61 - 2.59)	0.80 (0.40 - 1.62)	1.06 (0.64 - 1.75)
<b>Maternal age at delivery (years):</b>			
<35	1=reference	1=reference	—
≥35			
First trimester (weeks 1-12)	0.84 (0.43 - 1.63)	2.34 (0.85 - 6.45)	1.14 (0.67 - 1.96)
Second trimester (weeks 13-25)	0.60(0.25 - 1.43)	2.25 (0.73 - 6.88)	0.92 (0.48 - 1.75)
Third trimester (week 26 to <3 before birth)	0.60(0.30 - 1.23)	1.20 (0.58 - 2.49)	0.84 (0.51 - 1.38)
≤3 before to 42 days after birth	1.53 (0.91 - 2.57)	2.64 (1.41 - 4.96)	1.88 (1.26 - 2.78)
<b>Country of birth:</b>			
Sweden	1=reference	—	—
Other country	1.14 (0.72 - 1.83)	3.06 (1.45 - 6.44)	1.62 (1.09 - 2.41)
<b>Calendar period:</b>			
1990-2001	1=reference	1=reference	1=reference
2002-2008	1.55 (1.12 - 2.14)	2.64 (1.64 - 4.25)	1.86 (1.43 - 2.43)
<b>Marital status:</b>			
Cohabiting with father of child	1=reference	1=reference	1=reference
Other	0.81 (0.29 - 2.21)	1.34 (0.53 - 3.36)	1.03 (0.52 - 2.03)

\*Wald  $\chi^2$  for difference between IVF and no IVF.

†Wald  $\chi^2$ .

## 5.2.2 Paper IV

We used the same cohort of women as in study III, and the baseline characteristics of the study sample from 1990 to 2008 are thus given in table 4.

Less than 3% women emigrated and 0.5% died during follow-up. The mean follow-up time in the IVF and control group was  $8.6 \pm 4.6$  respectively  $8.6 \pm 4.9$  years, contributing to 201 498 respectively 1 000 368 person-years at risk (table 9).

**Table 9.** Follow-up time and drop-out frequency of the women in the study.

	IVF women		Control women	
	n	%	n	%
<b>Total number</b>	23 498		116 960	
<b>Possible to follow until end of follow-up</b>	22 911	97.5	113 158	96.8
<b>Emigrated</b>	471	2.0	3 215	2.8
<b>Deceased</b>	116	0.5	587	0.5
<b>Total years of follow-up</b>	201 948		1 000 368	
<b>Mean follow-up in years (<math>\pm</math>SD)</b>	8.6 ( $\pm$ 4.6)		8.6 ( $\pm$ 4.9)	

During this period, 823 women developed a first event (diabetes, n=201; hypertension, n=520; coronary heart disease, n=23; stroke, n=79). As shown in table 10, the proportion of women with hypertension and stroke was slightly greater in the IVF group as compared to the MBR controls.

Multivariable analysis adjusted for BMI, smoking, country of birth and educational level showed a higher incidence of hypertension among IVF women (HR 1.27, 95% CI 1.13-1.41) as compared to mothers after natural conception (table 11). There was a trend towards a higher risk of stroke (HR 1.27, 95% CI 0.96-1.68) but the incidence of coronary heart disease or diabetes did not differ after IVF pregnancies as compared to the controls (HR 0.72, 95% CI 0.44-1.17 and HR 0.96, 95% CI 0.81-1.14).

When looking at the whole group of women, those who were smoking more than 10 cigarettes on a daily basis had a much higher risk of coronary heart disease as compared to non-smokers (HR 5.46, 95% CI 3.55-8.40). Even women smoking less than 10 cigarettes daily had a higher risk compared to the non-smokers (HR 4.16, 95% CI 2.81-6.18).

**Table 10.** Total number and incidence per 10.000 person-years of diabetes, hypertension, coronary heart disease and stroke in women from the IVF and Medical Birth Register 1990-2008.

	IVF women			Control women		
	n	Incidence per 10.000 person-years	95% CI	n	Incidence per 10.000 person-years	95% CI
	23498			116960		
<b>Diabetes</b>	201	9.95	8.65-11.4	1 111	11.1	10.47-11.77
<b>Hypertension</b>	520	2.58	2.36-2.80	2 129	2.13	2.04-2.22
<b>Coronary heart disease</b>	23	1.14	0.74-1.68	185	1.85	1.60-2.13
<b>Stroke</b>	79	3.91	3.12-4.85	319	3.19	2.85-3.55
<b>Total number</b>	823	4.08	3.80-4.36	3744	3.74	3.62-3.86

Furthermore, as expected, an increase in BMI resulted in a higher incidence of CVD. A BMI higher than 30 increased the incidence of diabetes (HR 5.25, 95% CI 3.09-7.07), coronary heart disease (HR 5.23, 95% CI 2.34-11.72) and hypertension (HR 3.84, 95% CI 3.07-4.82), whereas stroke incidence was not affected (HR 1.15, 95% CI 0.70-1.90). All univariable and multivariable analyses are shown in table 11.

**Table 11.** CVD hazard ratios (HR) and 95% confidence intervals (95% CI) among Swedish women giving their first delivery from 1990 to 2008 with additional adjustments for BMI, smoking, country of birth and educational level.

		<b>Diabetes</b>	<b>Hypertension</b>	<b>Coronary heart disease</b>	<b>Stroke</b>
		HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
<b>Univariable analysis</b>					
MBR group	1=reference				
IVF group		0.90 (0.77-1.05)	1.24 (1.13-1.37)	0.65 (0.42-1.00)	1.24 (0.97-1.58)
<b>Univariable analysis with exclusion<sup>^</sup></b>					
MBR group	1=reference				
IVF group		0.90 (0.76-1.07)	1.26 (1.13-1.41)	0.64 (0.39-1.05)	1.22 (0.92-1.61)
<b>Multivariable analysis*</b>					
MBR group	1=reference				
IVF group		0.96 (0.81-1.14)	1.27 (1.13-1.41)	0.72 (0.44-1.17)	1.27 (0.96-1.68)
<b>Country of birth</b>					
Sweden	1=reference				
Other		2.14 (1.86-2.46)	0.99 (0.87-1.11)	1.21 (0.81-1.80)	1.40 (1.05-1.87)
<b>BMI before pregnancy</b>					
<20	1=reference				
20-24.9		1.00 (0.75-1.35)	1.39 (1.13-1.72)	1.46 (0.67-3.20)	0.89 (0.59-1.32)
25-29.9		2.02 (1.50-2.72)	2.23 (1.8-2.77)	2.37 (1.07-5.25)	1.07 (0.70-1.64)
>30		5.25 (3.09-7.07)	3.84 (3.07-4.82)	5.23 (2.34-11.72)	1.15 (0.70-1.90)
<b>Smoking</b>					
No	1=reference				
<10 cigarettes/day		0.88 (0.69-1.11)	1.06 (0.90-1.25)	4.16 (2.81-6.18)	1.12 (0.74-1.70)
≥10 cigarettes/day		1.31 (1.02-1.68)	1.25 (1.03-1.53)	5.46 (3.55-8.40)	1.83 (1.18-2.83)
<b>Education</b>					
Primary school		1.37 (1.15-1.62)	1.10 (0.95-1.28)	1.48 (1.00-2.21)	1.43 (0.8-2.58)
High school	1=reference				
More than 12 years		0.79 (0.68-0.91)	1.10 (1.00-1.21)	0.95 (0.64-1.42)	1.08 (0.85-1.37)

<sup>^</sup> Univariable analysis with exclusion of women lacking information on smoking and BMI

\* Multivariable analysis with additional adjustments for BMI, smoking, country of birth and educational level.

## 6 GENERAL DISCUSSION

Both venous and arterial thrombotic diseases pose major health problems. The four studies in this thesis comprise different perspectives on thromboembolism with aspects from the fields of internal medicine, cardiovascular epidemiology, gynaecology and laboratory medicine.

### 6.1 VENOUS THROMBOEMBOLISM DURING IVF

We identified an increased incidence of VTE during all trimesters of pregnancies after IVF as compared to during the trimesters of pregnancies after natural conception. The risk was in particular pronounced during the first trimester.

The distribution of PE and VTE during the trimesters after IVF (figure 12 and table 6) contrasted to that after natural conception. The risk of VTE and PE after natural conception is at its highest during the postpartum period<sup>192-194</sup>.

Numerous case reports of VTE during IVF pregnancies have been published and reviewed repeatedly<sup>25, 106, 195</sup>. One of the main reasons for the attention of these case reports is the localization of thromboses at unusual sites, such as in vessels of the upper extremities and neck. A general statement in these reviews has been that the risk of VTE is comparable to that of normal pregnancy<sup>106</sup>. However, this contention is clearly rejected by the present thesis as we found a significantly increased incidence of VTE during all trimesters and in particular during the first trimester. The close time relation to the IVF procedure suggests that changes induced by the procedure by itself could be of pathophysiological importance. A plausible initiator of adverse mechanisms could be the noticeable increase in endogenous oestrogen levels during the stimulation phase of treatment before the actual IVF procedure<sup>25</sup>.

In our study we did a separate analysis of PE cases. The risk of PE in women after IVF was increased almost sevenfold during the first trimester, although the absolute risk was low (2-3 additional cases of PE per 10 000 pregnancies). PE is a leading cause of maternal death<sup>95, 96</sup>, and our finding is therefore important to clinicians consulted by women who are pregnant after IVF.

Use of exogenous oestrogens has consistently been associated with an increased incidence of VTE, irrespective of the indication of its use. The first adverse report on the use of this hormone was in women using oestrogen containing contraceptive pills<sup>196</sup> and recently also in women using oestrogen after menopause<sup>197, 198</sup>. The risk of

VTE in the largest randomized study was doubled as compared to placebo (HR 2.06; 95% CI 1.57-2.70)<sup>197</sup>. Notably, increased cardiovascular events have been shown also in males receiving oestrogen due to testosterone dependent prostatic carcinoma<sup>199</sup>.

The validity of the Patient Register is generally considered to be high, and has previously been used to estimate the incidence of VTE during pregnancy during the time period 1990-93<sup>16, 189, 190</sup>. We found a similar incidence as Lindqvist et al.<sup>16</sup> in unexposed women during that time period in the present study. However, as earlier mentioned, VTE events in both exposed and unexposed women seem to increase during the first decade of the new millennium. A contributory reason for this could be the inclusion of outpatient diagnoses. There could also be an increased awareness of VTE during pregnancy. A high baseline index for suspicion of VTE during pregnancy is critical for diagnosis because many of the clinical signs and symptoms of VTE are common also in normal pregnancies<sup>194, 200</sup>. Improvement in diagnostic procedures, including a more extensive use of ultrasound examinations, has probably contributed to the increased incidence of VTE<sup>194</sup>.

A limitation of MBR is that it only includes women with deliveries. Thus, an obvious bias is that more complicated pregnancies resulting in fatal outcomes to the mothers were not included. This might, in fact, result in an underestimation of the true risk of VTE and in particular PE during IVF pregnancy.

A further potential weakness could be an influence of parity on the propensity to suffer thrombosis. However, primigravidae also had an increased risk of VTE as compared to normal pregnancy. Another bias could be the presence of thrombophilia including the antiphospholipid syndrome as this might influence fertility<sup>201, 202</sup>.

The risk of VTE increased as expected by level of BMI in the control group but this relation was not found in IVF women (figure 13). This latter finding was unexpected since increased BMI is known to increase the risk of VTE. We speculate whether direct action and production of oestrogen in fat tissue in obese women might cause a higher basic oestrogen level and therefore a less pronounced “oestrogenic change” during IVF treatment<sup>203</sup>. Furthermore, VTE might be overrepresented in women with OHSS due to capillary leakage and haemoconcentration<sup>102</sup> and OHSS is more prevalent in lean women<sup>101, 105</sup>.

## **6.2 HAEMOSTATIC DISTURBANCES DURING PREGNANCY AND IVF**

Studies of individual coagulation factors disclose a clear increase in VWF, FVIII, fibrinogen, as well as increased APC resistance, together with reduced AT, proteins C and S activity when ovarian stimulation occurs<sup>141, 146, 204-206</sup>. Our results, showing increased levels of FVIII, VWF, fibrinogen and nAPCsr during IVF are therefore not surprising. Totally, the individual factors seem to change towards a procoagulable state during IVF.

## **6.3 GLOBAL HAEMOSTASIS ASSAYS**

Study II is the first study in which the global haemostasis assays CAT, OHP and the fibrin gel permeability assay have been investigated and compared during IVF treatment. We were able to demonstrate a highly significant increase in ETP and OHP concomitant with an approximately 38 times increase in circulating oestradiol concentration from time of DR to HLS.

We investigated two parts of the coagulation process, i.e. thrombin generation (CAT assay) and fibrin formation (OHP assay). The OHP and CAT assays differ both in the end product of measurement but also in the use of different triggers of coagulation. The OHP assay uses thrombin whereas the CAT assay uses TF as trigger. As both methods have a global approach, their responses are dependent on several factors present in the plasma sample, making it impossible to specifically delineate the mechanism. The OHP assay showed that women undergoing IVF treatment had increased fibrin formation. The CAT data indicate that this may, at least in part, be due to increased thrombin generation.

### **6.3.1 Calibrated Automated Thrombogram**

Thrombin generation is augmented during IVF as demonstrated by the increase in both ETP and peak height, together with a decreased time to thrombin generation (lag phase) and time to peak of thrombin generation. Thrombin generation results as detected by the CAT method may provide information different from measurements of single markers of ongoing thrombin generation, i.e. F1+2 or TAT, which assess the *in vivo* thrombin formation. Thus, the CAT analysis may better reflect the physiologic prothrombotic tendency by stimulation with TF, which is considered to be the main coagulation trigger. Upon adding APC to the reagents in the CAT assay, the normalized APC sensitivity ratio (nAPCsr) significantly increased from DR to HLS which

indicates that IVF treatment causes an acquired form of APC resistance. This result is in line with Curvers et al. who were able to show that nAPCsr increased slightly at DR and significantly during HLS and remained even high during luteal support<sup>52</sup>.

Some clinical studies suggest that peak thrombin concentration is the most informative variable indicating procoagulability in an individual setting<sup>207</sup>. However, there was a large interindividual variability in both ETP and peak thrombin concentration in our IVF patients. This inter individual variability could in part be due to a different pathophysiology of infertility among these women, but other clinical factors may also have a considerable influence, i.e. twin pregnancy. The analysis of these variables during the high oestrogen level phase caused by normal pregnancy, preeclampsia and oral contraceptive use showed similar patterns<sup>78, 79, 208</sup>.

There are two different CAT assay approaches, i.e. the chromogenic method with defibrinated plasma and the fluorogenic method. We used the latter more sensitive method, which assesses thrombin generation in the presence of fibrinogen. Methodological differences are also the use of platelet rich versus platelet poor plasma. The fluorogenic method uses platelet poor plasma and the thrombin generation is therefore not affected by platelet function. Instead, a standard amount of procoagulant phospholipids are added to the platelet poor plasma sample.

### **6.3.2 Overall Haemostasis Potential**

During controlled ovarian hyperstimulation in study II, OHP increased significantly from the time of DR to HLS. Change in OHP was partly determined by change in FVIII level, as demonstrated by a statistically significant correlation between OHP on one side and the concentration of this coagulation protein on the other side. An additional measure in this global haemostasis assay - the Overall Coagulation Potential (OCP) - was significantly increased and correlated to ETP levels at the time of HLS reflecting enhanced fibrin generation in the IVF patients.

Previous studies by our group have confirmed the sensitivity of the OHP method to detect the presence of a procoagulable state when circulating oestrogen levels are elevated. Along with increased gestational age in pregnant women OHP and OCP increased and OFP decreased<sup>77</sup>. Furthermore, hormone replacement treatment with high-dose oral oestrogens in postmenopausal women caused an activation of coagulation and a decrease in fibrinolysis as measured by the OHP assay<sup>167</sup>.

To our knowledge, the study by Harnett et al.<sup>72</sup> was the only study utilizing a global haemostasis assay by thromboelastography in IVF patients. Aligned with their findings

of a significant decrease in clot formation time and an increase in clotting index at the time of oocyte retrieval, our results demonstrate that isolated high oestrogen concentrations, as produced by an IVF protocol, associate with a procoagulable state. This condition could potentially lead to thrombotic complications that can markedly affect the quality of life, and in fact, also in some cases be fatal to these women.

The studies of fibrinolytic variables during IVF are somewhat contradictory demonstrating both a down regulation and activation of fibrinolysis<sup>27, 56, 68</sup>. The overall fibrinolysis potential (OFP) and clot lysis time (CLT) investigated in the present study were not significantly altered during IVF procedure, and remained within the reference range both at DR and HLS. It thus seems that the fibrinolytic system is not significantly affected by the IVF treatment.

### **6.3.3 Fibrin network**

The non-significant decrease in fibrin gel permeability was surprising as increased thrombin generation may be expected to induce a tighter and more stable fibrin gel. However, we used a relatively high concentration of thrombin to initiate clot formation in our assay so endogenous thrombin generation should not influence the characteristics of the fibrin network formed. The observed increase in fibrinogen concentration could only partly influence the results of fibrin gel permeability. Fibrin polymerization rates as well as altered levels of fibrinogen-binding proteins (such as FXIII) are additional mechanisms that may affect fibrin gel porosity and deserve further investigations.

## **6.4 VON WILLEBRAND FACTOR AND ADAMTS13**

In our study we analyzed both VWF and ADAMTS13 to study their inter relationships. We found a rapid increase in VWF:Ag and VWF:RCoF activity levels together with increased FVIII levels and decreased ADAMTS13 antigen and activity levels in parallel with the on average 38 times higher oestradiol plasma levels during the middle stage of IVF treatment.

### **6.4.1 Von Willebrand factor**

VWF contributes to coagulation by stabilizing FVIII, which is an essential blood clotting factor. A high plasma level of FVIII is a risk factor for VTE<sup>204, 205</sup>. In our study, the rise of FVIII concentration by 31% is similar to previously reported data<sup>35</sup> in patients undergoing IVF, suggesting a procoagulant state during the course of ovarian

stimulation. Still, since the concentrations of VWF and FVIII are strongly related, it may be difficult to unravel separate effects of these proteins on a putative development of a procoagulable state during IVF. Clearly, other oestrogen-related mechanisms, such as direct effects on endothelial cells, may be active in the regulation of VWF levels observed during IVF <sup>206</sup>.

In fact, we know that the large multimers of VWF are necessary for an optimal haemostasis <sup>209</sup>. In our study we analyzed only the total level and activity of VWF, as the method of analyzing VWF multimers was not well standardized at our laboratory at that point of time.

VWF seems to add predictive power irrespective of risk score, both for bleeding and cardiovascular events <sup>210,211</sup>. Measurement of VWF levels are therefore of limited value in clinical practice since patients with a high level will have a high risk of both cardiovascular events and bleeding complications.

#### **6.4.2 ADAMTS13**

Study I is the first report of changes in circulating ADAMTS13 antigen and activity levels during IVF. As ADAMTS13 is the VWF-cleaving protease, we found an inverse correlation between VWF and ADAMTS13 at the time of high endogenous oestrogen concentration. With a correlation coefficient of 0.5 we could assume that around 25% of the variability in VWF levels in our study could be explained by changes in the levels of ADAMTS13.

Our results support the idea that oestrogen is of importance in the regulation of ADAMTS13 levels. However, it remains unclear if the decrease of ADAMTS13 levels in IVF patients is due to inhibition of its enzymatic activity, to consumption of the protease, or to increased clearance from plasma. Furthermore the concentrations of VWF:Ag at the time of HLS were inversely correlated to ADAMTS13 antigen and activity suggesting that changes in ADAMTS13 could partly influence VWF levels.

In our study we clearly showed that not only the antigen concentration of ADAMTS13 but also the activity of this enzyme was reduced during the high oestrogen levels in this phase of the IVF procedure. Measuring both ADAMTS13 antigen and activity helped us to understand whether or not the protease was fully active, and provides a new tool for understanding the physiology and pathophysiology of ADAMTS13 <sup>212</sup>.

ADAMTS13 is a molecule that influences haemostasis "outside" the tissue factor induced pathway of coagulation. This is of interest in the development of future and

new drug targets. Such strategies could have a potential to attenuate thrombosis without increasing bleeding.

## **6.5 LIFE STYLE ASPECTS**

Haemostatic factors may be influenced by lifestyle changes. An increase in physical activity has positive effects on thrombin generation as measured by F1+2 in plasma<sup>213</sup> and obesity is associated with elevated thrombin generation<sup>214</sup>. Three out of 31 women in the IVF group were obese (BMI>30), but obesity in general was not a large clinical problem in this patient group with a mean BMI of 24.1 kg/m<sup>2</sup>. Smoking has negative effects on fibrin network tightness<sup>215</sup>. Only two of our patients were smokers, and it is reasonable to assume that to stop smoking may beneficially influence the fibrin network tightness.

## **6.6 CARDIOVASCULAR DISEASE IN WOMEN**

The main result of study IV is a higher incidence of hypertension in women who had a child born after IVF than in women who delivered after natural conception during the time period 1990-2008. These IVF mothers also had a trend towards a higher incidence of stroke, while the incidence of coronary heart disease and diabetes did not differ between the two groups of women.

The main advantage of our study is the sample size and the prospective nature. To get a representative group of controls we included five control women for every IVF woman and were able to retrieve data about smoking, BMI, country of birth and education by matching of relevant registers. Furthermore, the result of our study is in accordance with the study of Parikh et al, which was conducted on Swedish women with self-reported data of subfertility in the Swedish MBR<sup>216</sup>.

Women in the IVF group were less often smokers, had a higher educational level and a lower proportion of women in this group were born abroad. Even though these life circumstances might indicate a reduced risk of CVD, the IVF women had a higher incidence of hypertension than control women. The number of CVD events, coronary heart disease (n=23) and stroke (n=79), in the 23.498 women in the IVF group was small. However, when looking at the effects of the other risk factors in the whole group of women (table 11), we found as expected that obese women (BMI>30) had a higher incidence of diabetes, coronary heart disease and hypertension.

We suggest that the number of women and the mean age in the present study were too low and the mean follow-up period of 8.6 years too short to induce a high enough cardiovascular event rate to reach reasonable statistical power. Due to the steady increase in numbers of IVF treated women, most of our included women had their IVF treatment after 2000. This resulted in a mean age at inclusion of 33 years, and a mean age at end of follow-up of only 41 years.

Despite the low mean age of the women, when stratifying the whole group into different subgroups of smoking, those who were smoking more than 10 cigarettes daily had an increased risk of coronary heart disease as compared to non-smokers.

Smoking is associated with a risk of a two years earlier onset of menopause<sup>217</sup> and smoking is also one of the most important determinants of coronary heart disease in women<sup>218, 219</sup>.

As in previous reports, preeclampsia was overrepresented in the IVF group, and the condition is a well-known risk factor for the development of CVD later in life<sup>220, 221</sup>. Actually, the risk has been found to be more than doubled as compared to those with uncomplicated pregnancy<sup>222</sup>. Preeclampsia occurs in 3-5% of normal pregnancies, and in our IVF group there were 7% women with preeclampsia as compared to 5.2% of the women in the control group.

Diagnoses of hyperlipidaemia and cholesterol levels are not included in study IV. Hyperlipidaemia diagnosis codes are not frequently used in registers in general and has thus a low validity. This is a limitation of our study since especially hypercholesterolaemia is an important CVD risk factor<sup>223</sup>. Differences in cholesterol levels between the two groups could have affected the results of the study. As the women in the IVF group could have a lowered risk of CVD due to life circumstances this could well affect lipid levels. Two other possible confounders in this study could be physical activity and alcohol intake, as these have an impact on the risk of CVD.

## **6.7 INFERTILITY**

In a recent report female infertility accounted for 64% of all infertile couples and 19% were due to male infertility. The remaining 17% had an unknown cause<sup>224</sup>. Unfortunately, we have no information about the causes of infertility in our 23,498 IVF women in studies III + IV, but male infertility cases may have diluted our results causing an underestimation of the true difference.

PCOS, prevalent in 6-8% of women in the reproductive ages <sup>225</sup>, accounts for up to 15% of female infertility and is associated with subclinical atherosclerosis as measured by carotid intima-media thickness and endothelial dysfunction <sup>226</sup>. The number of women with PCOS was higher in the IVF group than in the control group, but the proportion was low in both groups. We speculate that the condition is underreported in the registers.

Excess adiposity as assessed by BMI is associated with subfertility <sup>227</sup> and incident CVD <sup>228</sup>. However, adjustment for BMI neither nullified nor attenuated our CVD risk estimates, suggesting that factors other than BMI underlie the increased incidence of hypertension in the IVF group.

Another possible mechanistic link between infertility and CVD is hypothyroidism, which is linked both to infertility <sup>229</sup> and incident CVD <sup>230</sup>. However, in our material, the prevalence of hypothyroidism was low in both the study and control group. Therefore, we assume that this condition did not affect the result.

## **6.8 GENERAL REMARKS**

In summary, in study I+II we have shown haemostatic changes during IVF pre-treatment which may contribute to the higher incidence of VTE during the first trimester of IVF pregnancies as shown in study III.

This is the first study of CAT and OHP as well as of fibrin gel permeability in IVF patients establishing reference intervals of these variables which could differentiate them from normal pregnant women or female patients treated with exogenous oestrogens. Global assays of haemostasis can provide a possibility to assess the haemostatic balance during IVF when it is used as a complement to conventional risk factor evaluation for VTE.

We conclude that supraphysiologic oestrogen concentrations gained during IVF exert pronounced effects on blood clotting which potentially could lead to thrombotic complications.

After gaining new information from our studies in this thesis we suggest revising the National Guidelines regarding thromboprophylaxis during IVF and we recommend considering thromboprophylactic treatment more frequently to patients at risk during the first 12 weeks after embryo transfer.

To our knowledge, study IV in this thesis is the first prospective cohort study investigating whether CVD and related risk factors have a higher incidence in women

after IVF pregnancy as compared to women who delivered after natural conception. The IVF women in our study had a higher incidence of hypertension than control women in the years after delivery. Hypertension is a main risk factor for CVD but there is a long time delay between the initiation of vascular disease processes and manifest CVD such as coronary heart disease and stroke<sup>231</sup>. Therefore, we speculate that female infertility could be associated with a higher risk of CVD later in life. We recommend that clinicians should pay a close attention to and measure blood pressure in women who have passed an IVF treatment as they seem to have a higher propensity to develop hypertension. These women should be offered antihypertensive treatment if blood pressure increases above reference levels in order to prevent vascular damage.

## 7 CONCLUSIONS

- IVF treatment is associated with an increased incidence of VTE and PE during pregnancy.
- IVF treatment leads to an alteration of haemostasis towards a procoagulable state including:
  - Increased thrombin generation (CAT assay)
  - Increased fibrin formation (OHP assay)
  - Increased plasma levels and function of VWF
  - Decreased plasma levels and function of ADAMTS13
- Women undergoing IVF pregnancies have a higher incidence of hypertension and even a trend towards a higher incidence of stroke in the years after delivery compared to women who deliver after natural conception.
- Women undergoing IVF pregnancies did not seem to differ in the incidence of coronary heart disease and diabetes, as compared to women who delivered after natural conception. However, the time of follow-up in the cohort was too short to reject a long-term difference between the two groups.

## 8 FUTURE PERSPECTIVES

The higher incidence of VTE and PE during pregnancy after IVF treatment should result in more frequent use of thromboprophylaxis in these women.

Further prospective clinical studies are needed to establish the role of ETP and OHP as risk predictors of future thrombotic events during IVF treatment and as tools to monitor the IVF treatment.

Further research is also needed to increase the understanding of a possible pathophysiological role of ADAMTS13 in thrombosis. ADAMTS13 is an interesting drug target in the future with a potential to reduce thrombosis development without increasing bleeding.

Furthermore, additional mechanistic studies are needed to elucidate the long-term effects of IVF on global haemostasis during pregnancy, especially during the first trimester.

With the currently increasing age when couples start family planning in the Western world there will probably be even more infertile couples in the future. Since hypertension is more prevalent with increasing age and IVF treated women are older than the average pregnant woman, these women should be followed with regular blood pressure measurements after end of their pregnancies. A longer follow-up of an IVF cohort in the future could give the final answer whether infertile women might be prone to develop CVD later in life.

## 9 SVENSK SAMMANFATTNING

Venös tromboembolism (VTE) är blodproppar i ben eller lungor som orsakas bland annat av ett antal ärftliga, livsstils- och miljöfaktorer och där risken att insjukna ökar med stigande ålder. Sättet som en blodpropp bildas på är komplext och består av såväl blodlevring som aktivering av blodplättar. Blodlevringen skapar ett nätverk av fibrinrådar i vilket blodplättarna fastnar och bildar en propp.

Det kvinnliga könshormonet östrogen ökar risken för att drabbas av blodproppar. Under graviditeten stiger nivån av östrogen i blodet och risken för VTE ökar, särskilt i den senare delen av graviditeten när östrogennivån är som högst. En av tusen gravida kvinnor drabbas av VTE. Blodproppar som fastnar i lungorna kallas lungemboli och är den vanligaste dödsorsaken bland gravida kvinnor i västvärlden.

Syftet med doktorandprojektet var att undersöka om provrörsbefruktning (IVF) gör kvinnor mera blodproppsbenägna. Detta studerades både med laboratorietester och epidemiologiska studier. Vi undersökte även om hjärtkärlsjukdom är överrepresenterad i gruppen kvinnor som fött barn efter IVF jämfört med "vanliga" mödrar från det medicinska födelseregistret.

Jämfört med andra länder har Sverige unika möjligheter att göra epidemiologiska studier tack vare de landstäckande hälso- och sjukvårdsregistren. I registren kan man identifiera prospektivt insamlade data som kan kopplas till både slutenvårds- och öppenvårdsregister med hjälp av personnumret. IVF-registret är ett svenskt kvalitetsregister som är knutet till det medicinska födelseregistret, där alla kvinnor som fött barn ingår. Med hjälp av dessa register och kopplingen till sluten- och öppenvårdsregistret kunde vi identifiera 23 498 kvinnor som genomgått IVF-behandling under perioden 1990-2008. Bland IVF-behandlade kvinnor var det fyra gånger fler som fick VTE och sju gånger fler drabbades av lungemboli under första tredjedelen av graviditeten (trimestern) jämfört med 116 960 kontrollkvinnor från det medicinska födelseregistret (studie III). Eftersom kvinnor som genomgår IVF är friska, unga och blivande föräldrar, är det utomordentligt viktigt att minimera risker med behandlingen.

För att undersöka påverkan av IVF på blodproppsbildning och -upplösning (hemostasen) undersökte vi 31 patienter som genomgick IVF. Provtagningen utfördes vid två tillfällen; dels vid nedregleringsfasen (låga östrogennivåer) och dels vid stimuleringsfasen (höga östrogennivåer). Våra undersökningar fokuserade på metoder

som mäter balansen mellan blodproppsbildning och -upplösning (studie II). Genom att mäta förmågan till blodlevring hos den enskilda patienten med ett blodprov skulle man bättre kunna bedöma om kvinnan har en överrisk för blodproppar under IVF-behandlingen och den efterföljande graviditeten.

Vi undersökte även ett ämne från blodkärlen som har samband med proppbenägenhet (von Willebrands faktor) och dess reglerande enzym (ADAMTS13) vid samma provtagningstider (studie I). Studie I+II visade att kvinnor som genomgått IVF hade en generellt högre tendens att bilda blodproppar.

Infertilitet drabbar cirka 10 procent av alla par i världen. Orsaken till infertilitet kan bland annat bero på hormonrubbingar, strukturella hinder hos kvinnan eller nedsatt spermakvalitet hos mannen. En stor andel är oförklarad trots omfattande utredning vilket bidrar till att tre procent av alla barn i Sverige påbörjar sina liv i ett provrör på fertilitetskliniken.

Vi undersökte vidare om kvinnor som genomgått IVF-behandling hade en ökad förekomst av hjärtkärlsjukdomar jämfört med kontrollgruppen (studie IV). Vi följde alla kvinnor efter graviditeten från 1990 och fram till slutet av 2009 eller tills de drabbades av högt blodtryck, stroke, hjärtinfarkt eller diabetes. Eftersom kvinnorna inte hann uppnå så hög ålder under uppföljningsperioden (som var i genomsnitt åtta år) fokuserade vi på högt blodtryck, vilket är en av de vanligaste riskfaktorerna för hjärtkärlsjukdom och uppträder flera år innan man får en hjärtkärlhändelse. Kvinnorna som genomgått IVF-behandling hade en ökad risk att drabbas av högt blodtryck jämfört med kontrollgruppen från födelseregistret. Vi såg även en tendens till flera strokeinsjuknanden i IVF-gruppen.

Sammanfattningsvis finner vi ett klart samband mellan IVF-behandling och VTE, särskilt lungemboli. Detta är särskilt tydligt under de första tre månaderna av graviditeten. Vi har också visat att IVF är förenat med störning i blodlevringen. I studie IV fann vi ett samband mellan infertilitet och hjärtkärlsjukdom i form av ökad risk att utveckla högt blodtryck efter en IVF-graviditet. Om infertilitet och hjärtkärlsjukdom har ett samband bör denna patientgrupp följas upp med t.ex. regelbundna blodtryckskontroller efter graviditeten så att risken för senare hjärtkärlsjukdom kan minimeras.

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# Changes in von Willebrand factor and ADAMTS13 during IVF

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During IVF, circulating estradiol concentrations are strongly increased, and this may have direct effects on hemostasis. Elevated von Willebrand factor levels represent an important risk factor for arterial and venous thrombosis. ADAMTS13, also known as von Willebrand factor-cleaving protease, has an important regulatory function of von Willebrand factor but has not been studied during IVF. Blood was sampled from 31 women at maximal downregulation of estradiol synthesis using gonadotropin-releasing hormone analogues and during high-level stimulation of estradiol synthesis using follicle-stimulating hormone during the first phase of IVF. Von Willebrand factor antigen, von Willebrand factor ristocetin cofactor activity, factor VIII and ADAMTS13 antigen and activity levels in plasma were determined at the time of downregulation and at high-level stimulation. Estradiol increased from a mean of 154 pg/ml at downregulation to 5889 pg/ml at high-level stimulation (range 1620–19 500 pg/ml). Factor VIII increased from  $0.96 \pm 0.34$  to  $1.26 \pm 0.41$  kIU/l ( $P < 0.001$ ). Von Willebrand factor antigen and activity increased from  $0.75 \pm 0.22$  to  $1.06 \pm 0.40$  kIU/l ( $P < 0.001$ ) and from  $0.83 \pm 0.26$  to  $1.24 \pm 0.48$  kIU/l ( $P < 0.001$ ), respectively. ADAMTS13 antigen decreased from  $72.2 \pm 13.5$  to  $67.9 \pm 9.9\%$  ( $P < 0.05$ ,

$P = 0.01$ ) and ADAMTS13 activity from  $88.6 \pm 18.3$  to  $80.8 \pm 15.7\%$  ( $P < 0.01$ ). The increments in estradiol and factor VIII during IVF were paralleled by an increase in von Willebrand factor antigen and activity, and a decrease in circulating ADAMTS13 antigen and activity, respectively. This could in part explain why these patients have an increased risk of thrombotic events. *Blood Coagul Fibrinolysis* 22:127–131 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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## Introduction

IVF is the most common procedure performed to assist reproduction. Current studies demonstrate that the profound increase in estrogen attained during IVF exert direct effects on several hemostatic variables and may induce a hypercoagulable state [1–3]. The major complication of IVF is the ovarian hyperstimulation syndrome (OHSS), which can result in severe arterial or venous thrombotic complications [4].

The von Willebrand factor (VWF), previously named factor VIII (FVIII)-related antigen, is an adhesive plasma glycoprotein synthesized in megakaryocytes and vascular endothelial cells. It plays a major role in primary hemostasis serving as an adhesive link between platelets and the damaged vessel wall and indirectly contributes to coagulation by stabilizing circulating FVIII and protecting it from proteolysis [5]. In response to vascular damage, VWF is rapidly released in the form of ultralarge multimers mediating platelet adhesion. An important regulatory mechanism of VWF levels in plasma is the activity of a zinc-containing metalloprotease enzyme ADAMTS13, also known as von Willebrand factor-cleaving protease. This enzyme cleaves VWF at a single site in the A2 domain, thereby converting ultralarge VWF multi-

mers into lower molecular weight forms with reduced adhesive potential [6,7].

Increased circulating levels of VWF have been found in clinical conditions characterized by endothelial stimulation and dysfunction and are associated with risks of both arterial and venous thrombosis [8–10]. Both VWF and FVIII levels raise two-fold to three-fold during the second and third trimesters of pregnancy [11,12]. IVF treatment is also associated with an increase in circulating VWF levels followed by an increase in FVIII levels [2,13]. Additionally, high plasma concentrations of VWF have been reported to be of value in predicting whether patients are at risk of developing severe OHSS [14], and there is a negative association between plasma levels of VWF and the activity of ADAMTS13, which tends to be low in plasma when VWF is high [15–17]. Interestingly, a reduced function of ADAMTS13 is associated with thrombotic microangiopathies like thrombotic thrombocytopenic purpura [18–20]. There are some studies indicating a moderate decrease in this protease during pregnancy and puerperium [21]. However, ADAMTS13 has not been investigated previously in patients undergoing IVF. The present study was aimed to study possible changes in ADAMTS13 during IVF and

whether changes in VWF during IVF are related to changes in ADAMTS13.

## Methods

### Patients

Thirty-one women undergoing IVF treatment at the Fertility Unit of Karolinska University Hospital, Huddinge, Sweden, were included in the study.

In order to induce controlled ovarian hyperstimulation, the estrogen production was first downregulated by using a gonadotropin-releasing hormone (GnRH) agonist, buserelin acetate (Suprecur; Aventis Pharma, Mumbai, India). Starting at the 21st day of menstrual cycle, all patients received 300 µg buserelin acetate three times per day in the form of nasal spray. After the menstrual bleeding, 2 weeks later, an estradiol (E<sub>2</sub>) measurement was carried out to verify downregulation. Acceptable baseline hormonal concentration for initiating the IVF cycle was E<sub>2</sub> less than 150 pg/ml. After that, the GnRH dose was decreased to 300 µg twice a day.

Ovarian stimulation was then initiated by using recombinant human follicle stimulating hormone (FSH) administered subcutaneously, either as follitropin alpha (Gonal-f; Merck Serono, Geneva, Switzerland), or as follitropin beta (Puregon; Schering-Plough, Kenilworth, New Jersey, USA). The starting dose varied between 75 and 300 IU daily according to the woman's history and BMI. The response was followed up by an E<sub>2</sub> measurement 6 days later and by ultrasound scanning of the ovarian follicles at days 9–10 after the first FSH injection.

### Blood sampling

Blood samples were taken under fasting conditions twice during the IVF procedure: the first time at maximal downregulation of E<sub>2</sub> synthesis and the second time during high-level stimulation (HLS) of E<sub>2</sub> synthesis. Platelet-poor plasma was obtained by centrifugation at 2000g for 20 min and aliquoted plasma samples were stored at –80°C before being analyzed.

Blood tests, such as hemoglobin, platelet cell count, erythrocyte volume fraction, plasma creatinine and lipids, and plasma glucose, were performed at the time of the first blood sampling. Blood pressure, heart rate, weight, length and waist–hip ratios were also measured. The patients were interviewed regarding smoking habits and ongoing medication at inclusion. Patients with polycystic ovarian syndrome were excluded from the study. The study was approved by the ethics committee of the Karolinska Institutet.

### Laboratory methods

#### Von Willebrand factor and factor VIII

Von Willebrand factor antigen (VWF:Ag) concentrations were measured in plasma using the LIATEST from Diagnostica Stago (Asnieres, France). The measurement

procedure was according to the manufacturer's instructions [22,23]. The von Willebrand factor ristocetin cofactor (VWF:RCoF) activities were measured in plasma using the BC von Willebrand Reagent from Siemens Healthcare Diagnostics (Deerfield, Illinois, USA). FVIII was measured using the COAMATIC FVIII reagent from Haemochrom Diagnostica (Essen, Germany).

#### ADAMTS13

ADAMTS13 activity and antigen concentrations in plasma were analyzed using the TECHNOZYM ADAMTS13 kit which is based on the fluorogenic method described by Kokame *et al.* [18]. All reagents were obtained from Technoclone GmbH (Vienna, Austria) and their detailed measurement procedure followed. Results are reported as the percentage of those of a pool of plasma from more than 100 healthy donors. According to the manufacturer, the reference interval for ADAMTS13 antigen concentration is 75–110% and for ADAMTS13 activity 50–110% of the pool. The coefficient of variation (CV%) for duplicates should not exceed 15% according to the manufacturer. The assay was run using a Tecan Infinite M220 multireader (Männedorf, Switzerland).

The uncertainty has been estimated from duplicate measurements of samples in a recent study performed at our laboratory and was found to be 10.8% (SD), CV% 9.6, *N* = 48 for ADAMTS13 activity and 6.4% (SD), CV% 7.1, *N* = 48 for ADAMTS13 antigen [24].

#### Statistical analysis

Statistical analyses were performed using STATISTICA software, version 8 (Statsoft, Inc., Tulsa, Oklahoma, USA). We performed parametric tests for normally distributed variables. Data are presented as mean ± SD. E<sub>2</sub> concentrations are shown as the mean and the minimal respectively maximal concentrations (range). *P* less than 0.05 was considered statistically significant. The analysis of power in this study was performed *a priori* based on the assumption of detecting a 20% difference in the main endpoint. Our calculations showed that we would need approximately 16 patients assuming an  $\alpha$  of 0.05 and a power (1 –  $\beta$ ) of 0.80.

## Results

All patients were whites aged 25–38 years and with a normal resting blood pressure ( $\leq 140/90$  mm/Hg). None of them had a history of venous thrombosis. Basic characteristics of the patients are presented in Table 1.

E<sub>2</sub> increased from a mean of 154 pg/ml at downregulation to a mean of 5889 pg/ml at HLS (range 1620–19500 pg/ml).

VWF:Ag and VWF:RCoF activity increased by 41 and 49% (*P* < 0.001) from the time of downregulation to HLS, respectively. These changes were accompanied by a

**Table 1** Baseline characteristics of the 31 women enrolled in the study

	Patients	Normal range
Age (years)	33.0 ± 3.3	
BMI (kg/m <sup>2</sup> )	24.1 ± 3.6	<25
Waist-hip ratio	0.8 ± 0.1	<0.8
Causes and type of infertility		
Female [n (%)]	7 (22.6)	
Male [n (%)]	12 (38.7)	
Unknown [n (%)]	12 (38.7)	
Current smoking [n (%)]	2 (11.1)	
Triglycerides (mmol/l)	0.8 ± 0.4	0.45–2.6
Total cholesterol (mmol/l)	4.9 ± 0.7	3.3–6.9
LDL cholesterol (mmol/l)	2.9 ± 0.7	1.4–4.7
Creatinine (mmol/l)	68.9 ± 7.3	<90
Blood sugar (mmol/l)	4.7 ± 0.5	4.0–6.0
Hemoglobin (g/l)	129.0 ± 8.6	117–153
Hematocrit (%)	0.39 ± 0.02	0.35–0.46
Platelet count (×10 <sup>9</sup> per l)	251.2 ± 47.6	165–387

Data are presented as mean ± SD or n (%). LDL, low-density lipoprotein.

concomitant 6% decrease in ADAMTS13 antigen ( $P < 0.05$ ) and a 9% decrease in ADAMTS13 activity ( $P < 0.01$ ) (Table 2). The relative changes in ADAMTS13 antigen and activity from downregulation to HLS correlated significantly ( $r = 0.7$ ,  $P < 0.001$ ).

The concentrations of VWF:Ag at HLS were significantly and inversely correlated to the concentrations of ADAMTS13 antigen and ADAMTS13 activity at the same time point ( $r = 0.6$ ,  $P = 0.015$  and  $r = 0.5$ ,  $P = 0.026$ , respectively).

The FVIII levels increased 31% from the time of downregulation to HLS ( $P < 0.001$ ) (Table 2).

Twelve patients got pregnant and all except one were primigravida. Three patients developed OHSS. These three patients had a 64% increased VWF:Ag and 63% increased VWF:RCoF activity level from the time of downregulation to the time of HLS. FVIII increased 56% at the same period. These changes were accompanied by a 9% decrease in ADAMTS13 antigen and a 14% decrease in ADAMTS13 activity.

## Discussion

This is the first report of changes in circulating ADAMTS13 antigen and activity levels during IVF. We found increased VWF:Ag and VWF:RCoF activity

together with increased FVIII levels and decreased ADAMTS13 antigen and activity during the early stage of the IVF treatment. Furthermore, the concentrations of VWF:Ag at the time of HLS of E<sub>2</sub> production were inversely correlated to ADAMTS13 antigen and activity suggesting that changes in ADAMTS13 could partly influence VWF levels.

E<sub>2</sub> levels in plasma changed considerably within a short period during IVF. Our patients reached on average a 38 times higher E<sub>2</sub> level in plasma within a few days after the start of stimulation. IVF treatment is thus a unique model to study effects of profound and fast increases in endogenous estrogen on hemostatic variables.

It has previously been reported that hyperestrogenemia cause an increase in VWF concentrations in plasma [25]. This is supported by in-vitro findings of Harrison and McKee, who demonstrated that 17β-estradiol directly enhanced the synthesis of VWF in endothelial cells in a dose-dependent manner [26]. It is thus plausible that the increased level and activity of circulating VWF observed in the present study are caused by an estrogenic effect on the vascular endothelium [27].

We found a rapid increase in VWF:Ag and VWF:RCoF activity levels – at a mean of 45% – in parallel with increased E<sub>2</sub> concentrations during the first few weeks of IVF. The increase in VWF was smaller than what has been described during pregnancy [11,12]. However, it has to be considered that our measurements were performed within only a few days after the initiation of estrogen stimulation and starting from very low levels of E<sub>2</sub> attained during the preceding phase of downregulation. By contrast, the VWF levels during pregnancy were assessed after long-term stimulation by high levels of estrogens in the third trimester of pregnancy. Moreover, the variability between separate individuals with respect to increments in VWF following stimulated estrogen production is considerable.

It is not possible to deduce whether the observed changes in plasma levels of VWF could influence the development of OHSS in this particular group of patients. It is however known that an acute rise in VWF during the first 48 h of the acute coronary syndrome is a significant predictor of adverse cardiovascular events during the following 14 respectively 30 days [10].

Moreover, VWF contributes to coagulation by stabilizing FVIII, which is an essential blood-clotting factor. A high plasma level of FVIII is a risk factor for venous thromboembolism [28,29]. In our study, the rise in FVIII concentration by 31% is similar to that previously reported by Bremme *et al.* [2] in patients undergoing IVF, suggesting the presence of a procoagulant state during the course of ovarian stimulation. Still, as the concentrations of VWF and FVIII are strongly related, it may be difficult to unravel separate effects of these

**Table 2** Changes in von Willebrand factor, ADAMTS13 and factor VIII during IVF

	Downregulation	High-level stimulation	P
VWF:Ag activity (kIU/l)	0.75 ± 0.22	1.06 ± 0.40	<0.001
VWF:RCoF activity (kIU/l)	0.83 ± 0.26	1.24 ± 0.48	<0.001
ADAMTS13 antigen (%)	72.2 ± 13.5	67.9 ± 9.9	<0.05
ADAMTS13 activity (%)	88.6 ± 18.3	80.8 ± 15.7	<0.01
Factor VIII (kIU/l)	0.96 ± 0.34	1.26 ± 0.41	<0.001

Data are presented as mean ± SD; n = 31. Percentage = percentage of normal pooled plasma. VWF:Ag, Von Willebrand factor antigen; VWF:RCoF, von Willebrand factor ristocetin cofactor.

proteins on a possible development of a hypercoagulable state during IVF.

There is until now limited knowledge of effects of sex hormone on ADAMTS13 levels. In normal pregnancy, Mannucci *et al.* [30] have described a 32% decrease in ADAMTS13 activity levels in the second and third trimester. Thus, the authors suggested that estrogen could be one of the regulators of ADAMTS13 activity in plasma. In our study, we analyzed both ADAMTS13 antigen and activity in plasma, and our data clearly show that not only the antigen concentration of ADAMTS13 but also the activity of this enzyme was reduced during the extremely increased estrogen levels in this phase of IVF. Measuring both ADAMTS13 antigen and activity helps us to understand whether or not the protease is fully active and provides a new tool for understanding the physiology and pathophysiology of ADAMTS13 [31]. Our results support the idea that estrogen is of importance in the regulation of ADAMTS13 levels.

We also observed a significant inverse relationship between VWF:Ag and ADAMTS13 antigen and activity at the time of HLS with a correlation coefficient of around 0.5. We can thus assume that approximately 25% of the variability in VWF level in this study could be explained by changes in the levels of ADAMTS13. Clearly, other estrogen-related mechanisms, such as a direct effect on endothelial cells, may also be active in the regulation of the VWF levels observed during IVF [27].

In the present study, three patients developed OHSS. This is an occasional iatrogenic complication of ovarian stimulation usually occurring several days after embryo transfer. Clinical manifestations are massive extravascular fluid accumulation and hemoconcentration which appears to be caused by increased capillary permeability in the vascular endothelial cells. Mild cases of OHSS has been reported in up to 25% of the women undergoing IVF [32], and it is estimated that one out of 128 women with severe OHSS develop thromboembolic disease [33]. High plasma concentrations of VWF were found to be of value in predicting a risk of severe OHSS [14]. Ogawa *et al.* [4] demonstrated that the plasma levels of VWF exhibited a significant rise before the development of clinical manifestations of moderate to severe OHSS, and that the magnitude of the rise was associated with the clinical severity of OHSS. In our three patients who developed OHSS, there seemed to be a more pronounced increase in VWF:Ag and FVIII levels (mean increase of 64 and 56%, respectively) from downregulation to HLS as compared in the patients without the OHSS (mean increase of 45 and 31%, respectively). Furthermore, ADAMTS13 antigen and activity levels in these three patients decreased to a larger extent than in the patients without OHSS. However, it remains unclear whether this pronounced decrease in ADAMTS13 levels in patients with OHSS was due to inhibition of its enzymatic

activity, consumption of the protease or increased clearance from plasma.

To conclude, decreased circulating levels of ADAMTS13 together with a functional failure of this enzyme might result in higher levels of VWF and thus affect hemostasis in patients undergoing IVF. Further research is needed to increase the understanding of a possible pathophysiological role of ADAMTS13 in thrombosis. Furthermore, additional experimental studies are needed to elucidate the effects of estrogens on ADAMTS13.

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There are no conflicts of interest.

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## Regular Article

## Detection of a procoagulable state during controlled ovarian hyperstimulation for in vitro fertilization with global assays of haemostasis

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## ABSTRACT

**Introduction:** Controlled ovarian hyperstimulation during in vitro fertilization (IVF) causes profound increases in serum estradiol which may influence haemostasis and the ovarian hyperstimulation syndrome.

In the present study we investigated the effect of the standard IVF-stimulation protocol on coagulation and fibrinolysis as assessed by different global haemostatic assays.

**Materials and Methods:** Blood samples were drawn from 31 women during the down-regulation phase when estradiol secretion is inhibited, and before egg retrieval, i.e. when estradiol levels are at supraphysiological levels, in the following called high level stimulation phase. Haemostasis was assessed during both treatment phases with 1) the calibrated automated thrombogram which measures thrombin generation, 2) overall haemostasis potential which measures fibrin formation and degradation and 3) fibrin gel permeability measurements which measures the quality of the fibrin network.

**Results:** Estradiol increased from <150 pg/mL to 5889 pg/mL (range 1620–19500 pg/mL). We found both increased thrombin generation as measured by the calibrated automated thrombogram ( $p < 0.001$ ) and an increase in overall haemostasis potential ( $p < 0.001$ ) from time of down-regulation to high level stimulation.

**Conclusions:** The assays used indicated procoagulable changes in haemostasis during in vitro fertilization. Further studies should evaluate their potential in the prediction of thrombosis and hyperstimulation.

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## Introduction

Controlled ovarian hyperstimulation for in vitro fertilization (IVF) is a routine procedure in infertility treatment. A major complication of IVF is the ovarian hyperstimulation syndrome (OHSS) which has been associated with arterial and venous thrombotic complications in severe cases [1]. Precise figures of incidence are unknown because of lack of a systematic registration. Mild OHSS probably occurs in 8–23% of stimulated cycles, moderate forms in <1–7% and severe forms in ~0.5% of stimulated cycles [2,3]. High serum estrogen concentrations and an increased number of small ovarian follicles at the time of ovulation induction are factors known to relate to the development

of OHSS, but the predictive value of these factors for the development of OHSS is not high [4,5].

Previous studies demonstrate that supraphysiological increases in estrogen during IVF exert direct effects on individual haemostatic variables [6–9] and may induce a procoagulable state. Still, very little is known about the effects of short-term alterations of endogenous estrogen concentration during IVF treatment on the overall haemostatic balance of the individual. Although there is a wide spectrum of recently developed global haemostatic assays, none of them has so far been used to assess changes during IVF.

The calibrated automated thrombography (CAT) assay is a commercially available global haemostatic method which determines the time integral of thrombin generation after triggering with tissue factor, called endogenous thrombin potential (ETP) [10]. The CAT assay is based on the assumption that thrombin generation in examined plasma reflects the sum of the activities and concentrations of pro- and anticoagulant substances. An increasing body of evidence shows that CAT may be a useful tool in the diagnosis of acquired or congenital procoagulable states and is sensitive enough to detect some forms of hemorrhagic diathesis [11,12].

In contrast to CAT which is used to screen thrombin generation in the examined plasma, and thereby coagulation capacity only, we have

**Abbreviations:** ADAMTS13, A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; CAT, Calibrated automated thrombogram; DR, Down regulation; E<sub>2</sub>, Estradiol; ETP, Endogenous Thrombin Potential; HLS, High level stimulation; IVF, In vitro fertilization; Ks, Permeability coefficient; OCP, Overall Coagulation Potential; OFP, Overall Fibrinolysis Potential; OHP, Overall Haemostasis Potential; OHSS, Ovarian hyper stimulation syndrome; ttPeak, time to peak; VWF, Von Willebrand factor.

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developed the Overall Haemostasis Potential assay (OHP) [13] as a quantitative method for determination of the fibrin level in plasma associated with combined potential of coagulation and fibrinolysis. It is based on repeated spectrophotometric registrations of fibrin-aggregation in citrated plasma, to which small amounts of exogenous thrombin, t-PA and  $\text{CaCl}_2$  have been added. Our previous investigations have shown that this approach can be used to detect a variety of procoagulable disorders [13–15] as well as hypocoagulability due to deficiencies of procoagulants [14,16].

Moreover, by using the liquid-permeation technique to investigate fibrin gel permeability [17–19], the quality of the fibrin clot can be studied. The permeability coefficient (Ks) is an established parameter that provides information on the overall clot structure and reflects the size and shape of the pores in the clot. Low value of Ks indicates a tighter, less porous fibrin clot, which is more resistant to fibrinolysis [20] and may be associated with thrombotic complications [21,22].

The individual coagulation factors i.e. fibrinogen and FVIII are known to increase during IVF [4] and were also investigated. Moreover, these coagulation factors are known to correlate to the variables of the above mentioned global haemostasis assays [10,13] which supported their assessment in the present study.

The aim of the present study was to evaluate the effects of the standard IVF-stimulation protocol on coagulation and fibrinolysis as assessed by different global haemostatic assays. The effects of the IVF stimulation protocol on the final events in coagulation, i.e. the formation of a fibrin network, were investigated by studying the permeability of the fibrin gel.

## Material and methods

### Subjects

The present study was approved by the Regional Ethics Committee of the Karolinska Institutet and all participants gave their informed consent. Thirty-one consecutive women undergoing IVF treatment at the Fertility Unit of Karolinska University Hospital, Huddinge, were included in the study. At inclusion patients were interviewed regarding smoking habits, ongoing medication and own or family history of venous thrombotic diseases, diabetes and cardiovascular diseases. All patients were Caucasian aged 25–38 years (mean  $\pm$  SD =  $33 \pm 3.3$ ) and none of them suffered from arterial hypertension, diabetes mellitus, dyslipidaemia, or had a history of arterial or venous thromboses. Patients with polycystic ovarian syndrome or ongoing anticoagulation treatment were excluded from the study.

In order to induce controlled ovarian hyper-stimulation, the estrogen production was first down-regulated by using a gonadotrophin releasing hormone agonist, Buserelin® (Suprecur, Aventis Pharma, Frankfurt, Germany). Starting on the 21st day of menstrual cycle all patients received 300  $\mu\text{g}$  Buserelin® three times per day in a form of nasal spray. After the menstrual bleeding, two weeks later, an estradiol ( $\text{E}_2$ ) measurement was carried out to verify the down-regulation. Acceptable baseline hormonal concentration for initiating the IVF cycle was  $\text{E}_2 < 150 \text{ pg/ml}$ . After that, the GnRH dose was decreased to 300  $\mu\text{g}$  twice a day.

The ovarian stimulation was then initiated by using recombinant human Follicular Stimulating Hormone administered subcutaneously, either as follitropin alpha (Gonal-f, Merck Serono, Stockholm, Sweden), or as follitropin beta (Puregon, Schering-Plough, Gothenburg, Sweden). The starting dose varied between 75 and 300 IU daily according to the woman's history and body mass index. The response was followed up by an  $\text{E}_2$  measurement six days later, and by ultrasound scanning of the ovarian follicles at days 9–10 after the first FSH injection. It was repeated when necessary.

The trans-vaginal ultrasound-guided oocyte retrieval was planned when the largest follicles had reached the diameter of 18 mm. At that

stage, 250  $\mu\text{g}$  human recombinant chorion gonadotrophin, (Ovitrelle, Merck-Serono) or 5000–10 000 IU urine-purified human chorion gonadotrophin (Pregnyl, Schering-Plough) were given to induce the final maturation of the oocytes, which were retrieved 36–38 hours later. Therefore, the oocytes were retrieved 10 to 14 days after starting the Follicular Stimulating Hormone stimulation.

### Blood sampling

Blood samples were taken under fasting conditions twice during the IVF procedure: the first time at maximal down-regulation (DR) of  $\text{E}_2$  synthesis ( $< 150 \text{ pg/mL}$ ) and the second time during high level stimulation (HLS) of  $\text{E}_2$  synthesis before hCG is given. Blood samples were taken by venipuncture in a buffered sodium citrate medium (9 parts blood + 1 part sodium citrate, 0.129 mol/L; pH 7.4). Platelet-poor plasma was obtained by centrifugation at 2000 g for 20 minutes at room temperature, and plasma aliquotes were stored at  $-80^\circ \text{C}$ .

At the time of the first blood sampling we measured whole blood haemoglobin concentrations, platelet counts and white blood cell counts in venous blood, hematocrit, serum creatinine, sodium, potassium, CRP and liver transaminases in plasma, as well as plasma glucose concentrations.

## Laboratory methods

### Endogenous Thrombin Potential

The CAT assay was performed essentially as described by Hemker et al. [10] and according to the manual provided by Thromboscope B.V. (Maastricht, the Netherlands). Coagulation is initiated by recalcification in the presence of 5  $\mu\text{M}$  tissue factor and 4  $\mu\text{M}$  phospholipids and thrombin generation is continuously registered by measuring cleavage of a fluorogenic substrate. Four parameters were derived from the thrombin generation curves: lag time (initiation phase of thrombin generation), ETP (thrombin formation over time which is measured as "area under the curve"), peak height (i.e. peak thrombin concentration) and time to peak (i.e. time to peak thrombin concentration). The lag time was defined as the time to reach one-sixth of the peak height. The time until the thrombin concentration peak is reached, is described as the time to peak. The area under the curve (ETP) represents the total amount of thrombin generated over time.

### Determination of Overall Haemostatic Potential (OHP) in plasma

The OHP assay is based on repeated spectrophotometric registration of fibrin-aggregation in citrated plasma, to which small amounts of exogenous thrombin, t-PA and  $\text{CaCl}_2$  have been added [23]. For the overall coagulation potential (OCP),  $\text{CaCl}_2$  (final concentration 35 mmol/l) and thrombin (final conc. 0.09 IU/mL) were added to Tris buffer (66 mmol/l Tris and 130 mmol/l NaCl, pH 7). For OHP, t-PA (final conc. 660 ng/mL) was also added to the buffer for OCP. Seventy  $\mu\text{L}$  of plasma in each well of the microplate (NUNC, USA) were mixed with 10  $\mu\text{L}$  of platelet reagent (Unicorn Diagnostics Ltd, London UK), which is a platelet membrane preparation derived from washed normal human platelets ( $10^6 \text{ mL}^{-1}$ ). After that, 50  $\mu\text{L}$  of the buffer for determination of OCP or OHP, respectively, was added (final conc. of  $\text{CaCl}_2$  17 mmol/l and thrombin 0.04 IU/mL in both plus t-PA 300 ng/mL in the latter). Absorbance (Abs) at 405 nm was measured each minute for 40 min to construct the two fibrin-aggregation curves, OCP and OHP. The area under the curve was expressed by a summation of the Abs values (Abs-sum). The difference between the two areas reflects the overall fibrinolysis potential (OFF), calculated by  $\text{OFF} (\%) = ((\text{OCP}-\text{OHP})/\text{OCP}) \times 100$ .

### Flow measurement for determining fibrin gel permeability

As previously described [20], the fibrin gels were prepared by adding a 10  $\mu$ l TRIS buffer containing 0.42 mol/l  $\text{CaCl}_2$  and 8.3 IU/ml thrombin, giving final concentrations of 20 mmol/l and 0.4 IU/ml, respectively, to 200  $\mu$ l dialyzed plasma in a small plastic cylinder (2.5 cm long, opening area 0.1 cm<sup>2</sup>). The cylinder containing the fibrin gel was kept in a standing position in a wet box at room temperature overnight. Permeability (porosity) of the gel was measured by the volume of a buffer (pH 7.4, 0.02 mol/L TRIS, 0.02 mol/L imidazol, 0.1 mol/L NaCl) percolated through the gel under different hydrostatic pressures. We measured the eluted amount of buffer obtained with respective pressure and calculated the permeability coefficient (Ks) using a special equation given by Carr et al. 1977 [24].

FVIII was measured using the COAMATIC FVIII reagent from Haemochrom Diagnostica (Essen, Germany). Fibrinogen was analysed by Fibrin-Prest Automate from Diagnostica Stago (Asnieres, France).

### Statistical analysis

Statistical analyses were performed by parametric tests for normally distributed variables. Non-normal distributed data were log-transformed and checked to be normally distributed before analysis. Association was estimated by determining regression coefficients and correlations of variables with a normal distribution. Multiple regression was used when there was more than one independent variable. Data are presented as mean  $\pm$  SD.  $P < 0.05$  was considered statistically significant. The inter- and intra-assay CVs for all tests were less than 10 [20,25]. The analysis of power in this study was performed *a priori* based on the assumption to detect a 20% difference in both the endpoints ETP respectively OHP. Our calculations showed that we would need approximately 16 patients assuming an  $\alpha$  error of 0.05, to achieve a power (1- $\beta$ ) of 0.80.

### Results

Basic characteristics of the investigated subjects are presented in Table 1.

$E_2$  increased from  $< 150$  pg/mL at DR to  $5889 \pm 4723$  pg/ml at HLS (range 1620–19500). Fibrinogen and FVIII concentrations in plasma increased by 19% ( $p < 0.001$ ) and 30% ( $p < 0.001$ ) from time of DR to HLS. We observed a 32% increase in OHP ( $p < 0.001$ ) and a 27% increase in OCP ( $p < 0.001$ ) whereas OFP remained almost unchanged (a non significant decrease by 1.5% from time of DR to HLS was observed). Of the thrombin generation variables ETP ( $p < 0.001$ ) and peak height ( $p < 0.001$ ) were significantly raised at HLS as compared to DR. Concurrently there were decreases in lag time ( $p = 0.001$ )

**Table 1**  
Baseline characteristics of the 31 patients.

	Patients	Reference interval
Age, years	33.0 $\pm$ 3.3	
Body mass index, kg/m <sup>2</sup>	24.1 $\pm$ 3.6	<25
Waist-hip ratio	0.8 $\pm$ 0.1	<0.8
<i>Causes and type of infertility</i>		
Female, n (%)	7 (22%)	
Male, n (%)	12 (39%)	
Unknown, n (%)	12 (39%)	
Current smoking, n (%)	2 (11%)	
Triglycerides, mmol/l	0.8 $\pm$ 0.4	0.45–2.6
Total cholesterol, mmol/l	4.9 $\pm$ 0.7	3.3–6.9
LDL cholesterol, mmol/l	2.9 $\pm$ 0.7	1.4–4.7
Creatinine, mmol/l	68.9 $\pm$ 7.3	<90
Blood glucose, mmol/l	4.7 $\pm$ 0.5	4.0–6.0
Hemoglobin, g/L	129.0 $\pm$ 8.6	117–153
Hematocrit, %	0.39 $\pm$ 0.02	0.35–0.46
Platelet count, $\times 10^9$ /L	251.2 $\pm$ 47.6	165–387

and time to peak ( $p < 0.001$ ). The levels of ETP, peak height and time to peak in all 31 patients are shown in Fig. 1. Regarding fibrin gel permeability only a small (–9%) statistically non-significant reduction in permeability (Ks) was observed ( $p = 0.13$ ).

Although significant changes toward increased coagulation were observed in the variables of thrombin generation as well as fibrin formation, all remained within normal limits (Table 2). The significant results remained also after exclusion of the three patients who later developed OHSS. All changes in haemostasis variables during IVF are shown in Table 2.

The changes in  $E_2$  correlated negatively to the change in lagtime ( $r = -0.49$ ,  $p = 0.005$ ) and the change in time to peak ( $r = -0.47$ ,  $p = 0.008$ ). At HLS  $E_2$  correlated to peak height ( $r = 0.40$ ,  $p = 0.026$ ). There were no other relations between  $E_2$  and the global haemostatic markers.

Multiple regression analyses showed that the change in FVIII from DR to HLS explained a part of the change in OHP ( $r = 0.49$ ,  $p = 0.024$ ). The change in peak height could in part be explained by the change in fibrinogen and FVIII ( $r = 0.47$ ,  $p = 0.034$ ).

Multiple regression analyses performed at the time of HLS showed that plasma fibrinogen significantly explained a part of the variation in OHP (overall haemostatic potential) ( $r = 0.65$ ,  $p = 0.002$ ). In addition to fibrinogen ETP also explained a part of the variation in OCP ( $r = 0.70$ ,  $p < 0.001$ ). Fibrinogen and FVIII explained a part of the variation of peak height ( $r = 0.47$ ,  $p = 0.031$ ).

We found a strong correlation between ETP and peak thrombin height both at the time of DR and HLS ( $r = 0.85$ ,  $p < 0.001$  and  $r = 0.80$ ,  $p < 0.001$  respectively).

Twelve patients got pregnant and all except one were primigravidae.

Three patients suffered a mild OHSS as defined by the Practice Committee of the American Society for Reproductive Medicine [26]. They had higher mean levels of ETP, OHP and fibrin gel permeability than those without OHSS; however, conventional statistical tests were not performed due to the low number of cases.

### Discussion

This is, to the best of our knowledge, the first study in which the global haemostatic methods CAT, OHP and the fibrin clot permeability assay have been investigated and compared during IVF treatment. We were able to demonstrate a highly significant increase in ETP and OHP concomitant with an approximately 38 times increase in estradiol from time of DR to HLS. It thus seems that elevations in circulating estrogens during IVF cause a shift in the haemostatic balance in the direction of a procoagulable state. It is tempting to speculate that this change may confer an increased risk of thromboembolic complications.

IVF pretreatment is a unique model to study the effect of estrogen on haemostasis because endogenous estrogen levels change considerably within a short period of time. At the same time the mechanisms by which estrogen induces changes in haemostatic variables can be examined in a controlled manner during IVF, in contrast to studies with wide variations of estrogen dosages, routes of administration and patient populations.

The studies of individual coagulation factors disclose a clear increase in VWF, Factors VIII, V, fibrinogen, as well as increased APC resistance, together with reduced AT, proteins C and S activity when ovarian stimulation occurs [7,9,27–29]. Our results, showing increased levels of FVIII and fibrinogen during IVF are therefore not surprising. In addition, some markers reflecting fibrinolysis are reduced during IVF [30–32]. The recently published study by our group brought to knowledge that even reduced levels of ADAMTS13 antigen and activity contribute to the increased levels of VWF at the time of HLS [33]. Totally, the individual factors seem to change towards a hypercoagulable state during IVF.

Although several reports have highlighted a need for more rapid testing as an aid in the diagnosis and treatment of OHSS and thrombotic

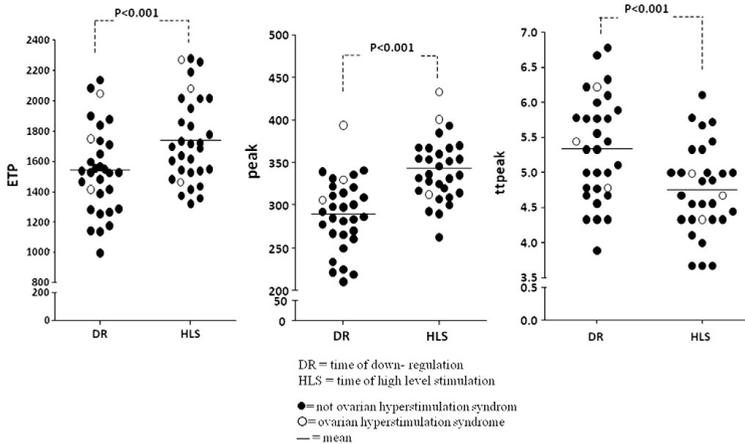


Fig. 1. Changes in ETP, Peak and Time to Peak in 31 patients.

diseases following IVF, only a limited number of haemostatic markers have been evaluated in individuals who eventually develop this complication [34]. Many of these tests are complicated, costly, and not widely available. To our knowledge, the study by Harnett et al. [35] was the only one utilizing a global haemostasis assay in IVF patients. They used thromboelastography in whole blood samples from 24 women at the time of down regulation and at the time of oocyte retrieval. A significant decrease in clot formation time and an increase in clotting index at the time of oocyte retrieval, indicated that supraphysiologic estrogen levels during IVF pretreatment were associated with a significant increase of the coagulable state. Aligning these findings, our results demonstrate that isolated high estrogen concentrations, as produced by an IVF protocol, are associated with an overall increase in haemostatic capacity.

Thrombin generation is augmented during IVF as demonstrated by the increase in both ETP and peak height, together with a decreased time to thrombin generation (lag phase) and time to peak of thrombin generation. A similar pattern has been observed when these parameters were analyzed in relation to the increased estrogen levels during normal pregnancy [36], preeclampsia [37] and oral contraceptive use [38].

Previous studies by our group have confirmed the sensitivity of the OHP method to detect the presence of a procoagulable state

when circulating estrogen levels are elevated. Along with increased gestational age in pregnant women OHP and OCP increased and OFP decreased [13]. Furthermore, hormone replacement treatment with high-dose oral estrogen in postmenopausal women caused an activation of coagulation and a decrease in fibrinolysis as measured by the OHP assay [39]. During controlled ovarian hyperstimulation in the present study, OHP increased significantly from the time of DR to HLS. Change in OHP was partly determined by change in FVIII level, as demonstrated by a statistically significant correlation between OHP on one side and the concentration of this coagulation protein on the other side. An additional variable of this global haemostatic assay - the Overall Coagulation Potential (OCP) - was significantly increased and correlated to ETP levels at the time of HLS reflecting enhanced thrombin generation in the IVF patients.

The studies of fibrinolysis parameters during IVF are quite opposing demonstrating a down-regulation of fibrinolysis at the time of oocyte retrieval on one side [27,30], as well as a fibrinolysis activation, with increased plasmin-antiplasmin (PAP) complexes and decreased PAI-1 on the other side [40]. The overall fibrinolysis potential (OFP) investigated in the present study was not significantly altered during IVF procedure, remaining within the reference range both at DR and HLS. It is possible that sample collection, preparation, and testing modality explained this difference. Still, our results demonstrate that supraphysiological estrogen levels during IVF exert more pronounced effects on coagulation than on fibrinolytic system, as observed by investigated global haemostatic markers.

A slight and non-significant decrease in fibrin gel permeability (Ks) was observed following HLS. This may be somewhat surprising as increased thrombin generation may be expected to induce a tighter and more stable fibrin clot. However, we used a relatively high concentration of thrombin to initiate clot-formation in our assay so endogenous thrombin generation should not influence the characteristics of the fibrin network formed. The observed increase in fibrinogen concentration observed, could only partly influence the results of fibrin clot permeability. Fibrin polymerisation rate as well as altered levels of fibrinogen-binding proteins (such as FXIII) are additional mechanisms that may affect fibrin clot porosity and deserve further investigations.

Despite the significant alterations in most of the investigated parameters in our study pointing towards enhanced coagulation, it

**Table 2**  
Plasma levels of haemostatic markers during the IVF treatment.

	DR	HLS	p-value	Reference interval
Factor VIII, %	1.0 ± 0.3	1.3 ± 0.4	<0.001	0.5 – 1.5
Fibrinogen, g/L	2.8 ± 0.7	3.3 ± 0.7	<0.001	2 – 4
Fibrin gel permeability, Ks	9.5 ± 3.5	8.6 ± 3.0	0.13	9.3 – 11.1
OHP, Abs sum	7.7 ± 2.9	10.2 ± 3.4	<0.001	4.2 – 14.5
OCP, Abs sum	15.3 ± 5.4	19.5 ± 5.5	<0.001	6.8 – 20.0
OFP, Abs sum	48.2 ± 11.7	47.5 ± 11.8	0.75	16.5 – 52.0
ETP, nM Ila	1542 ± 287	1739 ± 288	<0.001	1430 – 2273
Lag time, min	2.7 ± 0.6	2.5 ± 0.5	0.001	2.1 – 2.9
Peak, nM Ila	290 ± 42	343 ± 38	<0.001	270 – 395
ttPeak, min	5.3 ± 0.7	4.8 ± 0.6	<0.001	4.4 – 5.7

DR = down regulation.  
HLS = high level stimulation.

cannot be neglected that all variables remained within reference ranges. Nevertheless, there are potential scientific as well as clinical implications of our results. To start with, this is the first study of CAT and OHP parameters as well as fibrin clot permeability in this group of patients establishing reference intervals of these parameters which could differentiate them from normal pregnant women or patients treated with exogenous estrogens. More importantly, all investigated patients were healthy women without previous thromboembolic events or known thrombophilia and all of them underwent infertility treatment with the most widely accepted technique. Our results demonstrate that supraphysiologic estrogen concentrations gained during this treatment are associated with significant increases in the coagulable state which potentially could lead to thrombotic complications that can markedly affect the quality of life of these women. However, further experimental and prospective clinical studies are needed to establish the relevance of ETP and OHP as risk predictors of future thrombotic events and as tools to monitor the IVF treatment.

In conclusion, supraphysiologic estradiol concentrations during IVF-induced ovarian stimulation can lead to alteration of overall haemostasis towards a procoagulable state as demonstrated by global haemostatic markers and are related to increased thrombin generation and fibrin formation rather than decreased fibrinolysis or alterations in fibrin network characteristics.

#### Conflict of Interest Statement

No conflicts of interest to declare.

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## RESEARCH

# Incidence of pulmonary and venous thromboembolism in pregnancies after in vitro fertilisation: cross sectional study



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## Abstract

**Objective** To estimate the risk of pulmonary embolism and venous thromboembolism in pregnant women after in vitro fertilisation.

**Design** Cross sectional study.

**Setting** Sweden.

**Participants** 23 498 women who had given birth after in vitro fertilisation between 1990 and 2008 and 116 960 individually matched women with natural pregnancies.

**Main outcome measures** Risk of pulmonary embolism and venous thromboembolism (identified by linkage to the Swedish national patient register) during the whole pregnancy and by trimester.

**Results** Venous thromboembolism occurred in 4.2/1000 women (n=99) after in vitro fertilisation compared with 2.5/1000 (n=291) in women with natural pregnancies (hazard ratio 1.77, 95% confidence interval 1.41 to 2.23). The risk of venous thromboembolism was increased during the whole pregnancy (P<0.001) and differed between the trimesters (P=0.002). The risk was particularly increased during the first trimester, at 1.5/1000 after in vitro fertilisation versus 0.3/1000 (hazard ratio 4.22, 2.46 to 7.26). The proportion of women experiencing pulmonary embolism during the first trimester was 3.0/10 000 after in vitro fertilisation versus 0.4/10 000 (hazard ratio 6.97, 2.21 to 21.96).

**Conclusions** In vitro fertilisation is associated with an increased risk of pulmonary embolism and venous thromboembolism during the first trimester. The risk of pulmonary embolism is low in absolute terms but because the condition is a leading cause of maternal mortality and clinical suspicion is critical for diagnosis, an awareness of this risk is important.

**Trial registration** ClinicalTrials.gov NCT01524393.

## Introduction

Infertility affects more than 10% of couples worldwide.<sup>1</sup> Since the birth of the first “test tube baby” in 1978, in vitro fertilisation has been used increasingly to assist reproduction.<sup>2</sup> To date about

five million people have been born after in vitro fertilisation and this method is regarded as an effective and safe technique, with about a third of attempts resulting in pregnancy and a quarter in live births.<sup>3 4</sup>

It is well known that the risk of venous thromboembolism is increased during normal pregnancy. According to data from Sweden and Norway during the early 1990s venous thromboembolism occurred in slightly more than one out of 1000 pregnant women.<sup>5 6</sup> Venous thromboembolism in pregnant women after in vitro fertilisation has been reported in numerous case reports and in two small consecutive series, two out of 2500 (0.8/1000)<sup>7</sup> women and three out of 2748 (1.1/1000) women.<sup>8</sup> Notably, these estimates of incidence have been claimed to be comparable to those during natural pregnancy.<sup>9</sup> A recent report suggested that the incidence of venous thromboembolism after in vitro fertilisation was substantially increased during the first trimester but not in the other two trimesters.<sup>10</sup> No information exists on the risk of pulmonary embolism after in vitro fertilisation, which is important because embolism is a leading cause of maternal mortality.<sup>11 12</sup> Moreover, in the recently published report outpatient diagnoses were not included and no adjustments were made for the increased incidence of venous thromboembolism during the past decade.<sup>10</sup> Furthermore, no adjustments were made for the reported age difference in cases and controls, making the strength of the risk estimate of a 10-fold increase during the first trimester less exact.

We compared the risk of pulmonary embolism and venous thromboembolism in pregnant women after in vitro fertilisation with that of age and time matched women with natural pregnancy.

## Methods

This cross sectional study was based on the Swedish population between 1990 and 2008, with linkage of data from two national registers. The Swedish national patient register encompasses both inpatients and outpatients.<sup>13</sup> Inpatient data in Sweden became available nationwide in 1987. Outpatient diagnoses from hospitals started to be collected in 1997. Data in the patient register can be linked to other registries through the unique personal identity number assigned to Swedish residents.<sup>14</sup>

### Women who underwent in vitro fertilisation

From the Swedish in vitro fertilisation register at the National Board of Health and Welfare we retrieved information on mothers who had given birth after in vitro fertilisation. This register is now part of the Swedish medical birth register at the National Board of Health and Welfare and includes information on pregnancies after in vitro fertilisation since 1982. The birth register includes data that were collected and registered at the time the women were pregnant and includes validated information on the pregnancy, delivery, and neonatal periods for more than 99% of births in Sweden since 1973.<sup>15</sup> The birth register does not, however, include data on miscarriages or other pregnancy losses.

We restricted data retrieval to mothers of children born from 1990 and whose first child was born after assisted reproduction by in vitro fertilisation. Subsequent pregnancies in these women were therefore excluded from the study. Thus we identified 23 498 women who had given birth to their first child after in vitro fertilisation between 1990 and 2008.

### Women who did not undergo in vitro fertilisation

From the medical birth register each woman who had undergone in vitro fertilisation was matched with up to five women whose first child was born without in vitro fertilisation by calendar year of delivery (two years either way) and maternal age (one year either way). Subsequent pregnancies in these women were excluded from the study; the analysis therefore included only one pregnancy per patient. The matching resulted in 116 960 women who did not undergo in vitro fertilisation.

From the medical birth register we retrieved information on the women's country of birth, body mass index before pregnancy, marital status, smoking status, number of older siblings, singleton or multiple births, and estimated length of gestation. We calculated the dates of the trimesters from the length of gestation. The women's level of educational attainment was obtained from the register of education, Statistics Sweden, by record linkage.<sup>14</sup>

We obtained information on the diagnoses of venous thromboembolism including pulmonary embolism by linkage to the Swedish patient register.<sup>13</sup> The register comprises date of admission and discharge and the main diagnosis, with up to seven concomitant diagnoses. Inpatient care has been recorded nationwide since 1987. Outpatient visits at specialist clinics are included from 1997. Diagnoses were recorded according to the *International Classification of Diseases* (ninth revision before 1996 and 10th revision from 1997): codes 415B, 451B, 452, 453C-D, 453W, 453X, 673C, 671D-E, and 671F in ICD9 and I260, I269, I801-3, I808-9, I822-3, I828-9, O223, O225, O871, O873, O879, and O882 in ICD10. We therefore included all possible diagnoses of venous thromboembolism irrespective of location.

## Statistical analysis

The risk of venous thromboembolism and pulmonary embolism was assessed in women who had and had not undergone in vitro fertilisation during three periods: from 1 January 1987 to the calculated date of start of pregnancy, during pregnancy and delivery plus 42 days, and from day 43 to day 365 after delivery. We calculated proportional hazard regression on time to first event. To model and test the effect of in vitro fertilisation on venous thromboembolism in the different trimesters and delivery we used time dependent proportional hazard regression. We calculated 95% confidence intervals. In the time dependent model we tested effect modification of body mass index on the effect of in vitro fertilisation. Multivariate analysis taking parity, single or multiple births, smoking, education, maternal age, country of birth, calendar period, and marital status into account was carried out on the material stratified on body mass index and restricted to women with a body mass index <30.

All statistical calculations were done using SAS software, version 9.3. The regressions were carried out in the proportional hazards regression (PHREG) procedure of SAS.

## Results

Overall, 23 498 women were identified who had given birth after in vitro fertilisation. The median age of these mothers was 33 (interquartile range 31-36) years. Matching with women who had not had in vitro fertilisation resulted in an almost identical age distribution (median 33 (31-36) years, table 1⇓). The proportion of multiple births in the women who had undergone in vitro fertilisation was 16.9%. Of the women who had had in vitro fertilisation about 86% were born in Sweden, 6.7% were smokers, 53.2% had a body mass index <25, 8.1% were obese (body mass index >30), and 47.1% had attained university level education (>12 years).

The proportion of women who underwent in vitro fertilisation and experienced venous thromboembolism was 4.2/1000 (n=99) compared with 2.5/1000 (n=291) of the 116 960 matched women (table 2⇓). The risk after in vitro fertilisation increased during the whole pregnancy (P<0.001; hazard ratio 1.77, 95% confidence interval 1.41 to 2.23) and differed between the trimesters (P=0.002, fig 1⇓ and table 3⇓). In particular the risk was increased during the first trimester (1.5/1000 v 0.3/1000, hazard ratio 4.05, 2.54 to 6.46). The risk did not differ between the two groups of women before pregnancy (hazard ratio 0.85, 0.66 to 1.10) and during the year after delivery (1.29, 0.82 to 2.02, table 2).

Pulmonary embolism occurred in 19 women who underwent in vitro fertilisation (8.1/10 000) compared with 70 of the 116 960 matched women (6.0/10 000). The risk was increased after in vitro fertilisation (P<0.0034; hazard ratio 1.42, 0.86 to 2.36, fig 2⇓ and table 4⇓) and differed between the trimesters (P=0.0092). In particular the risk was increased during the first trimester (3.0/10 000 v 0.4/10 000, 6.97, 2.21 to 21.96).

Figure 3⇓ shows the time trend of diagnoses. The incidence of venous thromboembolism during pregnancy in women who did not undergo in vitro fertilisation was comparable to that of women in Sweden and Norway during the 1990s.<sup>5 6</sup> However, the incidence increased during the first decade of the new millennium, although it seemed mainly confined to outpatients.

No significant interaction was observed between body mass index and in vitro fertilisation on incidence of venous thromboembolism (P=0.21). The incidence in women who did not undergo in vitro fertilisation, however, increased as expected

by body mass index ( $P < 0.001$ ), but no such effect was observed in women after in vitro fertilisation ( $P = 0.46$ , fig 4 $\Downarrow$ ).

Further multivariate analysis taking calendar period, parity, single or multiple births, smoking, education, maternal age, country of birth, and marital status into account was carried out stratified on body mass index in two categories:  $< 25$  and  $25$ – $29.9$  (table 5 $\Downarrow$ ). Adjustment did not alter the significance of the main finding. The adjusted hazard ratio of venous thromboembolism during the first trimester was  $4.22$  (95% confidence interval  $2.46$  to  $7.26$ ). Multiple births seemed to have an influence on incidence of venous thromboembolism (adjusted hazard ratio  $2.12$ ,  $1.38$  to  $3.28$ ).

A separate multivariate analysis on women with first births after in vitro fertilisation compared with women not requiring assisted reproduction resulted in a hazard ratio of  $1.64$  ( $1.23$  to  $2.18$ ) during the whole pregnancy and  $3.50$  ( $1.81$  to  $6.80$ ) during the first trimester. The overall risk during 2001–08 compared with 1990–2001 did not decrease (table 5).

## Discussion

Pregnant women are at higher risk of venous thromboembolism after in vitro fertilisation, particularly during the first trimester. The risk of pulmonary embolism in women after in vitro fertilisation was increased almost sevenfold during the first trimester, although the absolute risk was low (2–3 additional cases of pulmonary embolism per 10 000 pregnancies).

Pulmonary embolism is, however, an elusive condition that is difficult to diagnose and is a leading cause of maternal death.<sup>11 12</sup> Our finding is therefore important to health professionals dealing with women who are recently pregnant after in vitro fertilisation.

The medical literature contains numerous case reports of venous thromboembolism during pregnancies after in vitro fertilisation, and these articles have been reviewed repeatedly.<sup>7 9 16</sup> One reason why these case reports attract attention is the unusual sites of the thromboses, such as the arms or neck. These reviews concluded that the risk of venous thromboembolism after in vitro fertilisation is comparable to that of pregnancies after natural conception,<sup>9</sup> different to our findings. In our study the incidence of venous thromboembolism in women after in vitro fertilisation significantly increased during the whole pregnancy and differed between the trimesters, in particular during the first trimester. This finding in the first trimester is in line with a recently published study.<sup>10</sup> However, the risk estimate in that study was almost double that of the present study—around 10-fold compared with fivefold. This difference in risk estimates probably resulted from the absence of matching by calendar period. From the Swedish medical birth register we matched each woman who had undergone in vitro fertilisation with five women who had not by both calendar period and age. Matching by age is important as women with pregnancies after in vitro fertilisation are on average older than those with natural pregnancies.<sup>10</sup> It is well known that the incidence of thromboembolism increases with age.<sup>17</sup> We also matched by calendar year of delivery, which avoided biased estimates as the number of pregnancies after in vitro fertilisation and the number of thromboembolic events both increase by calendar year. The in vitro fertilisation procedure has changed over time with the introduction of more patient friendly protocols, less vigorous stimulation with lower doses of gonadotropins and, particularly in Sweden, a decreasing rate of multiple births, from almost 30% to 5%.<sup>15</sup> Adjustments were also needed to take account of the increased incidence of venous thromboembolism noted recently.<sup>10</sup> We analysed and adjusted for several maternal factors known to affect pregnancy and vascular disease outcome

such as age, calendar year of delivery, body mass index, parity, smoking, country of birth, marital status, and education. The risk of venous thromboembolism increased by body mass index in women who did not undergo in vitro fertilisation but not in those who did (fig 4). The reason for this could not be explained. It could be speculated that differing oestrogen levels in lean and obese women might play a part. Furthermore, the ovarian hyperstimulation syndrome is more common in women with a low body mass index.<sup>18 19</sup> Since the ovarian hyperstimulation syndrome is closely related to venous thromboembolism this might also play a part.

## Strengths and limitations of this study

The patient register is generally considered to have high validity and was used to estimate the incidence of venous thromboembolism in pregnant women during 1990–93.<sup>4 13</sup> In the present study we found a similar incidence in women who did not undergo in vitro fertilisation during that period.

However, thromboembolic events in women regardless of whether they underwent in vitro fertilisation increased during the first decade of the new millennium. One reason for this could be the inclusion of outpatient diagnoses (fig 3). In addition, awareness of venous thromboembolism during pregnancy has increased over time. A high baseline index for suspicion of venous thromboembolism during pregnancy is critical for diagnosis because many of the clinical signs and symptoms are also common in normal pregnancies.<sup>20 21</sup> Improvement in diagnostic procedures, including a more extensive use of ultrasound examinations, has probably contributed to the increased incidence of venous thromboembolism.<sup>20 22 23</sup> We have no information on the severity of the venous thromboembolic events. Furthermore, improvements in diagnostic procedures and increased doctors' awareness could contribute to the increased incidence of venous thromboembolism.<sup>24</sup> A limitation of our study is that owing to a lack of relevant data we could not check these assumptions.

A further limitation is that we cannot rule out surveillance bias (detection bias) between the two groups of women. Surveillance bias is unlikely, however, because of the noticeable significant increase of thromboembolic events during the first trimester in pregnancies after in vitro fertilisation. A further argument against surveillance bias was the absence of an increased risk after delivery.

A weakness of the Swedish medical birth register is that it only includes women with deliveries. Thus an obvious bias is that more complicated pregnancies resulting in fatal outcomes to the mothers were not included. This could underestimate the true risk of venous thromboembolism and, in particular, pulmonary embolism during a pregnancy after in vitro fertilisation. Notably a major cause of maternal deaths during pregnancy is embolism.<sup>11 12</sup> Furthermore, the use of matched controls in the present study clearly indicated an increased risk of thromboembolism after in vitro fertilisation.

A further potential weakness could be an influence of parity on the propensity to acquire thromboses. However, this is opposed by the fact that also women with first births had an increased risk of venous thromboembolism compared with natural pregnancy. Another bias could be the presence of thrombophilia and the antiphospholipid syndrome as these might influence fertility.<sup>25 26</sup>

## Time distribution of thromboembolic events

The distribution of pulmonary embolism and venous thromboembolism during the three trimesters after in vitro

fertilisation (figs 1 and 2)) contrasted with that of natural pregnancies.<sup>17</sup> Notably, the risk of pulmonary embolism in natural pregnancies is highest in the postpartum period,<sup>17 20 27</sup> whereas in the present study the greatest risk in women after in vitro fertilisation was during the first trimester. The close time relation to the in vitro fertilisation procedure suggests that changes induced by the procedure itself could be of pathophysiological importance. A plausible initiator of adverse mechanisms could be the noticeable increase in endogenous oestrogen levels during the stimulation phase of treatment before the actual procedure.<sup>7</sup> During in vitro fertilisation the oestrogen level increases rapidly (10-fold to 100-fold) from the down-regulation phase to the stimulation phase.<sup>28</sup> Exogenous oestrogens have been repeatedly associated with an increased incidence of venous thromboembolism, irrespective of the indication for their use. The first reports were in women using oral contraceptives containing oestrogen and recently in women using oestrogen after the menopause.<sup>29-31</sup> In the largest randomised study to date the risk of venous thromboembolism in women using exogenous oestrogens compared with placebo was doubled (hazard ratio 2.06, 95% confidence interval 1.57 to 2.70).<sup>31</sup> An increased risk of cardiovascular events has also been shown in men receiving oestrogen for testosterone dependent prostatic carcinoma.<sup>32</sup>

## Conclusions

Our results show an increased risk of thromboembolism and, importantly, pulmonary embolism in pregnant women after in vitro fertilisation. Doctors should be aware of these increased risks because the symptoms of pulmonary embolism can be insidious and the condition is potentially fatal. Efforts should focus on the identification of women at risk of thromboembolism, with prophylactic anticoagulation considered in women planning to undergo in vitro fertilisation.

Contributors: PH, EW, and OH initiated the study. PH and AE had overall responsibility for the study and are the guarantors. All authors contributed to the study design. AE, EW, LB, and PH contributed to data analysis. All authors contributed to the interpretation of the data. PH led the writing of the report and wrote the first draft of the final report. All authors helped to prepare the final report and have seen and approved the final version. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

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Competing interests: All authors have completed the ICMJE uniform disclosure form at [www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) (available on request from the corresponding author) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; and no other relationships or activities that could appear to have influenced the submitted work.

Ethical approval: The study was approved by the research ethics committee of Karolinska Institutet, Stockholm, Sweden (Dnr 2010/267-31/4).

Data sharing: No additional data available.

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### What is already known on this topic

Embolism is an important cause of maternal mortality in developed countries

In vitro fertilisation (IVF) is increasingly used for assisted reproduction

Reports suggest that the risk of venous thromboembolism is not increased after IVF compared with natural pregnancies although a recent report showed an increased risk during the first trimester

### What this study adds

The risk of venous thromboembolism increased during all trimesters in women after IVF, in particular during the first trimester

The risk of pulmonary embolism significantly increased during the first trimester

## Tables

**Table 1 | Characteristics of pregnant women who underwent in vitro fertilisation (IVF) and women with natural pregnancies between 1990 and 2008, Sweden. Values are numbers (percentages) unless stated otherwise**

Characteristics	IVF pregnancies (n=23 498)	Natural pregnancies (n=116 960)
<b>Maternal age at birth:</b>		
19-24	322 (1.4)	1510 (1.3)
25-29	3764 (16.0)	18 438 (15.8)
30-34	10 129 (43.1)	50 575 (43.2)
35-39	8016 (34.1)	40 083 (34.3)
40-47	1267 (5.4)	6354 (5.4)
Mean (SD) age (years)	33.3 (4.0)	33.4 (3.9)
Median (interquartile range) age (years)	33 (31-36)	33 (31-36)
Born in Sweden	20 267 (86.2)	95 180 (81.4)
<b>Prepregnancy body mass index:</b>		
Missing data	3961 (16.9)	20 228 (17.3)
<25	12 490 (53.2)	62 464 (53.4)
25-29	5136 (21.9)	24 646 (21.1)
≥30	1911 (8.1)	9622 (8.2)
<b>Cigarette smoker:</b>		
Missing data	1793 (7.6)	7731 (6.6)
No	20 130 (85.7)	96 996 (82.9)
Yes	1575 (6.7)	12 233 (10.5)
<b>Education (years):</b>		
Missing data	48 (0.2)	1027 (0.9)
≤9	1789 (7.6)	11 741 (10.0)
10-12	10 588 (45.1)	50 527 (43.2)
>12	11 073 (47.1)	53 665 (45.9)
<b>Marital status:</b>		
Missing data	1800 (7.7)	7798 (6.7)
Married to or cohabiting with father of child	21 569 (91.8)	103 882 (88.8)
Single	51 (0.2)	3,166 (2.7)
Other	78 (0.3)	2114 (1.8)

Table 2| Venous thromboembolism and pulmonary embolism events in pregnant women after in vitro fertilisation (IVF) and women with natural pregnancies matched on age and calendar period of delivery. Values are numbers (percentages) of women unless stated otherwise

Events in relation to pregnancy	IVF pregnancies (n=23 498)	Natural pregnancies (n=116 960)	Proportional hazard regression (95% CI)
Venous thromboembolism:			
Prepregnancy	71 (0.30)	415 (0.35)	0.85 (0.66 to 1.10)
Pregnancy and delivery	99 (0.42)	291 (0.25)	1.77 (1.41 to 2.23)
Days 43-365 postpartum	24 (0.10)	95 (0.08)	1.29 (0.82 to 2.02)
Pulmonary embolism:			
Prepregnancy	21 (0.09)	103 (0.09)	1.04 (0.65 to 1.66)
Pregnancy and delivery	19 (0.08)	70 (0.06)	1.42 (0.86 to 2.36)
Days 43-365 days postpartum	3 (0.01)	26 (0.02)	0.60 (0.18 to 1.98)

**Table 3| Time to first venous thromboembolic event by trimester in pregnant women after in vitro fertilisation (IVF) and women with natural pregnancies matched on age and calendar period of delivery. Effect of different levels of effect modifier body mass index (BMI) is also given. Values are numbers (percentages) of women unless stated otherwise**

Variables	IVF pregnancies (n=23 498)	Natural pregnancies (n=116 960)	Hazard ratio (95% CI)	Proportional hazard regression (95% CI)*			
				Total	BMI <25	BMI 25-29.9	BMI >30
First trimester (weeks 1-12)	36 (0.15)	38 (0.03)	4.61 (2.95 to 7.21)	4.05 (2.54 to 6.46)	6.64 (3.6 to 12.23)	2.61 (0.97 to 7.07)	1.01 (0.22 to 4.6)
Second trimester (weeks 13-25)	23 (0.10)	63 (0.05)	1.00 (0.51 to 1.97)	1.11 (0.54 to 2.29)	1.04 (0.40 to 2.72)	2.13 (0.66 to 6.93)	—†
Third trimester (week 26 to <3 days before birth)	33 (0.14)	94 (0.08)	1.04 (0.64 to 1.69)	1.30 (0.77 to 2.19)	1.30 (0.61 to 2.80)	1.80 (0.81 to 4.01)	0.26 (0.04 to 1.91)
≤3 days before to 42 days after birth	49 (0.21)	192 (0.16)	1.69 (1.15 to 2.48)	1.59 (1.03 to 2.45)	1.66 (0.90 to 3.05)	1.55 (0.75 to 3.24)	1.45 (0.49 to 4.27)
P value for test of difference‡	—	—	—	<0.001	<0.001	0.07	0.53
P value for test of equal effect in all time periods§	—	—	—	0.002	0.0006	0.86	0.33

\*Women with complete data on BMI.

†Weeks 13-25 are merged with week 26 until three days before delivery owing to few events.

‡Wald  $\chi^2$  test for difference between IVF and no IVF.

§Wald  $\chi^2$ .

Table 4| Time to first event of pulmonary embolism during trimesters in pregnant women after in vitro fertilisation (IVF) and women with natural pregnancies matched on age and calendar period of delivery. Values are numbers (percentages) unless stated otherwise

Variables	IVF pregnancies (n=23 498)	Natural pregnancies (n=116 960)	Proportional hazard regression (95% CI)
First trimester (weeks 1-12)	7 (0.03)	5 (0.004)	6.97 (2.21 to 21.96)
Second trimester (weeks 13-25)	5 (0.02)	13 (0.01)	0.42 (0.05 to 3.20)
Third trimester (week 26 to <3 days before birth)	6 (0.03)	18 (0.02)	0.40 (0.10 to 1.68)
≤3 days before to 42 days after birth	11 (0.05)	49 (0.04)	1.79 (0.86 to 3.74)
P value for test of difference*	—	—	0.0034
Test of equal effect in all time periods†	—	—	0.0092

\*Wald  $\chi^2$  for difference between IVF and no IVF.

†Wald  $\chi^2$  test.

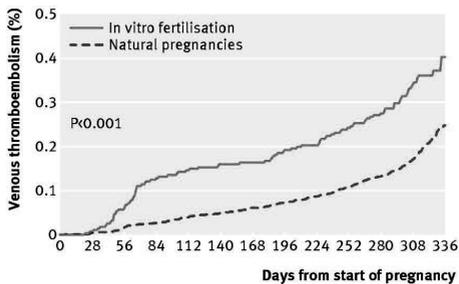
Table 5 | Multivariate analysis stratified on body mass index (BMI) in pregnant women with BMI <30: 23 498 women after in vitro fertilisation (IVF) and 116 960 women with natural pregnancies matched on age and calendar period of delivery

Variables	Hazard ratio (95 % CI)		
	BMI <25	BMI 25-29.9	Adjusted by conditioning on BMI
Natural pregnancies	1=reference	1=reference	1=reference
IVF pregnancies:			
First trimester (weeks 1-12)	5.21 (2.68 to 10.14)	2.40 (0.85 to 6.80)	4.13 (2.37 to 7.17)
Second trimester (weeks 13-25)	0.91 (0.34 to 2.46)	1.78 (0.53 to 5.93)	1.18 (0.55 to 2.51)
Third trimester (week 26 to <3 days before birth)	1.17 (0.53 to 2.58)	1.38 (0.57 to 3.33)	1.25 (0.69 to 2.25)
≤3 days before to 42 days after birth	1.10 (0.56 to 2.17)	1.27 (0.59 to 2.75)	1.16 (0.69 to 1.93)
P value for test of difference*	<0.001	0.77	<0.001
Test of equal effect in all time periods†	0.001	0.42	0.0017
No of older siblings:			
None	1=reference	1=reference	—
≥1	0.92 (0.65 to 1.30)	1.36 (0.58 to 3.19)	0.83 (0.63 to 1.1)
Single birth	1=reference	1=reference	—
Multiple births	2.52 (1.49 to 4.25)	1.33 (0.57 to 3.13)	2.06 (1.32 to 3.21)
Smoking at start of pregnancy:			
No	1=reference	1=reference	—
Yes	0.91 (0.49 to 1.68)	0.89 (0.45 to 1.77)	0.89 (0.56 to 1.41)
Education (years):			
≤9	1=reference	1=reference	—
10-12	1.16 (0.57 to 2.39)	0.55 (0.27 to 1.12)	0.88 (0.53 to 1.44)
>12	1.26 (0.61 to 2.59)	0.80 (0.40 to 1.62)	1.06 (0.64 to 1.75)
Maternal age at delivery (years):			
<35	1=reference	1=reference	—
≥35	—	—	—
First trimester (weeks 1-12)	0.84 (0.43 to 1.63)	2.34 (0.85 to 6.45)	1.14 (0.67 to 1.96)
Second trimester (weeks 13-25)	0.60(0.25 to 1.43)	2.25 (0.73 to 6.88)	0.92 (0.48 to 1.75)
Third trimester (week 26 to <3 before birth)	0.60(0.30 to 1.23)	1.20 (0.58 to 2.49)	0.84 (0.51 to 1.38)
≤3 before to 42 days after birth	1.53 (0.91 to 2.57)	2.64 (1.41 to 4.96)	1.88 (1.26 to 2.78)
Country of birth:			
Sweden	1=reference	—	—
Other country	1.14 (0.72 to 1.83)	3.06 (1.45 to 6.44)	1.62 (1.09 to 2.41)
Calendar period:			
1990-2001	1=reference	1=reference	1=reference
2002-08	1.55 (1.12 to 2.14)	2.64 (1.64 to 4.25)	1.86 (1.43 to 2.43)
Marital status:			
Married to or cohabiting with father of child	1=reference	1=reference	1=reference
Other	0.81 (0.29 to 2.21)	1.34 (0.53 to 3.36)	1.03 (0.52 to 2.03)

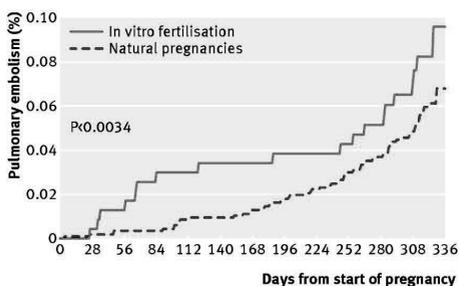
\*Wald  $\chi^2$  for difference between IVF and no IVF.

†Wald  $\chi^2$ .

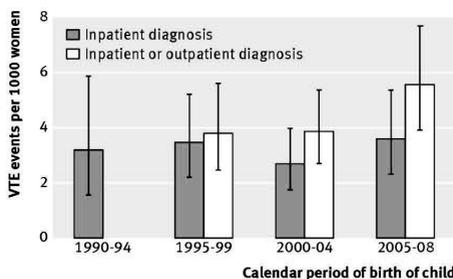
## Figures



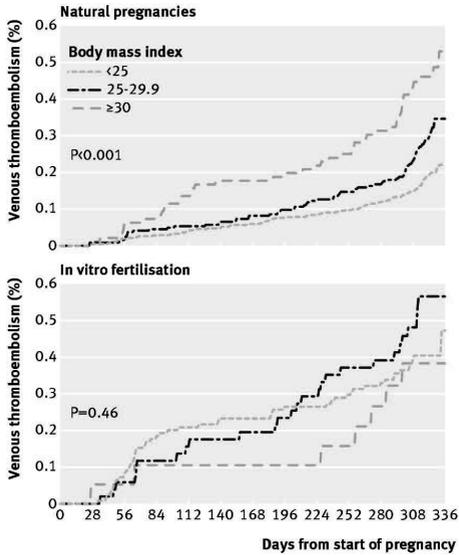
**Fig 1** Proportional hazard regression of venous thromboembolism in pregnant women after in vitro fertilisation (n=23 498) and in women with natural pregnancies (n=11 960) matched on age and calendar period of delivery



**Fig 2** Proportional hazard regression of pulmonary embolism in pregnant women after in vitro fertilisation (n=23 498) and in women with natural pregnancies (n=11 960) matched on age and calendar period of delivery



**Fig 3** Time trends of incidence of registered venous thromboembolic (VTE) diagnoses per 1000 pregnant women after in vitro fertilisation and women with natural pregnancies matched on age and calendar period of delivery in Swedish national patient register, National Board of Health and Welfare



**Fig 4** Proportional hazard regression of venous thromboembolism in three strata for body mass index (<25, 25-29.9, and ≥30) in pregnant women after in vitro fertilisation (n=23 498) and in women with natural pregnancies (n=116 960) matched on age and calendar period of delivery







**Increased incidence of hypertension in women after IVF pregnancy: A population-based cohort study from Sweden.**

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## **Abstract**

**Objective:** To investigate whether cardiovascular disease (CVD) and related risk factors have a higher incidence in women after IVF pregnancy as compared to women who delivered after natural conception.

**Design:** A cohort study in Sweden of all women who had given birth to a child after in vitro fertilization (IVF) between 1990 and 2008 and individually matched women from the Swedish Medical Birth Register. Inpatient and outpatient diagnoses of CVD and related risk factors in both groups were identified by linkage to the Swedish National Patient Register.

**Participants:** 23,498 women who had given birth to a child after IVF between 1990 and 2008 and 116,960 individually matched women.

**Main outcome measures:** Incidence rates of hypertension, diabetes, stroke and coronary heart disease in both groups.

**Results:** After a mean follow-up of 8.6 years in both groups, the incidence rate of hypertension was 2.58/10.000 person-years (n=520) in the IVF group as compared to 2.13/10.000 person-years (n=2129) in the control group. Multivariable analysis adjusted for women's BMI, smoking, education and country of birth showed that hypertension had a higher incidence in IVF mothers [Hazard Ratio (HR) 1.27, 95% Confidence Interval (CI) 1.13-1.41] as compared to mothers from the Medical Birth Register. There was a trend to a higher incidence of stroke (HR 1.27, 95% CI 0.96-1.68). However, the incidence of coronary heart disease and diabetes did not differ after IVF pregnancies as compared to controls (HR 0.72, 95% CI 0.44-1.17 respectively HR 0.96, 95% CI 0.81-1.14).

**Conclusion:** Hypertension was more prevalent after IVF pregnancies. This could indicate that infertile women could have a propensity to develop CVD later in life.

**Keywords:** Infertility, cardiovascular disease (CVD), In Vitro Fertilization (IVF), hypertension, stroke, diabetes

## **Introduction**

Infertility afflicts more than 10% of all couples worldwide (1). This condition has some relation to cardiovascular diseases (CVD). One is via the polycystic ovarian syndrome (PCOS), the most common cause of female infertility (2). Women with PCOS are characterized by a greater body mass and a sevenfold increased risk of myocardial infarction (3). Both obesity as well as underweight seems to be associated with reduced fertility (4). In addition, smoking, age and environmental factors also seem to impair the development and function of the reproductive organs, which could lead to hormonal imbalance and reduced fertility (5).

Endothelial dysfunction is an early marker of CVD (6). Possible associations of endothelial dysfunction in the ovarian vasculature and infertility have to our knowledge not as yet been studied. However, endothelial dysfunction in PCOS has been found in both humans and in animal models (7, 8). In addition, women with PCOS have menstrual irregularities, which are associated with subfertility (9) and increased risk of CVD (10).

Heart disease is the leading cause of death in women while stroke is the third most common cause of death (11).

Thus, the aim of our study was to assess whether CVD and related risk factors have a higher incidence in women after IVF pregnancy as compared to women who delivered after natural conception.

## **Methods:**

### Data sources

We performed a cohort study using linkage of Swedish population-based registers maintained at the Swedish National Board of Health and Welfare and Statistics Sweden (12, 13).

Sweden has excellent conditions for observational studies in the health care area, with high-quality population-based registers covering essentially all inpatient and now also outpatient care and birth records. Linking of the Swedish Medical Birth Register (MBR) and the Swedish IVF Register allowed us to compare the incidence of CVD after pregnancy in subjects who had undergone IVF to age and time period matched control women.

Individual records were linked across the registers using the unique personal identity number assigned to all Swedish residents, i.e. individuals either born in Sweden or immigrating to Sweden (14).

Information on women's country of birth, pre-pregnancy body mass index (BMI), family situation, cigarette smoking habits, number of older siblings and singleton/multiple births was retrieved from the MBR. MBR was initiated in 1973 to give information on maternal, pregnancy and infant factors (12). Up to 1998, more than 98% of all births in Sweden were registered in the MBR.

Women's education was retrieved from the Register of Education, Statistics Sweden (15), by linkage through the personal identity number (14).

Diagnoses of CVD and related risk factors were found by linkage to the Swedish Patient Register of the National Board of Health and Welfare. The register comprises dates of admission and discharge, and main diagnosis with up to seven contributing diagnoses. Inpatient care is recorded nationwide since 1987. Outpatient visits at specialist clinics are included from 2000. Diagnoses were recorded according to the International Classification of Diagnoses (ICD), edition 9 (ICD-9) from 1990 to 1996 and edition 10 (ICD-10) from 1997 onwards.

The National Causes of Death Register contains data concerning immediate, contributory, and underlying causes of death classified using ICD-10.

### Study population

All women undergoing successful IVF treatment between 1<sup>st</sup> of January 1990 and 31<sup>st</sup> of December 2008 were included. Each IVF woman was matched with up to five women from the MBR by calendar year of delivery  $\pm 2$  years and maternal age  $\pm 1$  year.

Mothers who had given birth to a child as a result of IVF were retrieved from the Swedish IVF Register, which is now a part of the Swedish MBR at the National Board of Health and Welfare and includes information on IVF pregnancies since 1982. The MBR includes prospectively collected and validated information from pregnancy, delivery, and neonatal period on more than 99% of all births in Sweden since 1973.

We restricted the retrieval to mothers of a child born as from 1990 and who had their first child born after assisted reproduction by IVF. Thus, we identified 23 498 women who had

given birth to a child after IVF during the time period 1990-2008 and 116,960 individually matched women.

#### Follow-up and outcomes

Follow-up started at the time of delivery. End of follow-up was 31<sup>st</sup> of December 2009 or the date of first registered event of the following: a CVD diagnosis (coronary heart disease or stroke) or a diagnosis of a related risk factor (hypertension and diabetes), emigration from Sweden, or death.

Hypertension was defined as ICD-9 codes 401-405, 642A-D and 642X, ICD-10 codes I10-13, I15, O10.0-4, O11.9, O13.9 and O16.9. Diabetes was defined as ICD-9 codes 250 and 648A and ICD-10 codes E10-11, E14 and O24. Codes that specifically differentiated between type 1 and type 2 diabetes were introduced first with ICD-10 in 1997.

Coronary heart disease was defined as unstable angina (ICD-9 code 411B, ICD-10 code I20.0), or myocardial infarction (ICD-9 codes 410 and 412, ICD-10 codes I21-23 and I25.2). Stroke comprised cerebral infarction (ICD-9 codes 433-434 and 438, ICD-10 codes I63 and I69.3-4) or intracerebral haemorrhage (ICD-9 code 431, ICD-10 code I61). The positive predictive values (i.e. validity) of myocardial infarction and stroke diagnoses in the Swedish hospital discharge register have been demonstrated to be around 95% when only primary diagnoses are considered (16, 17).

Hypothyroidism was defined as ICD-9 codes 243-44 and ICD-10 codes E02.9 and E03, whereas we used ICD-9 codes 642.4-7 and ICD-10 code O14 for preeclampsia. Polycystic ovarian syndrome was defined as ICD-9 code 256.4 and ICD-10 code E28.2.

The study was approved by the Research Ethics Committee of Karolinska Institutet, Stockholm, Sweden (Dnr 2010/267-31/4).

#### **Statistical analysis:**

Baseline variables and population characteristics are presented as means and standard deviations for normally distributed variables and as number and frequencies for categorical variables. BMI, educational level, smoking, country of birth, hypertension, diabetes, coronary heart disease and stroke were all analyzed as categorical variables.

The person-time for each woman was calculated from end of pregnancy to the month of diagnosis of the end-point (CVD or related risk factors), the month of death from other causes, emigration or end of follow-up (31<sup>st</sup> of December 2009).

We investigated the incidence of hypertension, diabetes, stroke and coronary heart disease in both univariable and in multivariable-adjusted analysis models. As the IVF group differed from the control group concerning educational level, smoking habits and country of birth, multivariable analyses were performed adjusting for those background factors and in addition BMI. Hazard ratios (HR) and 95% Confidence Intervals (95% CI) were estimated using proportional hazard regression analysis.

All statistical calculations were performed using the SAS software package, version 9.2, by SAS Institute Inc., Cary, NC, USA.

## **Results:**

The baseline characteristics of the study sample of women having their first delivery from 1990 to 2008 are given in table 1. The mean age of the women in the IVF group was  $33.3 \pm 4.0$  respectively  $33.4 \pm 3.9$  in the control group. Women in the IVF group had a higher educational level, were in a less frequency smokers and had a higher proportion of women who were born in Sweden as compared to the control group.

As shown in table 2, only a few women emigrated and just 0.5% died during follow-up. The mean follow-up time was  $8.6 \pm 4.6$  years contributing to 201 498 person-years at risk in the IVF group and a follow-up of  $8.6 \pm 4.9$  with 1 000 368 person-years at risk in the control group.

During this period, 823 women developed a first event (diabetes,  $n = 201$ ; hypertension,  $n = 520$ ; coronary heart disease,  $n = 23$ ; stroke,  $n = 79$ ). According to table 3, the proportion of women with hypertension and stroke was slightly greater in the IVF group as compared to the MBR controls.

Multivariable analysis adjusted for women's BMI, smoking habits, education and country of birth identified a higher incidence of hypertension among IVF women (HR 1.27, 95% CI 1.13-1.41) as compared to MBR controls. There was a trend to a higher incidence of stroke (HR 1.27, 95% CI 0.96-1.68) but the incidence of coronary heart disease and diabetes did not

differ after IVF pregnancies as compared to controls (HR 0.72, 95% CI 0.44-1.17 and HR 0.96, 95% CI 0.81-1.14).

As shown in table 4, when looking at the whole group of women, those who were smoking more than 10 cigarettes daily had a higher incidence of coronary heart disease as compared to non-smokers (HR 5.46, 95% CI 3.55-8.40). Even women smoking less than 10 cigarettes daily had a higher incidence of coronary heart disease as compared to non-smokers (HR 4.16, 95% CI 2.81-6.18). Furthermore, as expected, an increase in BMI resulted in a higher occurrence of CVD. BMI more than 30 increased the incidence of diabetes (HR 5.25, 95% CI 3.09-7.07), coronary heart disease (HR 5.23, 95% CI 2.34-11.72) and hypertension (HR 3.84, 95% CI 3.07-4.82), whereas stroke occurrence was unaffected (HR 1.15, 95% CI 0.70-1.90).

The results of all univariable and multivariable analyses are shown in table 4.

## **Discussion:**

The main result of this large, population based cohort study is a higher incidence of hypertension in women who had a child born after IVF than in women who delivered after natural conception in this Swedish population during the time period 1990-2008. IVF mothers also had a trend to a higher incidence of stroke, while the incidence of coronary heart disease and diabetes did not differ between the two groups of women.

To our knowledge, this is the first prospective cohort study investigating whether CVD and related risk factors have a higher incidence in women after IVF pregnancy as compared to women who delivered after natural conception.

The main advantages of this study are its sample size and its prospective nature. To get a representative group of controls we included five control women for each IVF woman and were able to retrieve data about smoking, BMI, country of birth and education by matching of relevant registers.

Women in the IVF group seemed to have life circumstances that should reduce the risk of CVD. This could be exemplified by a lower frequency of smokers, a higher educational level and a lower proportion of women born abroad.

However, IVF women had a higher incidence of hypertension than control women. Hypertension is a main risk factor for CVD but there is a long-term delay between the

initiation of vascular disease processes and manifest vascular diseases such as coronary heart disease and stroke (18).

The number of events with coronary heart disease (n=23) and stroke (n=79) in the 23,498 women in the IVF group was small. However, when looking at the effects of the risk factors in the whole group of women (Table 4), we found as expected that obese women (BMI>30) had a higher incidence of diabetes, coronary heart disease and hypertension.

We suggest that the number of women, the mean age and follow-up time in the present study were too low and the mean follow up period of 8.6 years too short to have a high enough cardiovascular event rate to reach statistical power. Due to increasing numbers of IVF-treated women, most of the included women had their IVF treatment after year 2000. As the mean age during inclusion was 33 years, the mean age at end of follow-up was only 41 years. Despite this low mean age, when stratifying the whole group into different subgroups of smoking, those who were smoking more than 10 cigarettes daily had an increased incidence of coronary heart disease as compared to non-smokers. Cigarette smoking is associated with a two year earlier onset of menopause (19) and smoking is an important determinant for coronary heart disease in women (20, 21).

The result of our study is in accord with the study of Parikh et al, which was conducted in Swedish women with self-reported data on subfertility in the Swedish MBR (22). They compared women reporting more than 5 years of subfertility with women who got pregnant within one year and found a significantly higher risk of CVD among subfertile women with a HR of 1.19 (95% CI 1.02-1.39). In contrast to Parikh, we separated the different subgroups of CVD and related risk factors into hypertension, diabetes, coronary heart disease and stroke. The strength of our study is thus the more specific diagnose codes. In the subgroup of stroke diagnoses we excluded subarachnoidal bleedings and subdural haematomas to get a more distinct group which is also related to hypertension as a risk factor.

As in previous reports, preeclampsia was overrepresented in the IVF group, and the condition is a well known risk factor for development of CVD later in life (23, 24). Actually, the risk is more than doubled as compared to those with uncomplicated pregnancy (25). Preeclampsia occurs in 3-5% of normal pregnancies, and in our IVF group there were 7.0% with preeclampsia as compared to 5.2% in the control group.

The incidence of hypertension could be higher in women after oocyte donation (26), but as this procedure was not allowed in Sweden until 2003 only a very low proportion in the IVF group had oocyte donation in the present study.

PCOS, prevalent in 6-8% of women in reproductive ages (27), accounts for up to 15% of women with female infertility and is associated with subclinical atherosclerosis as assessed by carotid intima-media thickness and endothelial dysfunction (28). The number of women with PCOS was higher in the IVF group than in the control group, but the proportion was low in both groups. We speculate whether this condition was under-reported.

Excess adiposity as assessed by BMI is associated with subfertility (29) and incident CVD (30). However, adjustment for BMI neither nullified nor attenuated our CVD estimates, suggesting that factors other than BMI underlie the increased incidence of hypertension in the IVF group.

Another possible mechanistic link between infertility and CVD is hypothyroidism, which is linked both to infertility (31) and incident CVD (32). However, in our material, the prevalence of women with hypothyroidism was low in both the study and control group. Therefore, we assume that this condition did not affect the result.

Limitations:

Our data are based on results from a register study. Therefore, it is not possible to determine causality from our results, and it is still plausible that increased blood pressure and infertility occur concomitantly, rather than one preceding the other.

Only women giving birth to a baby after IVF treatment are included in the IVF register. To get a complete picture of the situation, all women doing IVF treatments should preferably be included. This is today not possible with available registers.

Unfortunately, we have no information about causes of infertility in our women in the register. In a recent report 64% were due to female factors and male factors accounted for just 19% in all infertile couples. The remaining 17% had an unknown cause (33). In our study material with more than 20.000 IVF women, male infertility cases may have diluted the results causing an underestimation of the true difference.

Diagnoses of hyperlipidaemia and cholesterol levels are not included in our cohort study. Hyperlipidaemia diagnoses codes are not so well recorded in registers in general and has thus a low validity. Cholesterol levels are not at all included in the registers. This is of course a limitation of the study since hypercholesterolaemia is a major CVD risk factor (34). Differences in cholesterol levels between the two groups could have affected the results of the study. As the women in the IVF group could have a lowered risk of CVD due to beneficial life circumstances this could well be true concerning lipid levels. Two other possible confounders in this study could be physical activity and alcohol intake, as both could influence the risk of CVD.

Certainly, some of the study subjects used hormone replacement therapy (HRT) to prevent postmenopausal symptoms during the follow-up period. There are a lot of conflicting reports regarding HRT and CVD risk (35, 36). However, a mean age of 41 years at follow-up is well below the mean age of menopause in Sweden. Thus HRT should possibly not have affected our results.

### **Conclusion:**

Our results show that IVF pregnancy is associated with a higher incidence of hypertension in the years after delivery as compared to delivery after natural conception. This could indicate that infertile women might be prone to develop CVD later in life. The trend to a higher incidence of stroke seems to support such a contention. However, the incidence of manifest CVD is low in women at the present age distribution.

A longer follow-up of an IVF cohort in the future could give us a final answer.

Meanwhile, we recommend clinicians to pay a close attention to and measure blood pressure in women who have passed an IVF treatment as they seem to have a higher propensity to develop hypertension. These women should be offered antihypertensive treatment if blood pressure increases above reference levels in order to prevent vascular damage.

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**Table 1.** Characteristics of women from IVF register and Medical Birth Register (MBR) during the time period 1990–2008.

	<b>Women from IVF register</b>	<b>Women from MBR</b>
<b>Total number</b>	n=23,498	n=116,960
<b>Mothers age at birth of the child (years)</b>		
19-24	1.4%	1.3%
25-29	16.0%	15.8%
30-34	43.1%	43.2%
35-39	34.1%	34.3%
40-47	5.4%	5.4%
Mean age ( $\pm$ SD)	33.3 ( $\pm$ 4.0)	33.4 ( $\pm$ 3.9)
Median age (quartiles)	33 (31;36)	33 (31;36)
<b>Mothers born in Sweden</b>	86.2%	81.4%
<b>Prepregnancy Body Mass Index (BMI)</b>		
Missing	16.9%	17.3%
<25	53.2%	53.4%
25-29	21.9%	21.1%
$\geq$ 30	8.1%	8.2%
<b>Cigarette smoker</b>		
Missing	7.6%	6.6%
No	85.7%	82.9%
<10 cigarettes/day	4.9%	6.7%
$\geq$ 10 cigarettes/day	1.8%	3.8%
<b>Education (years)</b>		
Missing	0.2%	0.9%
$\leq$ 9	7.6%	10.0%
10-12	45.1%	43.2%
>12	47.1%	45.9%
<b>Single/multiple birth</b>		
Single birth	83.1%	97.4%
Multiple birth	16.9%	2.6%
<b>Polycystic ovarian syndrome, n (%)</b>	788 (3.4%)	563 (0.5%)
<b>Preeclampsia, n (%)</b>	1646 (7.0%)	6117 (5.2%)
<b>Hypothyroidism, n (%)</b>	447 (1.9%)	1790 (1.5%)

**Table 2.** Follow-up time and drop-out frequency of the women in the study.

Total number (n)	IVF women		Control women	
	n	%	n	%
Possible to follow until end of follow-up	22 911	97.5	113 158	96.8
Emigrated	471	2.0	3 215	2.8
Deceased	116	0.5	587	0.5
Total years of follow-up	201 948		1 000 368	
Mean follow-up in years( $\pm$ SD)	8.6 ( $\pm$ 4.6)		8.6 ( $\pm$ 4.9)	

**Table 3.** Total number and incidence per 10.000 person-years of diabetes, hypertension, coronary heart disease and stroke in IVF women and control women from the Medical Birth Register 1990-2008.

	IVF women			Control women		
	n	Incidence per 10.000 person-years	95% CI	n	Incidence per 10.000 person-years	95% CI
<b>Diabetes</b>	201	9.95	8.65-11.4	1 111	11.1	10.47-11.77
<b>Hypertension</b>	520	2.58	2.36-2.80	2 129	2.13	2.04-2.22
<b>Coronary heart disease</b>	23	1.14	0.74-1.68	185	1.85	1.60-2.13
<b>Stroke</b>	79	3.91	3.12-4.85	319	3.19	2.85-3.55
<b>Total</b>	823	4.08	3.80-4.36	3744	3.74	3.62-3.86

**Table 4.** Cardiovascular disease Hazard Ratios (HR) and 95% Confidence Intervals (95% CI) among Swedish women giving their first birth from 1990 to 2008 with additional adjustments for BMI, smoking, country of birth and educational level.

		<b>Diabetes</b> HR (95% CI)	<b>Hypertension</b> HR (95% CI)	<b>Coronary heart disease</b> HR (95% CI)	<b>Stroke</b> HR (95% CI)
<b>Univariable analysis</b>					
MBR group	1=reference				
IVF group		0.90 (0.77-1.05)	1.24 (1.13-1.37)	0.65 (0.42-1.00)	1.24 (0.97-1.58)
<b>Univariable analysis with exclusion<sup>^</sup></b>					
MBR group	1=reference				
IVF group		0.90 (0.76-1.07)	1.26 (1.13-1.41)	0.64 (0.39-1.05)	1.22 (0.92-1.61)
<b>Multivariable analysis*</b>					
MBR group	1=reference				
IVF group		0.96 (0.81-1.14)	1.27 (1.13-1.41)	0.72 (0.44-1.17)	1.27 (0.96-1.68)
<b>Country of birth</b>					
Sweden	1=reference				
Other		2.14 (1.86-2.46)	0.99 (0.87-1.11)	1.21 (0.81-1.80)	1.40 (1.05-1.87)
<b>BMI before pregnancy</b>					
<20	1=reference				
20-24.9		1.00 (0.75-1.35)	1.39 (1.13-1.72)	1.46 (0.67-3.20)	0.89 (0.59-1.32)
25-29.9		2.02 (1.50-2.72)	2.23 (1.8-2.77)	2.37 (1.07-5.25)	1.07 (0.70-1.64)
>30		5.25 (3.09-7.07)	3.84 (3.07-4.82)	5.23 (2.34-11.72)	1.15 (0.70-1.90)
<b>Smoking</b>					
No	1=reference				
<10 cigarettes/day		0.88 (0.69-1.11)	1.06 (0.90-1.25)	4.16 (2.81-6.18)	1.12 (0.74-1.70)
≥10 cigarettes/day		1.31 (1.02-1.68)	1.25 (1.03-1.53)	5.46 (3.55-8.40)	1.83 (1.18-2.83)
<b>Education</b>					
Primary school		1.37 (1.15-1.62)	1.10 (0.95-1.28)	1.48 (1.00-2.21)	1.43 (0.8-2.58)
High school	1=reference				
More than 12 years		0.79 (0.68-0.91)	1.10 (1.00-1.21)	0.95 (0.64-1.42)	1.08 (0.85-1.37)

<sup>^</sup> Univariable analysis with exclusion of women lacking information on smoking and BMI.

\* Multivariable analysis with additional adjustment for BMI, smoking, country of birth and educational level.

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