Proceedings of the Nutrition Society (2003), **62**, 479–493 DOI:10.1079/PNS2003251

Changes in haemostasis during normal pregnancy: does homocysteine play a role in maintaining homeostasis?

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Homocysteine, derived from the demethylation of the amino acid methionine, is either further catabolised by trans-sulfuration to cysteine or remethylated to methionine. Remethylation to methionine requires the cofactors, folate and vitamin B_{12}. Folate is an effective homocysteine-lowering agent and, thus, homocysteine and folate status are inversely related. Hyperhomocysteinaemia is a strong independent risk factor for venous thromboembolism (VTE) and is associated with adverse pregnancy outcomes such as pre-eclampsia, placental abruption, early pregnancy loss and neural-tube defects. Pregnancy is a risk factor for VTE as a result of prothrombotic changes in levels of haemostatic factors. However, despite this hypercoagulable state, the incidence of pregnancy-associated VTE is relatively low. Hyperhomocysteinaemia is associated with abnormalities in markers of coagulation activation, and recent research suggests that folic acid supplementation, as well as lowering homocysteine, lowers markers of coagulation activation and increases levels of coagulation inhibitors. Tissue factor (TF) is the initiator of blood coagulation *in vivo*, and homocysteine induces TF expression *in vitro*. During pregnancy, monocyte TF expression is lower than that in the non-pregnant state, and this lowering of TF may act to counterbalance increases in coagulation activation. Furthermore, despite a high folate requirement, several studies have reported that homocysteine is lower in normal pregnancy than in the non-pregnant state. Although the exact mechanism of homocysteine lowering during pregnancy is unclear, one possible outcome of lower homocysteine may be the protection of women from pregnancy complications and VTE, and thus lower homocysteine may contribute to maintaining homeostasis in haemostasis.

Homocysteine: Haemostasis: Pregnancy

Homocysteine

Homocysteine is a non-protein-forming thiol-containing amino acid derived from the demethylation of the essential amino acid methionine. Intracellular metabolism of homocysteine is regulated by two pathways, by which it is either further catabolised by trans-sulfuration to cysteine or remethylated to methionine. The trans-sulfuration pathway involves the enzyme cystathionine β-synthase and requires vitamin B_6 as a cofactor. Remethylation to methionine is catalysed by methionine synthase, which requires folate in the form of 5-methyltetrahydrofolate as a co-substrate and vitamin B_{12} in the form of methylcobalamin as a cofactor (Finkelstein, 2000). Methylenetetrahydrofolate reductase (MTHFR) is a crucial enzyme in the remethylation pathway, and is responsible for converting 5,10-methylenetetrahydrofolate to the co-substrate 5-methyltetrahydrofolate.

In the absence of renal disease and hyperproliferative disorders, elevated levels of plasma homocysteine are generally a result of either a genetic defect in one of the enzymes involved in homocysteine metabolism or a nutritional deficiency of one of the vitamins that acts as a cofactor or co-substrate (folic acid, vitamin B_{12} and vitamin B_6). Severe elevations in plasma homocysteine leading to homocystinuria are most commonly the result of a deficiency of the trans-sulfuration enzyme cystathionine β-synthase (Mudd *et al*. 1985), whereas mild elevations in homocysteine, known as hyperhomocysteinaemia, are associated with a common mutation in the MTHFR gene (Frosst *et al*. 1995). This autosomal recessive mutation is a C → T substitution at base pair 677 resulting in an alanine to valine substitution and, as a consequence, *in vivo* enzyme activity is impaired. Individuals who are homozygous for

Abbreviations: AT, antithrombin III; F1+2, prothrombin fragments 1+2; MTHFR, methylenetetrahydrofolate reductase; PAI, plasminogen activator inhibitor; TAT, thrombin–antithrombin; TM, thrombomodulin; TF, tissue factor; t-PA, tissue plasminogen activator; VTE, venous thromboembolism; vWF, von Willebrand factor.

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the 677C → T polymorphism (TT genotype; 5–18 % of the population) tend to have elevated homocysteine levels when compared with individuals who are heterozygous for the mutation (CT genotype) or without the mutation (CC genotype; Frosst *et al*. 1995; Brattström *et al*. 1998).

Homocysteine and folate status are inversely related (Kang *et al*. 1987; Andersson *et al*. 1992*a*; Selhub *et al*. 1993; Ueland *et al*. 1993), with extensive research showing that homocysteine can be lowered in response to folic acid supplementation (Homocysteine Lowering Trialists' Collaboration, 1998), and that this lowering of homocysteine can be achieved with a dose as low as 200 μg folic acid/d (Ward *et al*. 1997). In addition, several studies have demonstrated that vitamin B_{12} and vitamin B_6 are effective in lowering homocysteine, and indeed may enhance the homocysteine-lowering effect of folic acid (Ubbink *et al*. 1994; Brönstrup *et al*. 1998; Homocysteine Lowering Trialists' Collaboration, 1998; McKinley *et al*. 2001; Quinlivan *et al*. 2002).

The normal range of fasting homocysteine in adults is 5–15 μmol/l (Ueland *et al*. 1993), although Ubbink *et al*. (1995) proposed a range of 4·9–11·7 μmol/l, which represents the normal range in a population with optimum B-vitamin status. Plasma homocysteine concentrations increase with age and are higher in men than in women, with levels becoming more comparable after menopause (Andersson *et al*. 1992*a*; Nygård *et al*. 1995). Smoking and coffee consumption are also associated with elevated plasma homocysteine (Nygård *et al*. 1995, 1997, 1998). Homocysteine concentrations increase after moderate consumption of red wine and spirits, but not after moderate consumption of beer, and it is proposed that the vitamin B_6 in beer prevents the alcohol-induced rise in homocysteine (van der Gaag *et al*. 2000).

Folate and homocysteine in normal pregnancy

Folate and pregnancy

Pregnant women have a high folate requirement and are at an increased risk of folate deficiency as a result of increased folate catabolism and utilisation (McPartlin *et al*. 1993). Folate-responsive megaloblastic anaemia is reported in 2·5–5·0 % of unsupplemented pregnancies in the developed world, with as many as 25 % of women from otherwise well-nourished societies developing bone marrow megaloblastosis, which is indicative of subclinical folate deficiency (Chanarin, 1985). Folate status of the mother also has implications for her offspring. Rolschau *et al*. (1979) reported a positive correlation between folate status and birth weight, and between folate status and placental weight. Furthermore, in a recent study Rolschau *et al*. (1999) demonstrated that folic acid supplementation is associated with increased birth weight and a reduction in incidence of preterm labour and the number of infants with low birth weight or who were small-for-gestational age.

Spina bifida and anencephaly are severe congenital malformations referred to as neural-tube defects, resulting from incomplete closure of the spinal cord and cranium respectively in the fourth week of embryonic life. Confirming previous suggestions, the Medical Research Council Vitamin Study Research Group (1991) reported that periconceptual folic acid effectively prevents the recurrence of neural-tube defects. A subsequent study by Czeizel & Dudas (1992) confirmed that folic acid also prevents first-time occurrence of neural-tube defects. In response to this evidence national expert committees advised the current recommendations: 4–5 mg/d folic acid in tablet form for the prevention of neural-tube defect recurrence; 400 μg folic acid/d, to be commenced before conception and continued until the twelfth week of pregnancy for the prevention of first occurrence (Department of Health, 1992; Public Health Services, Centre for Disease Control and Prevention, 1992).

Homocysteine and pregnancy

Despite the increased risk of folate deficiency during pregnancy, several studies, predominantly of an observational design, have reported that homocysteine is lower in normal pregnancy than in the non-pregnant state (Kang *et al*. 1986; Anderson *et al*. 1992*b*; Bonnette *et al*. 1998; Walker *et al*. 1999; Quinlivan *et al*. 2000).

The reduction in homocysteine observed in pregnancy may not simply be attributed to a single factor, but is most likely to be a physiological effect of pregnancy, with several factors contributing. It is possible that hormonal changes in pregnancy play a substantial role in the regulation of homocysteine, given that hormone-replacement therapy reduces levels of homocysteine in post-menopausal women (Mijatovic *et al*. 1998*a,b*; van Baal *et al*. 1999; Somekawa *et al*. 2002). Furthermore, Morris *et al*. (2000), in the Third National Health and Nutrition Examination Survey, concluded that higher oestrogen status is associated with decreased homocysteine concentration, independent of nutritional status and muscle mass. For a comprehensive review of oestrogen and homocysteine, see Dimitrova *et al*. (2002).

Another possible cause of lower homocysteine during pregnancy may be the uptake of homocysteine by the fetus. Malinow *et al*. (1998) demonstrated a descending concentration gradient of plasma homocysteine from maternal vein to umbilical vein and to umbilical artery, suggestive of the likely incorporation of homocysteine into the fetal metabolic cycle and, thus, of utilisation by the fetus. Haemodilution is yet another possible factor resulting in lower homocysteine, as during normal pregnancy plasma volume expands, with levels at 12 weeks of gestation increased by 14 % over follicular-phase measurements (Bernstein *et al*. 2001). However, it is unlikely that haemodilution can account entirely for lower levels of homocysteine in normal pregnancy, as levels are at least 30 % lower in the first trimester than those of non-pregnant control subjects (Anderson *et al*. 1992*b*; Bonnette *et al*. 1998; Walker *et al*. 1999), indicating that some other factors must be involved. Albumin binds 70 % of the homocysteine in human plasma (Refsum *et al*. 1985), and as plasma albumin levels fall progressively over the duration of pregnancy (Anderson *et al*. 1992*b*; Walker *et al*. 1999), decreased plasma albumin may also contribute to lower homocysteine levels.

As folic acid is an established homocysteine-lowering agent (Homocysteine Lowering Trialists' Collaboration,

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1998), it is possible that folic acid supplementation during pregnancy may also lower homocysteine. However, there has been little research investigating the effect of folic acid on homocysteine levels during pregnancy. Although there is still some debate about which factors contribute, it is accepted that lower levels of homocysteine are found during normal pregnancy. The possible advantages of these lower homocysteine levels are discussed later.

Normal haemostasis

Haemostasis has evolved in order to maintain the integrity of the vasculature. Although it has been known since the time of Hippocrates and Aristotle that blood has the ability to clot, it was not until the 1730s that a Parisian surgeon, Jean-Louis Petit, related blood clotting to haemostasis (Owen, 2001). In the 19th century Rudolf Virchow (1860) first described the phenomena now known as deep-vein thrombosis and pulmonary embolism. When Morawitz (1905) reviewed the classic theory of coagulation there were four coagulation factors: fibrinogen; prothrombin; thromboplastin; Ca. Subsequently, the cascade (Macfarlane, 1964) and waterfall (Davie & Ratnoff, 1964) theories of blood coagulation described two alternative pathways, the 'extrinsic' and the 'intrinsic', which merged to form a 'common' pathway. The current revised scheme of blood coagulation is known as the tissue factor (TF) pathway (Nemerson, 1988; Rapaport & Rao, 1995; Fig. 1). Normal haemostasis is a complex network of interactions with positive and negative feedback loops, integrating blood vessels, platelets, coagulation factors, coagulation inhibitors and fibrinolysis, so that thrombin generation and subsequent fibrinolysis and remodelling are focused and finite.

Blood vessels and platelets are essential components of normal haemostasis. The endothelium of blood vessels has anticoagulant and fibrinolytic properties, as well as the ability to prevent platelet aggregation, and thus plays a vital role in maintaining blood flow. Platelets are activated in response to blood vessel damage or exposure to foreign surfaces, leading to shape change, secretion of platelet granule contents, platelet adhesion and platelet aggregation. Coagulation factors assemble and interact on the surface of the activated platelets, reinforcing the platelet plug with fibrin. George (2000) and van Hinsbergh (2001) reviewed the roles of platelets and the endothelium in normal haemostasis.

Coagulation factors and blood coagulation

Blood coagulation is initiated by TF, a transmembrane glycoprotein constitutively expressed by non-vascular cells such as alveolar epithelial cells of the lung (Drake *et al.* 1989), thus forming a haemostatic envelope. Within the vasculature, TF expression can be induced on monocytes and endothelial cells by the inflammatory cytokines tumour necrosis factor-α, interferon-γ and interleukin-1β (Schwagner

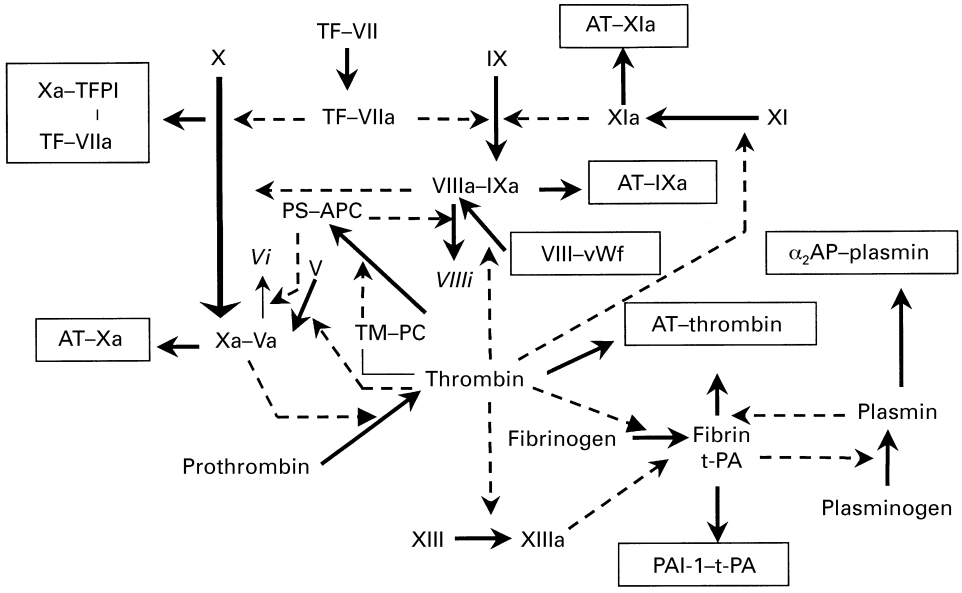

Fig. 1. Coagulation cascade: Tissue factor (TF) – factor VIIa complex initiates the coagulation cascade, activating factor X (X → Xa) and leading to thrombin generation. Thrombin activates factors XI, XIII, V and VIII, releasing factor VIII from its inactive complex with von Willebrand factor (vWF). Factor XIa activates IX, and sequentially the VIIIa–IXa and Va–Xa complexes result in further thrombin generation. Thrombin converts fibrinogen to fibrin, while XIIIa cross-links fibrin to stabilise the clot. Coagulation inhibitors: TF pathway inhibitor (TFPI) inactivates the TF–VIIa–Xa complex, while antithrombin (AT) inactivates thrombin and IXa. Thrombin binds thrombomodulin (TM), activating protein C (PC → APC). APC binds protein S (PS), degrading Va and VIIIa (Va → *Vi*; VIIIa → *VIIIi*). Fibrinolysis: Fibrin binds tissue plasminogen activator (t-PA), converting plasminogen to plasmin, which degrades fibrin to fibrin degradation products. Plasmin is inactivated by α_2-antiplasmin (α_2AP), while plasminogen activator inhibitor (PAI-1) inactivates t-PA. Inhibited complexes are represented in boxes (➔), changes of state; (– –➔), proteolytic activation–inactivation. (Modified from Hutton *et al.* 1999.)

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& Jungi, 1994) and interleukins 6 and 8 (Neuman *et al*. 1997), and by endotoxin (Colucci *et al*. 1983), C-reactive protein (Cermak *et al*. 1993) and homocysteine (Khajuria & Houston, 2000). When TF is exposed to blood, following vessel injury or possibly following cytokine induction of TF, it forms a proteolytically-active complex with factor VII. Factor VII, an inactive zymogen, circulates in plasma and is activated to VIIa when complexed with TF. The TF–VIIa complex catalyses the activation of factor IX to IXa, as well as catalysing a marked amount of factor X to Xa. Factor Xa activates factor V in sufficient amounts to activate prothrombin to thrombin, which in turn activates factors V, VIII and XI. Factor VIIIa forms a complex with IXa, which further activates X to Xa, resulting in the generation of more thrombin, leading to fibrin clot formation (Hutton *et al*. 1999; see Fig. 1).

Inhibitors of blood coagulation

Coagulation inhibitors are necessary to ensure that thrombin generation remains limited and localised. Antithrombin III (AT), heparin, heparin cofactor II, α_1-antitrypsin, α_2-macroglobulin and TF pathway inhibitor inhibit the serine proteases of the coagulation cascade, such as thrombin, Xa and TF–VIIa. Protein C, thrombomodulin (TM), protein S, C4b-binding protein and activated protein C inhibitor are all components of the protein C system. TM bound to thrombin activates protein C, which rapidly degrades factors VIIIa and Va on the phospholipid surface of activated platelets, a reaction that increases 10–20-fold when protein C combines with its cofactor protein S.

Fibrinolysis

Fibrinolysis controls fibrin deposition, thus maintaining a controlled procoagulant response. Fibrinolysis involves the conversion of plasminogen to plasmin by tissue plasminogen activator (t-PA). Plasmin cleaves fibrin and fibrinogen, yielding fibrin degradation products. α_2-Antiplasmin, a plasmin inhibitor, and plasminogen activator inhibitor (PAI) types 1 and 2 prevent excess fibrinogen degradation by plasmin. Endothelial cells are regulators of fibrinolytic activity, as they synthesise t-PA and PAI-1 (Hajjar, 1993).

Markers of haemostasis activation

Normal haemostasis requires a balance between coagulation and fibrinolysis. A shift in the haemostatic balance can result in either a tendency to bleed or hypercoagulability with increased risk of thromboembolism. The hypercoagulable condition is difficult to detect with routine laboratory assays, and requires the use of sensitive activation markers of coagulation and fibrinolysis such as prothrombin fragments 1+2 (F1+2), thrombin–antithrombin (TAT) complex (Bauer, 1999) and D-dimers (Whitaker *et al*. 1985). F1+2, cleaved from prothrombin after its activation by factor Xa, and TAT, formed during inactivation by its main inhibitor antithrombin, are markers of coagulation, whereas D-dimers are products of fibrin breakdown by plasmin and thus are markers of fibrinolysis.

Haemostasis in normal pregnancy

Normal pregnancy is associated with substantial changes in the haemostatic system, with pregnancy often referred to as a hypercoagulable state. Traditionally, it is thought that these changes are in preparation for the haemostatic challenge of delivery, with the haemostatic system returning to that of the non-pregnant state at approximately 4 weeks post delivery (Greer, 1994). In the present review alterations in coagulation factors, coagulation inhibitors, fibrinolysis and markers of haemostasis will be discussed in turn. Greer (1994) and Lyall & Greer (1996) reviewed alterations in platelets, platelet function and the endothelium during normal pregnancy.

Coagulation factors

During normal pregnancy factor XIII, fibrin-stabilising factor, increases in the early stages of pregnancy, returning to non-pregnant values in the third trimester (Persson *et al*. 1980; van Wersch *et al*. 1997). Levels of factor XII rise progressively during the gestation period (Hellgren & Blombäck, 1981; de Moerloose *et al*. 1998*a*; Donohoe *et al*. 2002), as do levels of factor X (Hellgren & Blombäck, 1981; Stirling *et al*. 1984). Conversely, levels of factor XI decrease during pregnancy (Hellgren & Blombäck, 1981), possibly as the result of increased factor XI consumption (Blombäck, 1991). However, given that activation of factor XI by thrombin is required to activate factor IX leading to further thrombin generation, it is possible that in normal pregnancy levels of factor XI are physiologically lowered to counterbalance the increases in other coagulation factors.

Factor VIII levels and coagulation activity rise progressively during pregnancy (Hellgren & Blombäck, 1981; Stirling *et al*. 1984; Chan *et al*. 1985; Bokarewa *et al*. 1997; Walker *et al*. 1997; Clark *et al*. 1998; Kjellberg *et al*. 1999), as do levels of von Willebrand factor (vWF) (Hellgren & Blombäck, 1981; Stirling *et al*. 1984; Chan *et al*. 1985). The vWF:VIII coagulation activity value remains constant until the third trimester, after which the value increases, suggesting an increase in proteolytic degradation of activated VIII in the later stages of pregnancy (Hellgren & Blombäck, 1981; Stirling *et al*. 1984). There is a gradual increase in factor VII during normal pregnancy (Stirling *et al*. 1984; de Moerloose *et al*. 1998*a*; Wright *et al*. 1998; Donohoe *et al*. 2002). The increase in factor V concentration in early pregnancy is followed by a decrease and stabilisation (Stirling *et al*. 1984), while factor V coagulation activity shows a gradual rise throughout gestation (Clark *et al*. 1998). Studies of prothrombin (factor II) levels in pregnancy have yielded inconclusive results, showing both increases (Stirling, 1984) and no change (Clark *et al*. 1998) during pregnancy. Fibrinogen levels show a steady increase during pregnancy (Hellgren & Blombäck, 1981; Stirling *et al*. 1984; Gatti *et al*. 1994; Francalanci *et al*. 1995; Cerneca *et al*. 1997; Kjellberg *et al*. 1999).

Finally, while levels of soluble TF remain constant during normal pregnancy (Bellart *et al*. 1998), monocyte TF activity and expression are lower in normal pregnancy than in non-pregnant control subjects (Øian *et al*. 1985; Holmes *et al*. 2002). As TF is the initiator of blood clotting *in vivo*,

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lower TF expression and activity on circulating monocytes may play an important role in protecting pregnant women from venous thromboembolism (VTE), despite increases in many of the clotting factors and the hypercoagulable state described earlier (Holmes *et al*. 2002).

Coagulation inhibitors

Levels of AT remain stable during pregnancy (Hellgren & Blombäck, 1981; Stirling *et al*. 1984; Gerbasi *et al*. 1990; Bremme *et al*. 1992; Gatti *et al*. 1994; Francalanci *et al*. 1995; Bellart *et al*. 1997; Cerneca *et al*. 1997), while heparin cofactor II, TM, α_1-antitrypsin and α_2-macroglobulin levels are raised during normal pregnancy (Stirling *et al*. 1984; Massouh *et al*. 1989; Bellart *et al*. 1997; de Moerloose *et al*. 1998*b*). Little is known about levels of TF pathway inhibitor during normal pregnancy. However, levels are lower during labour than in non-pregnant controls (Uszynski *et al*. 2001). Levels of total and free protein S gradually decrease throughout pregnancy (Comp *et al*. 1986; Malm *et al*. 1988; Fernandez *et al*. 1989; Bremme *et al*. 1992; Gatti *et al*. 1994; Faught *et al*. 1995; Cerneca *et al*. 1997; Clark *et al*. 1998; Kjellberg *et al*. 1999). The majority of studies report that levels of protein C remain constant during normal pregnancy (Bremme *et al*. 1992; Gatti *et al*. 1994; Faught *et al*. 1995; Cerneca *et al*. 1997; Clark *et al*. 1998; Kjellberg *et al*. 1999), yet acquired activated protein C resistance is reported in up to 57 % of normal pregnancies (Cumming *et al*. 1995; Mathonnet *et al*. 1996; Schlit *et al*. 1996; Bokarewa *et al*. 1997; Peek *et al*. 1997; Walker *et al*. 1997; Clark *et al*. 1998; Kjellberg *et al*. 1999; Shu *et al*. 2000). This increase in activated protein C resistance corresponds with increases in factor VIII and decreases in protein S and activated protein C inhibitor (Bokarewa *et al*. 1997; Walker *et al*. 1997; Clark *et al*. 1998; Shu *et al*. 2000). Normal pregnancy appears to be associated with bi-directional changes in levels or activity of coagulation inhibitors, and it is possible that these complex changes occur in order to maintain the coagulation–fibrinolysis balance during normal pregnancy.

Fibrinolysis

Lower levels of t-PA have been reported in normal pregnancy compared with the non-pregnant state (Wright *et al*. 1988; Cerneca *et al*. 1997; Kjellberg *et al*. 1999). In addition, levels of endothelial-derived PAI-1, the primary inhibitor of t-PA, are increased during the later stages of pregnancy, while placenta-derived PAI-2, detectable in the plasma during the first trimester, increases substantially throughout pregnancy (Wright *et al*. 1988; Estelles *et al*. 1989; van Wersch & Ubachs, 1991; Cerneca *et al*. 1997; Kjellberg *et al*. 1999). Plasminogen levels are increased during pregnancy, as are levels of the plasmin inhibitor α_2-antiplasmin (Hellgren & Blombäck, 1981; Wright *et al*. 1988; van Wersch & Ubachs, 1991). Overall, these changes suggest that the fibrinolytic system is impaired during normal pregnancy.

Markers of haemostasis activation

During normal pregnancy the changes in coagulation factors, coagulation inhibitors and components of the fibrinolytic system, as outlined earlier, are suggestive of a hypercoagulable state, with impaired fibrinolysis. Recent studies have concentrated on measuring markers of haemostasis activation, where TAT and F1 + 2 are measures of coagulation activation and fibrin degradation products and D-dimers are measures of fibrinolytic activation. During normal pregnancy, levels of TAT and F1 + 2 increase progressively, indicative of a substantial increase in coagulation activation (Bremme *et al*. 1992; Comeglio *et al*. 1996; Schlit *et al*. 1996; Cerneca *et al*. 1997; Clark *et al*. 1998; Reber *et al*. 1998; Eichinger *et al*. 1999; Kjellberg *et al*. 1999; Donohoe *et al*. 2002). Similarly, fibrinopeptide A, another marker of coagulation activation, is increased during pregnancy (Douglas *et al*. 1982; Gerbasi *et al*. 1990; Schlit *et al*. 1996; Bellart *et al*. 1998).

Despite the apparent impairment in fibrinolysis discussed earlier, normal pregnancy is associated with increases in fibrin degradation products and D-dimers (Gerbasi *et al*. 1990; Bremme *et al*. 1992; Francalanci *et al*. 1995; Schlit *et al*. 1996; Bellart *et al*. 1998; Eichinger *et al*. 1999; Kjellberg *et al*. 1999; Donohoe *et al*. 2002), indicative of a substantial increase in fibrinolytic system activation. As previously stated, normal haemostasis requires a balance between coagulation and fibrinolysis, and despite the marked changes in haemostasis associated with normal pregnancy, the incidence of VTE remains relatively low. According to Eichinger *et al*. (1999), levels of coagulation and fibrinolytic indices in healthy pregnant women in the third trimester are similar to, or higher than, those found in patients following a deep-vein thrombosis or pulmonary embolism, yet none of the women in this study developed clinical symptoms of VTE. Eichinger *et al*. (1999) also demonstrated that the endogenous thrombin potential, an indicator of the potency of plasma to generate thrombin in response to a thrombogenic stimulus (Hemker & Beguin, 1995), remained unchanged throughout pregnancy. Moreover, the fibrinopeptide A:D-dimer ratio also remains constant throughout pregnancy, demonstrating a constant coagulation–fibrinolysis balance during normal pregnancy (Bellart *et al*. 1998). In contrast, a high fibrinopeptide A:D-dimer ratio, suggestive of hypofibrinolysis, was observed in women with pre-eclampsia (Bellart *et al*. 1999), thus highlighting the importance of the coagulation–fibrinolysis balance during normal pregnancy.

Thrombosis in pregnancy

Venous thromboembolic disease in pregnancy

Pregnancy and the puerperium are known risk factors for venous thrombosis (Rosendaal, 1999). Virchow's (1860) triad postulates that the principal factors underlying venous thrombosis are hypercoagulability, venous stasis and vascular damage, all of which occur during pregnancy (Greer, 1999). The hypercoagulable state is evident throughout pregnancy, venous stasis of the lower limbs occurs by the end of the first trimester (Macklon *et al*. 1997), and the potential for vessel damage is present during delivery. Although maternal death is rare, pulmonary embolism remains the leading cause of maternal death in the UK (Confidential Enquiries into Maternal Deaths, 2000).

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The incidence of VTE during normal pregnancy is estimated to be 5·5–6·0 times higher than that in the general female population of child-bearing age (McColl *et al*. 1997), with the reported incidence of pregnancy-associated VTE estimated at between 0·62 and 1·88 per 1000 deliveries (Macklon & Greer, 1996; McColl *et al*. 1997; Gherman *et al*. 1999; Lindqvist *et al*. 1999; Chan *et al*. 2001; Simpson *et al*. 2001). The incidence of deep-vein thrombosis and pulmonary embolism is estimated at 0·71 and 0·15 per 1000 deliveries respectively, with the majority of deep-vein thrombosis events occurring in the antenatal period (McColl *et al*. 1997; Gherman *et al*. 1999). Although Gherman *et al*. (1999) reported that almost half antenatal deep-vein thrombosis events were detected before 15 weeks of gestation, the puerperium should be regarded as the period of greatest risk (McColl *et al*. 1997).

Clinical risk factors linked with pregnancy-associated VTE include advanced maternal age, high parity, weight, multiple birth, major current illness, operative delivery and pre-eclampsia (Macklon & Greer 1996; McColl *et al*. 1999; Simpson *et al*. 2001). Personal or family history of thromboembolic disease is a recognised risk factor for VTE, with evidence of a genetic predisposition to VTE emerging. In one study approximately 30 % of patients with confirmed VTE associated with pregnancy were found to have a heritable thrombophilia (McColl *et al*. 1997). Inherited or congenital thrombophilia, discussed by Rosendaal (1999), includes deficiencies of AT, protein C and protein S, and the presence of factor V Leiden, prothrombin 20210, and homozygosity for the thermo-labile variant of MTHFR. McColl *et al*. (1999) reviewed the role of inherited thrombophilia in pregnancy-associated VTE. Venous thrombosis is a multicausal disease, with interaction between genetic and acquired risk factors (Rosendaal, 1999), yet 28 % of pregnancy-associated VTE are not related to either a clinical risk factor for thrombosis or a thrombophilic defect (McColl *et al*. 1997). Thus, it is possible that other unknown factors may contribute to the increased risk of VTE in pregnancy, highlighting the need for further research into changes in haemostasis during pregnancy.

Homocysteine, methylenetetrahydrofolate reductase status and venous thromboembolism in pregnancy

Hyperhomocysteinaemia is a known risk factor for venous thrombosis (Falcon *et al*. 1994; den Heijer *et al*. 1996; Eichinger *et al*. 1998; Ray *et al*. 2001). Little is known about the relationship between homocysteine and pregnancy-associated VTE, and given that homocysteine can be lowered by folic acid supplementation (Homocysteine Lowering Trialists' Collaboration, 1998), it is apparent that further studies investigating homocysteine and pregnancy associated-VTE are required.

In relation to the 677C → T MTHFR polymorphism and risk of VTE, it is still unclear if homozygosity for this mutation is a risk factor, with some studies reporting an association (Gemmati *et al*. 1999*a*; Salomon *et al*. 1999; Couturaud *et al*. 2000), while others found no association (Kluijtmans *et al*. 1998; Gemmati *et al*. 1999*b*; Alhenc-Gelas *et al*. 1999; Ray *et al*. 2001). Given that hyperhomocysteinaemia is a risk factor for VTE, it is not yet apparent why the relationship between the 677C → T MTHFR polymorphism and risk of VTE remains controversial. Kluijtmans *et al*. (1998) discussed several possible explanations; however, a meta-analysis of the data would be beneficial in determining any relationship. Similarly, in pregnant women it is unclear whether the 677 → T MTHFR polymorphism confers a risk of VTE, with McColl *et al*. (2000) finding no association, while Grandone *et al*. (1998) reported an increased risk of pregnancy-related VTE in homozygous individuals, thus highlighting the need for further studies.

Thrombophilia and pregnancy complications

Complications of pregnancy such as pre-eclampsia, fetal loss, placental abruption and intrauterine growth retardation are associated with both acquired and congenital thrombophilia (Dekker *et al*. 1995; Preston *et al*. 1996; Grandone *et al*. 1998; Greer, 1999; Gris *et al*. 1999; Kupferminc *et al*. 1999). Evidence that heparin and low-dose aspirin therapy are effective in increasing rates of live births in women with a history of recurrent miscarriage (Rai *et al*. 1997) highlights the importance of procoagulant changes in the pathophysiology of recurrent miscarriage (Greer, 2001). Furthermore, in anti-phospholipid syndrome, a hypercoagulable state, intrauterine growth retardation and fetal distress leading to premature birth or fetal death are attributed to utero-placental insufficiency that is the result of multiple placental thrombi and infarcts (Gharavi *et al*. 2001). The role of inherited and acquired thrombophilia in severe pregnancy complications has been investigated with conflicting results. Many *et al*. (2001) reported an increased rate of placental vascular lesions in women with severe complications and thrombophilia, whereas other researchers found no significant difference between the placentas of women with adverse pregnancy outcomes with or without a thrombophilic defect (Mousa & Alfirevicl, 2000; Sikkema *et al*. 2002). It may be that factors other than those normally investigated in a thrombophilia screen are responsible for the abnormal placental pathology associated with adverse pregnancy outcome, such as increased production of the fibrinolytic inhibitor PAI-1 (Sheppard & Bonnar, 1999). These data, together with recent reports of increased procoagulant microparticles in women with a history of pregnancy loss (Laude *et al*. 2001), suggest that excessive coagulation in general may be responsible for adverse fetal outcome (Greer, 2001).

Homocysteine, methylenetetrahydrofolate reductase status and pregnancy complications

The link between homocysteine, MTHFR and neural-tube defects is well established (Steegers-Theunissen *et al*. 1994; Mills *et al*. 1995; Whitehead *et al*. 1995). However, for the purposes of the present review, prominence will be given to pregnancy complications and homocysteine–MTHFR status where the probable link is a thrombotic mechanism. Placental vasculopathy resulting in placental infarcts compromises the ureto-placental circulation and is thought to contribute to pregnancy complications. Recently, van der Molen *et al*. (2000) reported that elevated homocysteine

concentration and homozygosity for the 677C → T MTHFR mutation are risk factors for placental vasculopathy. Furthermore, there is substantial evidence that elevated homocysteine concentration is associated with common pregnancy complications and adverse pregnancy outcome (de Vries *et al*. 1997; Ray & Laskin, 1999; Vollest *et al*. 2000), such as pre-eclampsia (Dekker *et al*. 1995; Rajkovic *et al*. 1997; Powers *et al*. 1998; Cotter *et al*. 2001), placental abruption (Goddijn-Wessel *et al*. 1996) and recurrent early pregnancy loss (Wouters *et al*. 1993; Quèrè *et al*. 1998; Nelen *et al*. 2000).

As with MTHFR genotype and risk of VTE, the association between risk of pregnancy complications and homozygosity for the 677C → T MTHFR mutation is more uncertain. In a study by Kupferminc *et al*. (1999) in which the study population comprised women with any one of the obstetrical complications (pre-eclampsia, placental abruption, intrauterine growth retardation or stillbirth), the frequency of TT genotype individuals was higher in cases than in controls. Grandone *et al*. (1997) reported an association between homozygosity for the 677C → T MTHFR mutation and the occurrence of pre-eclampsia and, although several other researchers have not found this association (Powers *et al*. 1999; Kaiser *et al*. 2001; D'Elia *et al*. 2002), a meta-analysis by Ray & Laskin (1999) associated the TT genotype with a moderate risk of pre-eclampsia. Similarly, several authors found no association between placental abruption or fetal loss and homozygosity for the 677C → T MTHFR mutation (Brenner *et al*. 1999; Gris *et al*. 1999; Foka *et al*. 2000), while a meta-analysis reported that the TT genotype is a probable risk factor (Ray & Laskin, 1999).

Homocysteine and haemostasis

Coagulation factors

There is increasing evidence that elevated levels of homocysteine are associated with prothrombotic changes in haemostatic factors. Rodgers & Kane (1986) reported increased factor V activation in association with homocysteine-treated endothelial cells. In patients with acute coronary syndrome elevated plasma homocysteine has been associated with elevated factor VIIa (Al-Obaidi *et al*. 2000), although other researchers have been unable to demonstrate any correlation between factor VII activity and homocysteine in population-based studies (Kario *et al*. 2001; Kuch *et al*. 2001). The association between homocysteine and fibrinogen also remains unclear, with some researchers reporting an association (von Eckardstein *et al*. 1994; Kuch *et al*. 2001), and others finding no association (Folsom *et al*. 1998; Yarnell *et al*. 2000; Kario *et al*. 2001; Kuch *et al*. 2001). Kuch *et al*. (2001) studied two populations, Czech and German, and reported more pronounced associations between homocysteine and coagulation factors in the Czech population, which has a higher proportion of cardiovascular disease, and thus proposed that the association may become more apparent with underlying conditions that enhance and produce hypercoagulable states. In folic acid-supplementation studies Undas *et al*. (1999) reported no change in fibrinogen and prothrombin levels despite a reduction in homocysteine concentration, whereas other researchers reported decreases in both homocysteine and fibrinogen following supplementation (Naruszewicz *et al*. 2001; Mayer *et al*. 2002; Mayer Jr *et al*. 2002).

As well as playing a role in the coagulation pathway, vWF is an established marker of endothelial damage. Plasma vWF is increased in patients with hyperhomocysteinaemia (Freyburger *et al*. 1997; de Jong *et al*. 1997), and several studies have shown positive correlations between homocysteine and vWF (de Valk-de Roo *et al*. 1999; Becker *et al*. 2000; Yarnell *et al.* 2000; Kuch *et al.* 2001), although Kario *et al*. (2001) did not find any correlation. Similarly, results from folic acid-supplementation studies have not been consistent, with some studies demonstrating a decrease in vWF in association with homocysteine lowering (van den Berg *et al*. 1995; Mayer Jr *et al*. 2002), while other studies found no change in vWF levels (Constans *et al*. 1999; Thambyrajah *et al*. 2000, 2001). It may be that the inconsistency is the result of higher baseline homocysteine levels in studies in which homocysteine lowering was observed, possibly equating to damaged endothelium and higher baseline vWF levels, which were lowered or normalised on removal of the damaging stimulus, homocysteine. Furthermore, as study populations consisted of varying disease states, it is possible that, in some conditions, factors other than hyperhomocysteinaemia are involved in the pathophysiology of the prothrombotic state, thus leading to inconsistent results in supplementation studies.

In relation to TF, the initiator of blood clotting *in vivo*, low concentrations of homocysteine have been shown to induce TF activity in endothelial cells (Fryer *et al*. 1993), while physiologically-relevant concentrations of homocysteine induce TF expression on monocytes (Khajuria & Houston, 2000), thus presenting a plausible mechanism whereby homocysteine may induce thrombosis. Using a rat model, Durand *et al*. (1997) showed that hyperhomocysteinaemia enhances platelet aggregation and macrophage-derived TF activity. Moreover, homocysteine was positively correlated with plasma levels of TF in patients with IHD (Marcucci *et al*. 2000). However, only a randomised placebo-controlled folic acid-supplementation study designed to investigate whether homocysteine lowering by folic acid is accompanied by a reduction in TF, will conclusively establish a cause–effect relationship between homocysteine and TF *in vivo*.

Coagulation inhibitors

Evidence is also emerging that elevated levels of homocysteine may result in prothrombotic changes in coagulation inhibitors. TM acts as a cofactor for thrombin-catalysed activation of protein C, and *in vitro* studies have demonstrated that homocysteine inactivates TM activity, therefore reducing its anticoagulant properties (Lentz & Sadler, 1991; Hayashi *et al*. 1992). TM is also a marker of endothelial damage and, in agreement with *in vitro* experiments (Hayashi *et al*. 1992), *in vivo* studies of patients with hyperhomocysteinaemia reported increased levels of TM when compared with normohomocysteinaemic patients (Hofmann *et al*. 1998; Brunelli *et al*. 2000), although this outcome has not been reported in all studies (de Jong *et al*. 1997;

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Kario *et al*. 2001). Results from folic acid-supplementation studies have shown that lowering of homocysteine is associated with a reduction in TM levels (van den Berg *et al*. 1995; Constans *et al*. 1999). The effects of homocysteine on TM demonstrate two mechanisms whereby homocysteine may contribute to thrombosis risk: increased endothelial damage; reduced anticoagulant properties.

In agreement with an early study reporting decreased AT activity in homocystinuric patients (Palareti & Coccheri, 1989), Nishinaga *et al*. (1993) demonstrated that homocysteine suppresses anticoagulant heparan sulfate expression, thus reducing the capacity of endothelial cells to bind and activate the anticoagulant AT. However, several *ex vivo* studies found no correlation between plasma homocysteine concentrations and AT levels (Brattström *et al*. 1989; Bienvenu *et al*. 1991, 1993). Although Palareti & Coccheri (1989) demonstrated that folic acid and pyridoxine treatment resulted in a return of AT activity to normal in homocystinuric patients, folic acid supplementation for treatment of hyperhomocysteinaemia has been reported to increase AT in some (Mayer *et al*. 2002; Mayer Jr *et al*. 2002), but not all (Undas *et al*. 1999), intervention studies.

Homocysteine also interferes with the protein C pathway. *In vitro*, supraphysiological concentrations of homocysteine reduce protein C activation by acting as a competitive inhibitor to thrombin (Rodgers & Conn, 1990) and by irreversibly inactivating protein C (Lentz & Sadler, 1991). More recently, Undas *et al*. (2001) demonstrated that homocysteine is rapidly incorporated into factor Va, resulting in impaired inactivation of factor Va by activated protein C, and that this process can occur at physiologically-relevant concentrations. Furthermore, Lentz *et al*. (1996) showed that the aortic endothelium from monkeys with hyperhomocysteinaemia activated protein C *in vitro* less effectively than that of control animals. However, in human studies elevated levels of homocysteine do not appear to affect protein C activation (Cattaneo *et al*. 1998), and folic acid treatment has no effect on the activity of protein C (Undas *et al*. 1999). It is unclear why there is a discrepancy between *in vitro* and *in vivo* results. One explanation may be that circulating activated protein C measured *in vivo* reflects protein C activation occurring in the microcirculation, and that there is a different relationship for activated protein C measured *in vitro*, which reflects localised activity (Cattaneo *et al*. 1998).

Overall, it is not clear why some studies link elevated homocysteine with impaired anticoagulation, whereas others show no association, but it may be possible that in certain disease states factors other than, or including, homocysteine influence thrombotic mechanisms, and thus inconsistent results may be related to the different populations studied.

Fibrinolysis

Elevated homocysteine also appears to result in prothrombotic changes within the fibrinolytic system. *In vitro*, homocysteine impairs the ability of endothelial cells to generate plasmin by modulating annexin II, inhibiting t-PA-annexin II assembly on the cell (Hajjar & Jacovina, 1998), and thus reducing t-PA activity by 60 % (Hajjar, 1993). Ling & Hajjar (2000) proposed that the subsequent reduction in plasmin generation could underlie the prothrombotic activity associated with homocysteine *in vivo*. Although these results, together with those of *in vitro* experiments which reported no effect of homocysteine on t-PA mRNA levels (Midorikawa *et al*. 2000), suggest that homocysteine affects the activity rather than the expression of t-PA, results from *in vivo* studies are inconclusive. Several studies reported no significant difference in levels of t-PA antigen between individuals with and without hyperhomocysteinaemia (de Jong *et al*. 1997; Božič *et al*. 2000), and folic acid supplementation of hyperhomocysteinaemic patients resulted in no significant change in t-PA concentration (van den Berg *et al*. 1995). However, other studies have reported an association between homocysteine and t-PA concentration (Bienvenu *et al*. 1993; Lindgren *et al*. 1996; Kristensen *et al*. 1999). Furthermore, t-PA activity of hyperhomocysteinaemic patients was not found to be significantly different from that of normohomocysteinaemic patients (Božič *et al*. 2000). In order to increase our understanding of the effect of homocysteine on t-*PA in vivo*, further studies of the effect of homocysteine lowering on both t-PA antigen and activity are necessary.

In vitro, homocysteine increases PAI-1 gene expression (Midorikawa *et al*. 2000), yet the effect of homocysteine on PAI-1 *in vivo* remains unclear. There was no significant difference in either PAI-1 antigen or activity for hyperhomocysteinaemic individuals compared with normohomocysteinaemic individuals (de Jong *et al*. 1997; Božič *et al*. 2000), and there was no association between homocysteine and PAI-1 activity (Bienvenu *et al*. 1993). However, in renal transplant recipients Marcucci *et al*. (2001) reported a positive correlation and a reduction in PAI-1 levels and homocysteine concentration following folic acid supplementation. In a folic acid-supplementation study of a similar size involving haemodialysis patients, lowering homocysteine levels did not result in a reduction in PAI-1 (Kunz *et al*. 1999). Again, one possible explanation for these apparently inconsistent results may be that in different disease states the mechanisms contributing to the prothrombotic state are complex, such that lowering homocysteine in one population may lower PAI-1 levels, whereas in a different population other factors continue to impinge on fibrinolysis.

There is some evidence that homocysteine may impair fibrinolysis, which could represent one mechanism whereby homocysteine contributes to the increased risk of thrombosis. However, in view of the disparity in results from studies to date, there is clearly a need for further investigation of homocysteine lowering *in vivo* in order to fully elucidate the link between elevated homocysteine and fibrinolytic factors.

Markers of haemostasis activation

It is apparent from the evidence discussed earlier that elevated homocysteine is associated with changes in coagulation factors, coagulation inhibitors and fibrinolytic factors. However, in order to determine whether hyperhomocysteinaemia is associated with the hypercoagulable state, changes in the sensitive activation markers of coagulation and fibrinolysis, F1 + 2, TAT and D-dimer, must be examined.

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Homocysteine is positively correlated with TAT in patients with IHD (Marcucci *et al*. 2000) and, although mildly increased homocysteine levels are associated with elevated levels of TAT in plasma, folic acid supplementation does not modify TAT levels (Freyburger *et al*. 1997). Treatment of hyperhomocysteinaemia with folic acid and vitamin B_{12} has been reported to reduce TAT, F1 + 2 and also increase bleeding time (Undas *et al*. 1999). A positive correlation has been observed between homocysteine and F1 + 2 in patients with acute coronary syndromes, but not in patients who presented with chest pain that was not of cardiac origin (Al-Obaidi *et al*. 2000) or in asymptomatic populations (Kario *et al*. 2001; Kuch *et al*. 2001). In large population studies a positive correlation was found between homocysteine and D-dimer, demonstrating a relationship between homocysteine and fibrinolytic activity (Yarnell *et al*. 2000; Kuch *et al*. 2001). However, increased fibrinolysis is suggestive of increased coagulation and, therefore, it would be of greater relevance to observe the effect of elevated homocysteine on the coagulation–fibrinolysis balance, perhaps by measuring the fibrinopeptide A:D-dimer ratio in subjects with elevated homocysteine, and observing the effect of homocysteine-lowering treatment.

It is difficult to interpret the effect of elevated homocysteine on haemostasis as many *in vitro* studies use supraphysiological concentrations, and *in vivo* and *ex vivo* studies examine a variety of different populations with complex disease states. Thus, further prospective studies are required in order to fully establish whether homocysteine is associated with the hypercoagulable state as a causative agent or whether elevated levels of homocysteine are a product of other, unknown, mechanisms involved in the pathophysiology of thrombotic diseases.

The present review has sought to examine the effect of homocysteine on haemostasis, and also describes alterations in normal haemostasis and in homocysteine during normal pregnancy. Although to date there has been little research into the effect of homocysteine lowering in pregnancy on haemostatic factors, it is possible that this physiological lowering of homocysteine plays a homeostatic role in the regulation of haemostatic factors during an otherwise hypercoagulable period.

Concluding statement

In normal haemostasis there is a balance between coagulation factors and coagulation inhibitors, and between fibrinolytic factors and fibrinolytic inhibitors, with normal haemostasis ultimately being the result of a balance between coagulation and fibrinolysis. Although normal pregnancy is associated with many changes in haemostasis, with a potential tilt towards the hypercoagulable state, there is evidence that this balance is maintained (Bellart *et al*. 1998; Eichinger *et al*. 1999). In addition to increases in certain coagulation inhibitors and a decrease in factor XI (discussed earlier), the reduction in the expression of monocyte TF, the initiator of blood clotting *in vivo*, may play a major role in redressing the haemostatic balance (Holmes *et al*. 2002). As homocysteine induces monocyte TF expression at physiological concentrations *in vitro* (Khajuria & Houston, 2000), it may be that reduced levels of plasma homocysteine during normal pregnancy play a role in down regulating TF expression and, thus, in maintaining homeostasis in haemostasis. The reason for lower homocysteine in pregnancy is unknown, but as elevated homocysteine is associated with prothrombotic changes in haemostasis and consequently with increased risk of pregnancy complications, one outcome of lower homocysteine may be the protection of the mother and fetus from VTE and pregnancy complications.

As folic acid is effective in lowering homocysteine, it is essential to investigate homocysteine metabolism in pregnancy and to establish the role of folate in homocysteine metabolism at the different stages of pregnancy. Finally, longitudinal prospective studies in pregnant women at risk of VTE or with pregnancy complications are necessary in order to fully evaluate the link between homocysteine and thrombotic complications associated with pregnancy.

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https://doi.org/10.1079/PNS2003251 Published online by Cambridge University Press